

USING ADENO-ASSOCIATED VIRAL VECTORS TO MODEL HBV SUBGENOTYPE A1 REPLICATION IN CELL CULTURE AND IN VIVO

By

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THESIS

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Declaration

DECLARATION

I declare that thesis hereby submitted to the University of Witwatersrand for the Doctor of Philosophy degree in medicine has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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07 June 2021

.....
Surname initials ,tittle & signature

.....
Date

DEDICATION

I would like to dedicate my work in memory of my grandparents Tshinakaho and Tshamaano Limani who have contributed a lot throughout my life and studies, and to the Almighty God of Major 1 for the strength and courage that He gave me throughout my study.

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ABSTRACT

Development of new animal models is critically important to study the biology of hepatitis B virus (HBV) and evaluate the intrahepatic effects of therapeutics aimed at curing chronic HBV infection. Currently, chimpanzees are the only animals that are infectable by HBV and recapitulate the entire HBV replication cycle. However, the use of chimpanzees and other great apes in biomedical research has been banned in a number of countries. As a consequence, there is an urgent need to develop new models of HBV infection and replication. This study aimed to use adeno-associated viruses (AAVs) to model HBV replication in cell culture and in vivo. To achieve this, recombinant AAVs bearing greater-than-genome-length sequences of the A1 HBV subgenotype (AAV-A1) or Subgenotype D3 (AAV-D3) were produced, whereby AAV-D3 served as a positive control. Subgenotype A1 infection is of particular concern to South Africa where it has the highest prevalence and is associated with a much greater risk of developing HBV-related hepatocellular carcinoma. Vectors packaged in AAV2 (AAV2-A1 and AAV2-D3) or AAV8 (AAV8-A1 and AAV8-D3) capsid were produced in large scale for in vitro or in vivo studies, respectively. Following transduction of liver-derived cells with AAV2-A1 or AAV2-D3, significant levels of HBV surface antigen (HBsAg) were detected in culture supernatants. Evaluation of the in vivo safety of AAV vector dose used to deliver HBV genome sequences was carried out using Cytometric bead array and RT-qPCR. Administration of AAV8-A1 or AAV8-D3 to mice did not induce expression of inflammatory markers as assessed by cytometric bead array analysis (IL-12p70, IL-6, TNF, IL-10, MCP-1, INF- γ) or RT-qPCR (*IFN- β* , *OAS-1* and *IFIT1*). In addition, alanine transaminase enzyme levels remained within the normal range in animals receiving AAV8-A1 or AAV8-D3, indicating no liver toxicity. Sustained expression of HBsAg and HBcAg were detected

Abstract

in mice infected with AAV8-A1 or AAV8-D3 for a period of 6 months. The HBcAg levels in the liver of mice injected with AAV8-A1 were higher in the nucleus, whereas AAV-D3 injected mice livers had more cytoplasmic HBcAg localisation. The relaxed circular (RC) and double stranded linear HBV DNA intermediates were detected in mouse livers using Southern blot. The Northern blot analysis revealed the 3.5 kb, 2,4kb and 2, 1 kb transcripts. These DNA intermediates and RNA transcripts serve as indicators of HBV replication. Detection of viral particle equivalents using qPCR confirmed HBV replication in mice injected with AAVs bearing HBV genomes. There was no significant liver inflammation or cirrhosis detected as a result of infecting the mice with HBV genome bearing AAVs.

The possibility of using the AAV-HBV A1 murine model to screen anti-HBV therapeutics was explored. AAV8 expressing artificial primary-microRNA that target the HBV X region (scAAV8 miR 3/5,8,9) efficiently silenced viral gene expression from AAV-A1 or AAV-D3, both in cell culture and in mice. These data showed for the first time that AAVs can be successfully used to model HBV subgenotype A1 replication. Most importantly, this model can be applied to screening novel anti-HBV therapeutics to enhance development of innovative treatments of chronic HBV infection.

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LIST OF ABBREVIATIONS

Ac No	-	Accession number
ALT	-	Alanine aminotransferase
Amp-	-	Ampicillin
AAV	-	Adeno-associated virus
bp	-	Base pair
BCP	-	Basic core promoter
CBA	-	Cytometric bead array
CRISPR	-	Clustered regularly interspaced short palindromic repeat
dH ₂ O	-	Distilled water
DNA	-	Deoxyribonucleic Acid
DMEM	-	Dulbecco's modified Eagle's medium
DR1	-	Direct repeat 1
DR2	-	Direct repeat 2
Enh	-	Enhancer
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme linked immunosorbent assay
FBS	-	Foetal bovine serum
<i>g</i>	-	Gravitational force
eGFP	-	Enhanced green fluorescent protein
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
HCC	-	Hepatocellular carcinoma
HEK293	-	Human Embryonic Kidney 293

List of Abbreviations

HBsAg	-	Hepatitis B surface antigen
HBV	-	Hepatitis B virus
HBcAg	-	Hepatitis B core antigen
Huh7	-	Human hepatoma 7
IFIT1	-	Interferon-induced protein with tetratricopeptide repeats 1
IFN	-	Interferon
IL	-	Interleukin
kDa	-	Kilo Dalton
LB	-	Luria Bertani
NTBC	-	2-(2-nitro-4-trifluoro-methylbenzoyl)1,3-cyclohexedione
MCS	-	Multiple Cloning Site
M _r	-	Relative Molecular weight
MCP-1	-	Monocyte Chemoattractant Protein-1
miR	-	microRNA
NMRI	-	Naval Medical Research Institute
NTCP	-	Sodium taurocholate cotransporting polypeptide
OAS-1	-	Oligoadenylate synthetase-1
ORF	-	Open reading frame
OD	-	Optical density
ORF	-	Open Reading Frame
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PEG	-	Polyethylene glycol
PG	-	Polyglutamate

List of Abbreviations

pgRNA	-	Pre-genomic RNA
Pol	-	Polymerase
PEI	-	Polyethyleneimine
Poly (I:C)	-	Polyinosinic:polycytidylic acid
PCR	-	Polymerase Chain Reaction
qPCR	-	Quantitative polymerase chain reaction
RNA	-	Ribonucleic acid
RNAi	-	RNA interference
RT-qPCR	-	Reverse transcription quantitative polymerase chain reaction
rcDNA	-	Relaxed circular DNA
SDS	-	Sodium dodecyl sulphate
TAE	-	Tris/Acetic Acid/EDTA
TE	-	Tris/EDTA
TALEN	-	Transcription-activator-like effector nuclease
TNF	-	Tumour Necrosis Factor
VPE	-	Viral particle equivalent

LIST OF SYMBOLS

α - Alpha

β - Beta

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Viral hepatitis occurs as a result of viral infection of the liver and concomitant inflammation and damage (Kumar et al., 2007). Viruses that cause hepatitis also known as hepatitis A, B, C, D, and E. Hepatitis A and E viruses typically spread through ingestion of contaminated food or water. Hepatitis B (HBV), C and D viruses spread parenterally and the most common mode of transmission is sexually and through use of contaminated needles and blood products (Thuener, 2017, Meng et al., 2002, Lu et al., 2003). Hepatitis B and D viruses may also spread through contact with other body fluids such as semen and infected blood (Mol et al., 2016). Perinatal HBV infection may also occur during the time of birth through contact with infected blood (Sun et al., 2012). Hepatitis A and E viruses typically cause only acute or short-term infections. Hepatitis B, C, and D viruses can cause acute and chronic infections (Riaz et al., 2011). Acute hepatitis is a short-term infection with symptoms lasting from several weeks to 6 months (Lee, 1997). Chronic hepatitis results from a long-lasting infection of over six months and can lead to high morbidity and mortality as a result of complications such as aggressive hepatitis, cirrhosis, hepatocellular carcinoma (HCC) and eventually liver failure (Cheng et al., 2012, Kulik and El-Serag, 2019). Hepatitis B and C viral infections are independent risk factors for the development of cirrhosis, a condition in which liver cells die, regenerate and results in scarring of liver (Tsai et al., 1994). Chronic HBV infection plays a significant role in liver damage (Suhail et al., 2014). Many individual infected by HBV develop liver cirrhosis or hepatocellular carcinoma, a cancer characterised by massive

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inflammation and fibrosis (Müller et al., 2020). HCC is the most common primary liver malignancy and is a leading cause of cancer-related death worldwide (El-Serag, 2020). Unlike other causes of chronic hepatitis, HBV is unique in that it can cause HCC without evidence of cirrhosis (Arora et al., 2013). HBV carriers have 10–25% lifetime risk of developing HCC (Crissien and Frenette, 2014). Thus, complications from hepatitis B are a major public health issue (Lim et al., 2012).

Globally, two billion people have been infected with HBV, with 257 million currently chronically infected (Al-Sadeq et al., 2019). About 700 000 people die each year as a result of HBV-related cirrhosis and HCC (El-Serag, 2012). HBV infection is particularly common in sub-Saharan Africa, east and south-east Asia, and the western Pacific islands, where complicating cirrhosis and HCC occur with high frequency. In South Africa, HBV infection is a critical problem, about 2.5 million people estimated to be chronically infected (Spearman and Sonderup, 2014).

HBV Infection in young children and adults vary prominently because children's immune system is not fully matured compared to adults (Guzman-Cottrill et al., 2013). Infection in children below the age of 1 year is usually asymptomatic and quite often leads to chronicity (Liang et al., 2009, Seeger and Mason, 2000). A patient is characterized as having Chronic HBV infection when hepatitis B surface antigen (HBsAg), established marker of HBV infection is detectable in the serum for period of more six months (Krajden et al., 2005). The impact of chronicity has been demonstrated to contrast between various genotypes and host attributes, for example, sex, males were more likely to have chronic when contrasted to female (Yang et al., 2010).

1.2 HBV genome organisation, structure, protein translation and replication

HBV is a prototype member of the family of hepatotropic DNA viruses, the Hepadnaviridae (Liaw and Chu, 2009). It has a partially double-stranded relaxed circular DNA (rcDNA) of approximately 3.2 kilobase pairs (kb) in size [(Fig 1.1) (Tuttleman et al., 1986)]. The DNA genome consists of four overlapping reading frames encoding the reverse transcriptase/polymerase (P), core (C), three surface proteins (S), the X protein (HBx) and the hepatitis B e antigen (HBeAg) (Tuttleman et al., 1986). Expression of HBV genes is controlled by four separate promoters, namely; the basic core, preS1, preS2 and X promoters (CHEN and OU, 1995). The *cis* enhancer elements, where the enhancer I (EnhI) is located upstream of the X promoter, and enhancer II (EnhII), located upstream of the basic core promoter (BCP), and they promote liver-specific viral gene expression (Doitsh and Shaul, 2004). The other elements include two direct repeats, namely DR1 and DR2, which play a significant role in the synthesis of plus DNA strand (Habig and Loeb, 2006). The *polymerase* Open Reading Frame (ORF) encodes for the DNA polymerase enzyme. The C ORF consists of the *precore* and the *core* regions, collectively called *precore*. *Core* region encodes HBV core antigen (HBcAg) which produce the viral capsid (Günther et al., 1996, Steven et al., 2005). HBcAg is an intracellular 21-kDa protein that assembles around the genome thereby encapsidating it, yielding the nucleocapsid. The *precore/core* region encodes for *precore* protein, which is processed to HBeAg which is a useful clinical marker of active viral replication. The *precore/core* mRNA encodes a hydrophobic signal peptide that guides the *precore* protein to the endoplasmic reticulum (ER), where it undergoes post-translational modification resulting in N- and C-terminal cleavage to release the 18-kDa

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monomeric HBeAg (Bruss and Ganem, 1991, Chen et al., 2005). The S ORF, which is divided into three regions with three different start codons but a single stop codon, encodes three proteins, namely: small, middle and large HBsAg (Prange and Streeck, 1995). The largest of these, the preS1 protein (large HBsAg or LHBs) is the product of translation initiation from the first start codon, while initiation at the second initiation codon produces preS2 or the middle HBsAg (or MHBs) (Heermann et al., 1984). Initiation at the third start codon produces the small HBsAg (SHBs or S) (Gerlich, 2013). The surface proteins collectively are important in production of new viral envelope which protects the nucleocapsid. The X ORF codes for the HBx protein which targets the cellular structural maintenance of chromosome (SMC) 5/6 complex for degradation and enables transcription from cccDNA. The HBx binds to the DBB domain of ubiquitin ligase 1 to render SMC5/6 unstable and subsequently facilitates HBV gene expression (Decorsiere et al., 2016, Murphy et al., 2016). The SMC5/6 complex is a limiting factor responsible for suppressing the expression from episomal HBV DNA (Decorsière et al., 2016, Niu et al., 2017).

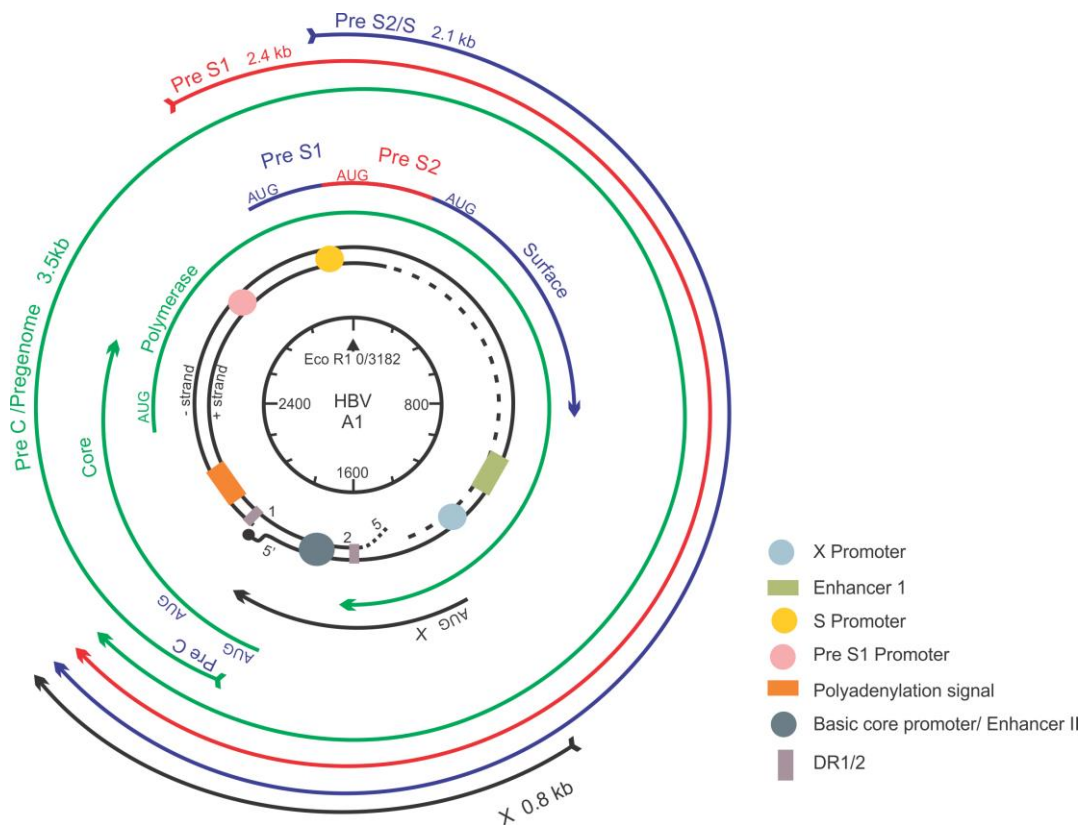


Figure 1.1: HBV genome organisation.

The internal circle represents the genome position relative to *Eco* RI. Partially double stranded genome is shown with RNA primer and polymerase protein attached. Open reading frames (ORFs) are indicated by the green, black and red coloured lines. The outer most black, blue and red coloured lines represent the viral RNA transcripts with shared polyadenylation site [adapted from (Moolla et al., 2002)].

HBV Infection of hepatocytes starts with interaction of the Dane particle with glycosaminoglycans situated on the cell surface (Sureau and Salisse, 2013). This is followed by the high affinity binding to the sodium taurocholate co-transporting polypeptide (NTCP) through the preS1 domain of the large surface antigen (Zhong et al., 2013). The virion then enters the hepatocyte through host factor-induced endocytosis (Urban et al., 1998). The viral nucleocapsid is released into the cytoplasm after endocytosis. The rcDNA-containing capsid is transported into the nucleus where it is disassembled to release the rcDNA (Will et al., 1987). This is followed by the repair of rcDNA to the covalently closed circular DNA (cccDNA)

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which serves as a template for transcription of the pre-genomic (pgRNA) and three subgenomic RNA transcripts (Zoulim and Locarnini, 2009).

The three subgenomic RNA transcripts are the 2.4 kb, 2.1 kb and 0.7 kb RNAs, which are translated respectively into envelope proteins preS1, preS2/S and X protein (Nassal, 2015). The pregenomic RNA is translated into the HBcAg and viral DNA polymerase (Quarleri, 2014, Yuh et al., 1992). The HBcAg form capsid around the pgRNA. Within the capsid, the pgRNA is reverse transcribed to the minus DNA strand (Habig and Loeb, 2003). As the pgRNA is reverse transcribed, the viral polymerase RNase H activity degrades it and a small RNA oligonucleotide remains. The small oligonucleotide translocates to the 3' end of the minus strand to its 5' end, it then functions as primer for synthesis of the plus strand (Summers and Mason, 1982). The DNA-containing mature nucleocapsid is either transported to the nucleus to form additional cccDNA or enters the ER where it acquires surface antigen containing envelope to form mature virions before being secreted from the cell by exocytosis (**Fig 1.2**) (Bartenschlager and Schaller, 1992, Will et al., 1987, Bartusch et al., 2017). Recently, a compartment of the late endocytic pathway, the multivesicular body (MVB), has been shown to participate in the final stages of HBV maturation and release. Internalization in vesicles is believed to induce fusion between the viral envelope and the cell-derived vesicular membrane, however the mechanism remains unknown (Tsukuda and Watashi, 2020). .

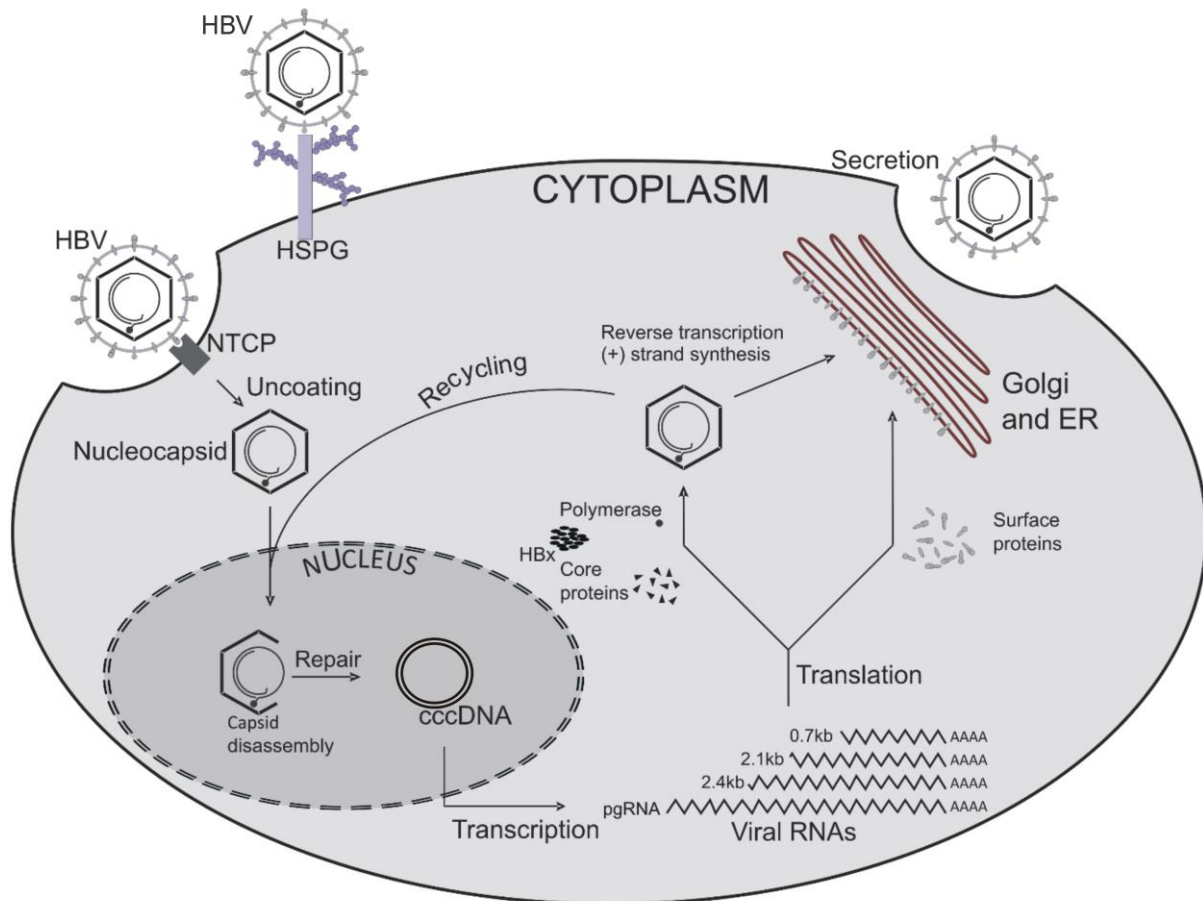


Figure 1.2: Schematic diagram showing Hepatitis B virus replication cycle.

Attachment of the hepatitis B virion to sodium-taurocholate co-transporting polypeptide (NTCP) and other receptors, heparan sulphate proteoglycans (HSPG) initiates infection process and entry through endocytosis. The nucleocapsid is transported to the nucleus, where the rcDNA is converted to covalently closed circular (cccDNA) which serves as the transcriptional template for subgenomic and pregenomic RNAs. The pregenomic RNA (pgRNA) is translated into the core protein and the viral polymerase. The subgenomic RNAs are translated into surface proteins and the X protein. The polymerase enzyme binds to the pgRNA and facilitates encapsidation. The pgRNA is reverse transcribed and the negative strand of DNA is formed, the negative DNA strand serves as a template for positive DNA strand synthesis. The mature nucleocapsid that contains rcDNA is formed. The nucleocapsid is recycled back into the nucleus and maintain cccDNA pools or binds to the endoplasmic reticulum, thereby receiving its envelope embedded with surface proteins. The HBV virion is secreted from the hepatocyte through exocytosis.

1.3 HBV genotypes

The viral polymerase lacks proofreading ability, resulting in high nucleotide sequence heterogeneity in the rcDNA (Sanjuán and Domingo-Calap, 2016). Hence the HBV

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genome has been estimated to evolve at an error rate of approximately 10^3 to 10^6 nucleotide substitutions/site/year (Osiowy et al., 2006). HBV has been classified phylogenetically into 9 genotypes dependent on an intergroup difference of more than 7.5% across the complete genome (Yu et al., 2010, Tatematsu et al., 2009, Norder et al., 2004). There is a putative tenth genotype, J, segregated from a solitary individual and anticipated to be a genotype C recombinant (Tatematsu et al., 2009). However, further characterisation of this genotype is required. Genotypes A-D, F, H, and I are grouped up to 35 subgenotypes based on intergroup nucleotide difference. The genotypes and other subgenotypes have different geographical spread and differ in clinical manifestation.

Genotype A is highly predominant in Africa, Europe, India, and America. Genotype A consists of subgenotype A1 and A2, whereby subgenotype A1 is prevalent in Africa and A2 is mainly found in Europe (Kimbi et al., 2004). Acute infection in children infected with genotype A is prone to chronicity (Lin and Kao, 2011) and HBeAg seroconversion occurs earlier when compared to genotype D (Kimbi et al., 2004). Infection with genotype A1 is also associated with high chances of developing HCC. This is mediated by deletions within the preS2 domain that harbors the T and B cell epitopes, hence facilitating immune escape and increasing the hepatocarcinogenic potential (Mak and Kramvis, 2020). When contrasted with subgenotype A2 and D3, subgenotype A1 bears sequence divergence within the BCP/Core protein region, which decreases the expression of HBeAg (Kramvis et al., 2018). Subgenotype A1 shares significant nucleotide sequence identity with other genotypes (Lago et al., 2014), however, the high sequence identity does not necessarily translate into similar clinical manifestation and response to treatment (Kramvis et al., 2018). This idea reinforces findings that nucleotide variability in

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precore/core (preC/C) sequences of subgenotype A1 and genotype D explains the difference in HBeAg expression and efficiency of pgRNA packaging (Ren et al., 2010).

Recently, it was demonstrated that genotype A and D responded poorly to tenofovir as compared to genotype C and B (Bannister et al., 2018). Genotypes B and C are the most common in the Asia-Pacific region (Okamoto et al., 1988, Tamada et al., 2012). HBV genotype C is associated with higher HCC risks than genotype B (Chan et al., 2004). Core promoter sequence divergence in HBV genotype C is associated with HCC (Chou et al., 2015). Basal core promoter A1762T/G1764A sequence variation and pre-S1 and S2 deletion is associated with higher viral load in patients infected with genotype C (Chen, 2016). Genotype D is prevalent in Africa, Europe, the Mediterranean region and India (Tatematsu et al., 2009). Acute infection with genotypes D leads in higher rates of chronicity than genotypes B and C (Lin and Kao, 2011). These distinctions in clinical manifestation and reaction to treatment might be because of subtle viral genomic sequence variation, even though host elements can also play part. Hence it is important to develop models of HBV disease that take into account various genotypes like in the current study where genotypes A1 and D3 are evaluated.

Genotype E to J appear to be more confined geographically (Magnius and Norder, 1995, Norder et al., 1994). Genotype E is limited to West and central Africa and Saudi Arabia. Genotype F is found in Central and South America (Huy et al., 2008). Genotype G has been reported in France, Germany and the Americas (Huy et al., 2008). Genotype H is found in Central America and Mexico (Magnius and Norder, 1995). Genotype I was isolated in Vietnam and Laos. Genotype J was identified in Japan (Tatematsu et al., 2009).

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1.4 Current anti-HBV vaccines and therapies

1.4.1 HBV vaccines

There is a universal and an effective recombinant HBV vaccine which provides 90-95% efficacy in vaccinated individuals (Margolis, 1998). SA was amongst the first 10 countries that participated and implemented a universal vaccine, however despite the high risks of perinatal and early childhood transmission, the birth dose has not been administered (Kane, 1995, Spearman and Sonderup, 2014). Despite availability of a vaccine, it is not effective in breaking perinatal infection once chronic infection is established. This factor contributes to high mortality rate associated with HBV infection. Reduced response to vaccine has also been observed in immunocompromised individuals (Spearman and Sonderup, 2014).

1.4.2 Anti-HBV drugs

Several people with acute HBV infection usually recover without treatment, but patients with chronic hepatitis B require anti-viral treatment. Two classes of anti-HBV drugs on the market used to treat chronic infection are Nucleot(s)ide analogues and immunomodulators. The five Nucleot(s)ide analogues approved by the US Food and Drug Administration are lamivudine, adefovir, tenofovir, telbivudine and entecavir (Tao et al., 2020). Lamivudine is a commonly used nucleoside analogue and the first line treatment for chronic hepatitis B, particularly in developing countries (Dienstag et al., 2003, Lai et al., 1998, Yuen et al., 2005). Nucleot(s)ide analogues are reverse transcriptase inhibitors that function by disrupting viral replication, reducing hepatic cccDNA but not eliminating it (Grimm et al., 2011). As a result of HBV error prone DNA replication mechanism, drug-resistant HBV mutants, particularly to lamivudine, frequently arise leading to treatment failure and progression to liver disease (Zoulim,

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2011). The approved immune modulators are IFN- α and its pegylated form PEG-IFN- α . IFN- α and PEG-IFN- α stimulate the host's immune system to clear the virus. The drawbacks of using interferon-based therapies are that only a small number of individuals, approximately 40% of treated patients, exhibit long-term clinical benefits and the drug causes significant side effects and is expensive (Ryan et al., 2015). These limitations necessitate the development of novel anti-HBV therapies.

1.4.3 Gene therapy

Use of gene therapy as a novel strategy to incapacitate HBV replication has demonstrated potential and created substantial curiosity. These gene therapies include the use of gene silencing and gene editing technologies such as RNA interference (RNAi) (Ely et al., 2009, Ely et al., 2008a), transcription activator-like effector nucleases (TALENs) (Bloom et al., 2013) and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system (Scott et al., 2017). Recently, the use of RNase H inhibitor, Nucleocapsid assembly and core inhibitors, HBsAg release inhibitors and NTCP inhibitors have gained popularity as curative approach to target HBV. Currently available RNase H inhibitors include α -hydroxytropolones (Cai et al., 2014); Nucleocapsid assembly and core inhibitors include core protein allosteric modifiers (CpAMs) (Fung and Lok, 2004); HBsAg release inhibitors include Nucleic acid polymers (NAPs) (Schöneweis et al., 2018) and the NTCP inhibitors include Myrculudex. Some of the inhibitors have progressed to clinical trials (Maepa et al., 2021). This study focuses on the RNAi-based therapeutics, which are the most explored with some candidates having reached clinical trials (Springer and Dowdy, 2018, Yuen et al., 2019, Huang, 2017).

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RNAi is a naturally occurring post-transcriptional gene silencing mechanism present in eukaryotic cells (Ngo et al., 1998, Lohmann et al., 1999, Kennerdell and Carthew, 1998). The most understood and explored RNAi pathway for gene therapy is the microRNA (miR) pathway. It starts in the nucleus, whereby a miR-encoding gene is transcribed into multi stem loop primary miR (pri-miR) that carries the sequence which is homologous to the target sequence. This is followed by cleavage of the pri-miR by the nuclear microprocessor complex comprising DROSHA and DiGeorge critical region 8 (DGCR8) to form precursor miR (pre-miR) (Bernstein et al., 2001). Subsequently, the RAN-GTP-dependent exportin transports the pre-miR through the nuclear pore to the cytoplasm and it is then processed into miR duplex by the RNase III enzyme DICER (Bohnsack et al., 2004, Yi et al., 2003). The miR duplex is then incorporated into a cytoplasmic RNA-induced silencing complex (RISC) and the guide strand is selected (Han et al., 2004). The single strand from the duplex is selected to guide the complex to the cognate RNA sequence (Bartel, 2004, Hammond et al., 2000, Lim et al., 2005, Martinez et al., 2002). When the guide strand achieves full hybridisation the RNA is degraded, whereas if partially bound translation is inhibited (McCaffrey et al., 2003, Choi et al., 2015, Hutvagner and Zamore, 2002). The RNAi pathway can be manipulated by introducing artificial sequences resembling RNAi intermediates into the cell to reprogramme the pathway and result in targeted gene silencing (Wang and Seeger, 1992). This can be achieved by introducing promoter-driven pri-miR or pre-miR mimics or synthetic short interfering RNAs (siRNAs) that mimic the miR duplex (Giladi et al., 2003, Klein et al., 2003, Hamasaki et al., 2003, Konishi et al., 2003). RNAi-based therapies have shown promising outcomes against other viral infections such as HIV-1 infection

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(Novina et al., 2002, van den Berg et al., 2020), human papillomavirus and human cytomegalovirus (HCMV) (Xiaofei et al., 2012).

Features of HBV that make RNAi-based therapy a suitable anti-HBV strategy are the overlapping ORFs of the viral genome and the single transcriptional termination signal that produces transcripts with similar 3' ends. This means that a single RNAi trigger can result in the degradation of all viral messenger RNAs and pregenomic RNA if that trigger is directed to the 3' end.

Several expressed and synthetic RNAi activators have been used to directly target HBV transcripts with high specificity, profoundly reducing the production of viral proteins and HBV viral replication (Giladi et al., 2003). Studies, both *in vitro* and *in vivo*, have demonstrated that HBV replication can be inhibited by synthetic siRNA duplexes. However, siRNA result in transient therapeutic effects (Marimani et al., 2013). Unlike synthetic siRNAs, expressed RNAi activators result in persistent gene silencing, hence they are better candidates for targeting chronic HBV infection (Maepa et al., 2017). Though efficient HBV gene silencing by short hairpin RNAs (shRNAs), mimics of pre-miR expressed from RNA polymerase (Pol) III promoters have been reported, constitutive transcription may result in harmful side effects (Grimm et al., 2006). Using known pri-miR sequences, artificial pri-miRs (apri-miRs) have been developed. Use of apri-miRs is very useful because it allows expression from RNA polymerase II promoters, which enable better transcriptional control of RNAi activators (Ely et al., 2009). Moreover, the pri-miRs exist as polycistronic sequences in nature, this feature may be adapted to produce multi-targeting miRs from a single DNA cassette (Ely et al., 2009). Expressed RNAi activators are compatible with viral vectors such as adenoviral vectors (Ad) and adeno-associated

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viral (AAV) vectors, which enables efficient delivery into the liver (Maepa et al., 2017, Mowa et al., 2014).

Recent approval of patisiran, an RNAi-based drug for treatment of hereditary transthyretin amyloidosis raises hope for RNAi-based therapies in the fight against HBV infection (Solomon et al., 2019). These RNAi-based therapies may play an important role in future therapeutic regimens aimed at improving HBsAg seroclearance and eliminating the need for lifelong therapy. To advance RNAi-based therapy against HBV infection, animal models that can recapitulate HBV infection and replication are important. Improved understanding of the molecular biology of HBV replication has been beneficial but establishing a suitable and convenient model that reproduces all stages of HBV infection remains a challenge.

HBV genotype is not only predictive of clinical outcomes but has also been associated with response to antiviral therapy. Patients infected with genotype A or D respond better to interferon-based therapy compared to patients infected with either genotype C or B (Kramvis, 2014). Furthermore, when a gene therapy tool such as RNAi was employed to knockdown HBV, some discrepancy was observed amongst different HBV genotypes. For example, in a study that aimed at evaluating efficacy of expressed shRNAs against genotypes A-H, twenty one shRNAs were designed to target conserved regions (Sun et al., 2010). Six of the shRNAs were highly effective and in some cases could tolerate mismatches between the guide and target. One of the effectors, sh10, reduced viral DNA by $\geq 95\%$ despite having one mismatch in targets of genotypes A, B and E and two mismatches to cognates of genotype G and H. This efficacy equaled that of silencers with perfectly complementary targets. In another study, forty shRNAs were designed to target conserved sequences of genotypes A-I (Zhang et al., 2010). Four shRNAs were effective against genotypes

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A-D and I, and inhibited HBV gene expression by up to 90%. However, the inhibitory efficacy of these shRNAs varied significantly against the different genotypes.

Using polycistronic RNAi activators, which produce multiple guides, may be a useful means of overcoming problems of silencing varied sequences with one effector (Ely et al., 2009). Overall, it is clearly important that anti-HBV therapy regimens be tested in the panel of described HBV genotypes to be able to achieve broad efficacy in most chronic carriers of HBV.

1.5 Models of HBV infection and replication

Investigations of HBV biology and development of novel therapies relies on suitable cell culture and animal model systems that efficiently support virus infection and/or replication. Such systems should ideally provide conditions that resemble those of natural host cells, such as the cell-type specific signalling and metabolic pathways. There are several cell culture and animal models which have been developed for screening of new therapies and the study of HBV infection, replication, and gene expression.

1.5.1 Cell culture models of HBV infection

1.5.1.1 Primary human hepatocytes

Primary human hepatocytes are the most used culture system for HBV infection studies as they support the complete replication cycle of HBV (Hu et al., 2019, Rumin et al., 1996, Gripon et al., 1988). Studies that demonstrated that culturing of an adult primary human hepatocytes in the presence of dimethyl sulfoxide (DMSO) render the cells susceptible to HBV infection represented the original proposition of an *in vitro* cell line capable of recapitulating HBV infection (Gripon et al., 1988, Galle

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et al., 1994). Since then, another *in vitro* system based on primary human foetal hepatocytes has also been described to support HBV infection. These cells were reported to have greater viability and proliferative capacity than adult primary hepatocytes (Ochiya et al., 1989). The experimental processes for infecting primary cell lines have also been improved by addition of PEG in cell cultures maintained in the presence of 2 % DMSO (Gripon et al., 1993, Ortega-Prieto et al., 2018).

The recent development of 3D microfluidic systems for primary human hepatocytes culturing allowed maintenance of HBV infection capability for 40 days and at low multiplicity of infection (MOI) without the need for PEG or DMSO (Ortega-Prieto et al., 2018). Despite these exciting developments, primary human hepatocytes have a shortcoming in that they undergo rapid differentiation within few days of isolation. This has to be circumvented by co-culturing with non-parenchymal cells that enables maintenance of the human foetal hepatocytes features (Zhou et al., 2014). However, they appear to be unstable *in vitro*, resulting in limited durability of infection (Witt-Kehati et al., 2016). Furthermore, the cells are scarce, expensive, do not proliferate in culture, have a limited life span of 1 month or shorter, vary among batches, and have low infection efficiency, limiting their common use (Zhou et al., 2014).

1.5.1.2 Immortal human liver cell lines

Even though primary human hepatocytes have been applied as the gold standard for HBV infection, challenges associated with their use resulted in several studies resorting to immortal liver cell lines. The HuS-E/2 immortalised cell line represents one of the cell lines that are closely related to the human primary hepatocytes. They are a derivative of human primary hepatocytes expressing human telomerase reverse transcriptase (hTERT) and human papillomavirus (HPV) proteins (E6 and

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E7). HuS-E/2 differentiation is induced by addition of DMSO, and the cells become susceptible to HBV infection and results in HBV replication for 28 days (Huang et al., 2012a).

Recently, human hepatocytes-like cells (HLCs) derived from either human embryonic stem cells (hES) or induced pluripotent stem cells (iPCs) have been used for HBV infection (Xia et al., 2017, Nie et al., 2018). However, hepatocytes derived from stem cells carry mixed populations of mature and foetal hepatocytes that express α -fetoprotein with minimal levels of the NTCP (Sakurai et al., 2017). This affects the formation of cccDNA and results in short-term HBV infection (Sa-ngiamsuntorn et al., 2019).

Well-characterised and commonly used immortalised liver cell lines include hepatoma derived HepaRG, hepatoblastoma derived HepG2 and hepatocellular derived Huh 6 and Huh 7 cell lines.

1.5.1.3 HepaRG cell line

The HepaRG human liver cell line is derived from a hepatoma in a patient infected with hepatitis C virus (Gripon et al., 2002, Hantz et al., 2009). Upon differentiation, the HepaRG cell line resembles primary human hepatocytes with respect to hepatocyte-specific markers such as the expression of cytochrome P450 enzymes, liver-specific transcription factors, and transporter proteins like NTCP (Ni and Urban, 2017a). This cell line has been shown to be susceptible to HBV only when cultures are propagated and maintained in the presence of corticoids and DMSO (Gripon et al., 2002). HepaRG cell line exhibits lower susceptibility to HBV infection when compared with primary human hepatocytes cells (Ni and Urban, 2017a, Gripon et al., 2002). This limitation can partially be overcome by constitutive expression of the

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NTCP (Ni and Urban, 2017a). In differentiated HepaRG cells, HBV infection is characterised by long-term persistence without cccDNA amplification (Hantz et al., 2009). The close resemblance of the cells to normal human hepatocytes represents a suitable tool to model HBV infection/replication (Gripon et al., 2002, Hantz et al., 2009). The main drawback limiting the use of the HepaRG cell line is that HBV infection is inefficient even with high MOI and cell differentiation is time consuming (requires 4 weeks) (Verrier et al., 2016). The cells are also subject to licensing to use, thus also limiting common use.

1.5.1.4 HepG2 and Huh6/7 cell lines

The hepatoblastoma derived (HepG2) and hepatocellular carcinoma derived (Huh6 and Huh7) cell lines represent the most commonly used *in vitro* systems to model HBV replication and transcription (Sureau et al., 1986, Bchini et al., 1990, Mabit et al., 1994, Witt-Kehati et al., 2016). These human hepatoma cell lines are not susceptible to HBV infection but the greater-than-genome-length HBV can be introduced into the cells by ectopic integration or transfection (Sureau et al., 1986, Sells et al., 1987a). Upon transfection, the cells are able to support viral production and replication leading to secretion of virions that are virologically and morphologically indistinguishable from the viral particles produced from the infection of HBV (Guha et al., 2004, Hu et al., 2019). The cells are relatively stable genetically and virtually unlimited in supply, making them convenient systems as models of HBV replication. However, the cells lack the HBV hNTCP receptor and are unable to elicit innate immune response (Shen et al., 2018).

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1.5.1.5 NTCP expressing- HepG2 and Huh cell lines

The identification of the NTCP receptor and the demonstration that NTCP expression in nonpermissive liver-derived cells confers HBV susceptibility resulted in several studies developing NTCP based *in vitro* HBV infection models (Iwamoto et al., 2014, Qiao et al., 2018, Zhou et al., 2019, Ni and Lempp, 2014). The HepG2 cell line expressing the NTCP (HepG2-hNTCP-C4) has since been commonly used as a tool to model HBV infection (Iwamoto et al., 2014, Qi et al., 2016, Sun et al., 2017). Huh7 cells have also been engineered to express human NTCP (Huh7D^{hNTCP}). DMSO treatment of Huh7D^{hNTCP} results in rapid transformation into a non-dividing, well-differentiated polarised phenotype, thus supporting HBV infection (Zhou et al., 2017). These cell lines have been heavily used as *in vitro* models that facilitate discovery and development of antiviral drugs against HBV infection (Durantel et al., 2004, Li et al., 2014, Iwamoto et al., 2014, Xiao et al., 2019). NTCP-expressing HepG2 or Huh 7 cells are capable of recapitulating the whole HBV replication cycle, however, a high MOI is required to achieve infection (Miyakawa et al., 2018).

The HepAD38 is a widely used HepG2-derived cell line carrying HBV pgRNA encoding sequence under the control of a tetracycline-inducible promoter. Induction of viral pgRNA expression results in the accumulation of subviral particles in the cytoplasm containing DNA intermediates, characteristics of viral replication, followed by secretion of viral particles in the supernatant (Ladner et al., 1997). The HepAD38 cells produced and accumulated cccDNA (Zhou et al., 2006). This phenomenon was exploited in the development of high throughput assay for screening novel antiviral drugs targeting HBV cccDNA using HBeAg as a reporter protein (Ladner et al., 1997). Since the HepAD38 cell line can produce high levels of HBV DNA, it is useful for analyses of the viral replication cycle that depend on viral DNA synthesis in a

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synchronised fashion. In addition, this cell line has been used in the development of a high-throughput, cell-based assay that permits the large-scale screening of diverse compound libraries for new classes of inhibitors of HBV replication.

1.6 Animal models

Although a large number of established *in vitro* systems have been instrumental in understanding HBV biology and drug discovery, these systems have various maintenance and higher economical limitations and more importantly, they do not recapitulate the *in vivo* environment. Hence the establishment of animal models that support HBV replication are essential. Both non-human primate and non-primate models of HBV infection have been described.

1.6.1 Non-human Primate Models

Chimpanzees are the only primate animals other than humans that are fully susceptible to HBV infection (Guidotti et al., 1999). These animals develop acute and chronic HBV infection and their immune response is similar to that observed in humans (Maynard et al., 1972, Barker et al., 1975, Eren et al., 2000, Wieland, 2015b). The chimpanzee model has been used widely to elucidate molecular mechanism by which host cells clear HBV infection (Wieland, 2015b, Wieland et al., 2004), the effect of the viral inoculum size on the kinetics of viral spread and immunological priming (Asabe et al., 2009), and in the evaluation of potential therapeutic agents for treatment of HBV infection (Eren et al., 2000, Lanford et al., 2013). A number of recombinant antigens have been established as adequate protective vaccines against HBV infection following extensive evaluation in the chimpanzee model (McAuliffe et al., 1980, Pancholi et al., 2001, Wieland, 2015b).

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Rhesus macaques (*Macaca mulatta*) are naturally not permissive to HBV infection. Hence few studies have used viral vectors to deliver HBV genome into macaques or primary macaque hepatocyte cells. Recently, a helper-dependent adenoviral (HDAd) vector was used to mediate expression of human NTCP (hNTCP) in rhesus macaques. This rendered the animals infectable by HBV and supported HBV replication for a period of 6 weeks (Burwitz et al., 2017a). This has shown to be a promising HBV infection model, however further optimisation is required to ensure long-term HBV expression. Interestingly, a recent study reported naturally occurring transmissible chronic HBV infection among cynomolgus macaques (*Macaca fascicularis*). Whole HBV genome-sequencing data analyses revealed that the *M. fascicularis* HBV had 99% sequence identity to genotype D subtype ayw3 clinical isolate and carried a substitution in position 67 of preS1. It was then predicted to be transmitted from human to *M. fascicularis* (Dupinay et al., 2013). These results represent the biggest leap in the development of macaques for *in vivo* modelling of HBV infection and replication. However restrictions on use of primates, high costs and ethical concerns have limited their use as a preclinical model (Wieland, 2015a).

1.6.2 Tree shrew Model

Because tree shrews (*Tupaia belangeri*) possess many features valuable in biomedical research, attempts have been made to employ them as models for HBV infection (von Freyend et al., 2011). The primary hepatocyte cells from the northern tree shrew were shown to be susceptible to HBV infection *in vitro* and *in vivo*. However, infection of the tree shrew with HBV causes mild and transient infections with low viral titres (Zhao et al., 2013). Furthermore, tree shrews are difficult to handle in captivity and are not easily available.

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1.6.3 Animal models of HBV related viruses

Models based on HBV-related viruses, like the duck and woodchuck models have been developed for HBV studies. The duck HBV (DHBV) genome has a 40% homology to HBV genome (Schaefer, 2007). The DHBV model played an important role in elucidating HBV replication mechanisms (Shih et al., 2015). However, significant differences exist between the two viruses. DHBV, for example, uses the carboxypeptidase (DCPD) and not the NTCP as a receptor for viral entry (Dandri and Petersen, 2017). This makes the DHBV model a poor substitute to model HBV infection. The genome organisation of woodchuck hepatitis virus (WHV) is closely related to HBV (Dandri and Petersen, 2017). However, the mechanism of hepatocarcinogenesis differs between woodchucks and humans, and there is no HBeAg seroconversion and associated reduction of viral replication in chronically infected woodchucks. Therefore, the accuracy of extrapolation from woodchucks to human may be difficult (Ferrari et al., 1990, Hsu et al., 1992). Woodchucks are difficult to breed and maintain in captivity. Furthermore, the 35% nucleotide differences between HBV and WHV constitute significant challenges for evaluating the efficacy of anti-HBV drug and vaccine candidates (Sprengel et al., 1985).

1.6.4 Mouse models of HBV infection

Several mouse models of HBV have been established, and novel mouse models that enable assessment of the viral infection process and efficacy of antiviral agents are being developed continuously. The advantages of using the mouse model include ease of genetic manipulation, low cost and wide availability.

1.6.4.1 Chimera mouse model

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Chimeric mouse models with a humanised liver provide a unique tool to study hepatic diseases and develop antiviral therapy. The first human chimeric model to be developed was the HBV trimera mouse model (Ilan et al., 1999). In this model, the fragments of human liver infected with HBV are transplanted beneath the kidney in lethally irradiated severe combined immunodeficiency (SCID) mice. Use of this model is limited by short-term expression of HBV genes of up to six weeks and difficulty in maintaining the functions of hepatocytes for long periods of time [(Sun and Li, 2017).

The other two human chimeric mouse models are the urokinase-type plasminogen activator (uPA) transgenic mice (Heckel et al., 1990) and the fumarylacetoacetate hydrolase (Fah) knockout mice. The uPA transgenic mouse model was generated by first expressing a toxic uPA under the albumin (alb) promoter, which then induces subacute liver failure in newborn mice (McKenzie et al., 2008). The uPA mice were then backcrossed with immune deficient mice such as Severe combined immunodeficiency (SCID) or recombinant activating gene 2 (Rag2) knockout mice (Meuleman et al., 2005). This is followed by human hepatocytes introduction through intra-splenic injection, which can then grow in the liver. Currently, this model is used to evaluate genotype-dependent differences in HBV gene expression and replication. However, the fact these animals are fragile to work with as they become infertile and are susceptible to potentially fatal bleeding, and kidney disorders makes it difficult to use this model (Mercer et al., 2001, Dandri et al., 2001).

To overcome the challenges associated with uPA mouse model, the Immune-deficient NOD-Rag1^{-/-}IL2RgammaC-null (NRG) Fah Knockout (FRG) mouse model was developed by crossbreeding the Fah^{-/-} mice with Rag2^{-/-}Il2rg^{-/-} mice (Azuma et al., 2007, Li et al., 2017, Bergeron et al., 2001). The deficiency of FAH, an enzyme

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which functions in the tyrosine catabolic pathway, results in accumulation of toxic fumarylacetoacetate metabolite which induce liver injury (Vogel et al., 2004). In this model, human hepatocytes are engrafted through intrasplenic injection, after they are injured by urokinase-expressing adenovirus. The engrafted human hepatocytes can then proliferate in the FRG mouse liver (Allweiss et al., 2016). Nevertheless, because of their severe immunodeficiency, they cannot be used for immunopathogenesis and vaccine development studies (Bility et al., 2013, Winer et al., 2017). Recently, a human liver chimera mouse model based on the engraftment of hepatocyte-like cells (HLCs) generated from human induced pluripotent stem cells (hiPSCs) was developed and used to study HBV infection (Paganelli et al., 2013, Helsen et al., 2016, Ni and Urban, 2017b). The model was developed by engrafting the hiPSC-HLCs into *Fah^{-/-}Rag2^{-/-}IL-2R γ ^{-/-}* SCID (FRGS) mice. The absence of the *Fah* gene leads to accumulation of toxic metabolites in mouse hepatocytes causing these cells to die off. This allows the injected human hepatocytes to repopulate in the mouse. *Fah* was used to enhance hepatic maturation and maintain the hepatic differentiation state of hiPSC-HLCs (Yan et al., 2017, Xia et al., 2017). The model supported long-term HBV infection; however, its use is also limited by its immunodeficient nature, limiting its application in immunopathogenesis and vaccine development studies.

1.6.4.2 Transgenic mice carrying HBV genes

Transgenic mice expressing individual HBV genes have been developed mainly to evaluate the function and oncogenic potential of each HBV protein. For example, the microinjection technique was used to introduce HBV DNA fragments into fertilised mouse eggs to produce transgenic mouse models expressing the envelope proteins (Chisari et al., 1986). As a result of an inefficient process to secrete the HBV

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proteins, the envelope proteins in this transgenic mouse model result in the accumulation of HBsAg in the hepatocyte ER. As the ER expands due to progressive accumulation of envelope proteins, the hepatocytes become enlarged, undergoing coagulative necrosis with the mice developing hepatocellular hyperplasia. This is characterised by focal hepatocellular degeneration and necrosis, lobular macrophagic inflammation, and increased serum transaminase activity, eventually resulting in hepatocellular carcinoma (Chisari et al., 1987).

The use of transgenic mouse model has led to the discovery of HBV X protein being responsible for liver tumourgenesis. The expression of HBV X protein in transgenic mice induces liver damage that ends in hepatocarcinogenesis (Koike et al., 1994, Slagle et al., 1996). The tolerogenic potential of the HBV precore and HBcAg proteins was examined using HBcAg/HBeAg-expressing transgenic mice crossed with T cell receptor transgenic mice expressing receptors for the HBcAg/HBeAg. The study revealed the potential role of HBeAg as the protein that biases virus-host interaction toward chronicity by down-regulating host T-cell responses to nucleocapsid antigens (Chen et al., 2005).

Studies have progressed to transgenic mouse models carrying the complete HBV replicons. These HBV transgenic mouse models involve the integration of a replicative linear HBV DNA into the host genomic DNA. Microinjection of HBV genomes into mouse embryos resulted in development of transgenic mouse lineages that enabled high level replication of HBV (Marion et al., 2003). Noteworthy was the observation that expression of HBV genes could occur in both the liver and kidney, implying that HBV replication is not tissue-specific upon bypassing viral entry (Ge et al., 2004). This model created the opportunity to examine the influence of viral and host factors on HBV pathogenesis and to assess the antiviral potential of

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pharmacological agents (Gao et al., 2004). The model was used to demonstrate that acute viral hepatitis B is cleared through noncytopathic antiviral mechanisms involving purging HBV replicative intermediates from the cytoplasm and the covalently cccDNA from the nucleus of infected cells (Chisari et al., 1985, Chisari et al., 1987). Furthermore, this transgenic mouse model has been widely used to consider the adequacy of novel antiviral quality therapeutics against hepatitis B in the liver (Carmona et al., 2006, Maepa et al., 2017).

Although transgenic mice are the most commonly used animal model and have generated most of the information available in HBV biology and antiviral drug developments, they are not without shortfalls. Viral entry and spread cannot be studied in transgenic mice, as HBV does not infect mouse hepatocytes. Also, transgenic mice show variability in HBV replication from mouse to mouse, HBV replication markers varies over time in the same mouse and the markers of HBV replication are usually too high. The transgenic mice are immunologically tolerant to viral antigens, hence do not mimic the hostile immune pressure the virus experience in humans (Inuzuka et al., 2014).

1.6.4.3 HBV genome episomes-bearing mouse models

To overcome some of the limitations associated with the use of transgenic mice, vectors have been used to introduce HBV genome in the livers of mice. Notable vectors used to transfect HBV DNA genome into the mice liver are the naked plasmids introduced by hydrodynamic transfection and viral vectors. The current study adopts the latter approach where adeno-associated viral vectors mediate HBV gene expression .

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1.6.4.4 Hydrodynamic transfection of HBV DNA

Hydrodynamic injection (HDI) creates a local, high-pressure environment to transfect various tissues with plasmid DNA and other substances. Hydrodynamic tail vein injection, for example, is a well-established method by which the liver can be transfected (Sells et al., 1987b, Sells et al., 1988). The naked HBV replicative DNA was introduced into mice liver through injecting a huge volume into the tail vein of mice (Yang et al., 2002, Huang et al., 2006). HDI of plasmid carrying HBV genome into the mouse liver results in high levels of RNA and DNA HBV replicative intermediates (Liu et al., 1999). The injection can be performed in immunocompetent mice; therefore, host immune responses against HBV can be observed (Yang et al., 2002, Huang et al., 2006). Although the model can be used for studying the pathogenesis of HBV and its immune response, it results in transient liver damage as a result of high-pressure injection, hence is not a good model of HBV chronic infection.

1.6.4.5 Adenovirus vectors for HBV DNA transfection

Adenoviral vectors have been extensively used as a gene transfer tool because of its high transduction efficiency both *in vitro* and *in vivo* (Shayakhmetov et al., 2004). Natural tropism for the liver makes adenoviral vectors good candidates for liver targeted gene transfer and most relevant for HBV infection studies. Adenoviral vectors carrying a HBV genome (Ad-HBV) were proven to deliver HBV genome in the liver of mice and results in efficient HBV gene expression (Huang et al., 2012b, von Freyend et al., 2011). The intravenous injection of HBV genome containing adenoviral vectors results in HBV replication for a period of three months. However, transduction of Ad-HBV in mice results in a strong immune response against the

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adenoviral vectors (Sprinzl et al., 2001). This makes the use of Ad- HBV model difficult to screen the effect of anti-HBV therapeutics.

1.6.4.6 Adeno-associated viral vectors for HBV DNA transduction

Adeno-associated virus (AAV) based vectors are currently one of the safest and the leading platform for gene delivery for the treatment of a variety of human diseases. The efficacy of these vectors have been well demonstrated, for example, Glybera is the first AAV based gene therapy product to be taken into the market to treat lipoprotein lipase deficiency (Büning, 2013, Ylä-Herttuala, 2012). Recently, AAV-mediated gene delivery for treatment of cardiovascular, muscular, metabolic, neurological, haematological and ophthalmological diseases have reached clinical trials (Li and Samulski, 2020).

AAVs belong to the genus *Dependoparvovirus* within the family Parvoviridae. AAV serotypes (AAV1-AAV12) differ broadly in transduction efficiencies and tissue tropisms and thus hold enormous potential as vectors for human gene therapy. AAV was originally discovered as a cell culture contaminant (Atchison et al., 1965) and the well characterised serotype AAV2 serves as a prototype (Gao et al., 2004). Amongst the AAV serotypes, AAV2 and AAV8 serotypes have been the most commonly used as vectors and proven to have a liver specific tropism.

AAV is a single stranded DNA parvovirus with a genome comprising of *rep* and *cap* genes flanked by two inverted terminal repeats (ITRs) [Fig 1.3 (Srivastava et al., 1983)]. The 125 nucleotides sequence of the ITRs contain palindromic sequences (A-A', B-B') and single non-palindromic region (D), that form internal T-shaped hairpin structures that serve as a primer during DNA replication. The 20 bases D sequence remain unpaired and is important for genome packaging (Fig 1.3). The

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ITRs contain the Rep binding site (RBS) and the terminal resolution site (TRs) which are important for replication and packaging of AAV genome (Koczot et al., 1973). The four proteins are encoded by *rep* gene, namely: Rep78, Rep68, Rep50 and Rep42. Their expression is driven by p5 and p15 promoters and are essential for viral genome replication (Gaj et al., 2013). The rep protein quartet has helicase and ATPase activity. The rep 78 and 68 exercise a specific binding activity site within the genome ITRs rep binding element (RBE) position and a site-specific endonuclease activity at the terminal resolution site (trs) which are required for AAV genome replication, packaging, chromosomal integration, and rescue (Nony et al., 2003). The p40 promoter is located adjacent to the left ITR and it drives expression of four overlapping polypeptides viral proteins VP1, VP2, VP3 and assembly activating protein (AAP) from the *cap* gene (**Fig 1.4**). The VP1, VP2 and VP3 proteins exist in a 1:1:10 molar ratio on the capsid (Daya and Berns, 2008). The assembly activating protein (AAP) is translated from an alternative ORF within the *cap* gene with a non-canonical CTG start codon . The AAP is essential, it directs the capsid proteins to the nucleus and also function in assembling the viral capsid.

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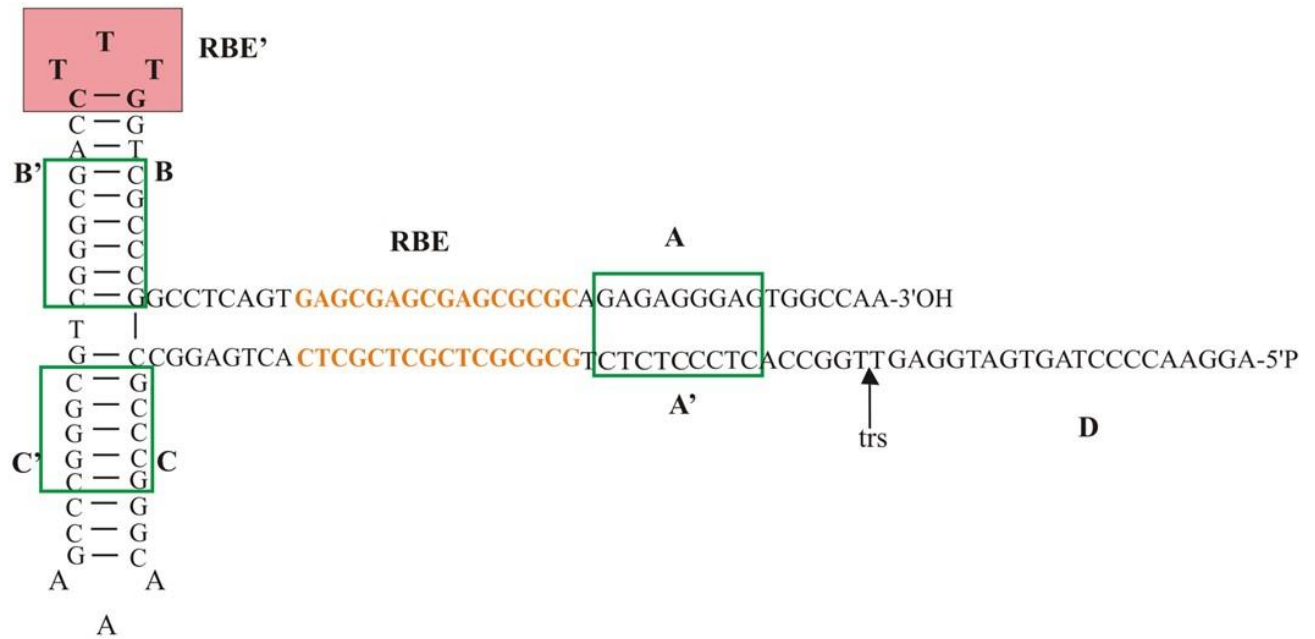


Figure 1.3: Mapping sequences of the wild type AAV2 inverted terminal repeat (ITR).

The first 125 nucleotides of the ITRs contain palindromic sequences (A-A', B-B' and C-C', green boxes) that form internal T-shaped hairpin structures that serves as a primer during DNA replication. The other 20 bases remain unpaired and are called the D sequence important for genome packaging. The Rep binding element (RBE, coloured orange) allows large Rep protein binding to exercise endonuclease activity on the terminal resolution site (black arrow). Whereas RBE' at the tip of T-structure (highlighted in red) functions to stabilises large Rep protein interaction with ITR. Adapted from (Berns, 2020)

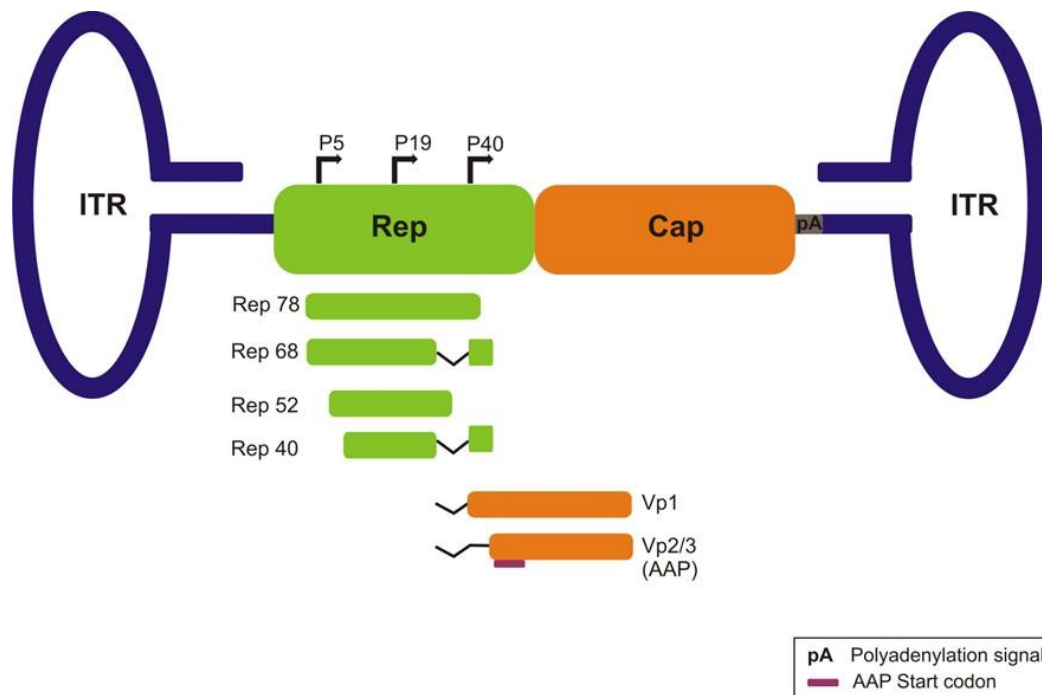


Figure 1. 4: Schematic representation of wild type AAV-2 genome structure and transcription.

The *rep* and *cap* genes are flanked by inverted terminal repeats (ITRs) with poly A signal downstream of the *cap* gene. The p5 and p19 promoters drive the transcription of the *rep* gene, whereas p40 promoter initiates the expression of the *cap* gene. The p5 promoter initiates the transcription of the rep 78 and rep whereas the rep 52 and rep 40 proteins are transcribed from the p19 promoter. The p40 promoter is responsible for transcription of The VP1,VP2 and VP3 structural proteins [adapted from (Samulski and Muzyczka, 2014)].

The AAV replication cycle occurs in two forms : the lytic and lysogenic cycle. AAV2 enters the cells through binding to two receptors namely: heparin sulphate proteoglycan receptor and co-receptor fibroblast growth factor receptor (Daya and Berns, 2008). The avβ5 has been identified as an additional cellular molecule, which participated in viral attachment and internalisation (Qing et al., 1999). Thereafter, the AAV is endocytosed through clathrin-coated vesicles (Duan et al., 1999, Joo et al., 2011, Liu et al., 2013). In the absence of helper virus, there is limited expression of Rep78/68 and viral replication, and expression is repressed. AAV genome mainly

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persists episomally, and to a lesser extent it then integrates within a 4 kb region near a site on human chromosome 19q13.42 known as AAVS1 (Kotin et al., 1990, Schnepp et al., 2005). With the aid of a helper virus, the lytic cycle is initiated, where both the p5 and p9 promoter are activated and de-repression of *rep* gene occurs and in turn Rep protein expression enables AAV genome rescue. During replication, the second-strand synthesis occurs at AAV hairpin 3'OH end, which then act as primer for extension of the daughter strand by the DNA polymerase. After AAV genome has been replicated, the Rep78/68 binds to the RBE situated inside the ITRs and makes site and strand explicit scratches inside the TRS.

The nick created within the TRS regenerates a 3' hydroxyl end which can be extended to replicate the downstream ITR. The palindromic sequences in the ITRs of the AAV duplex regenerates hairpin structures from which re-initiation can start. Re-initiation and extension generate a double stranded full length AAV genome and a single stranded full length AAV displacement product. Various factors such as host-derived proteins like DNA polymerase δ complex, replication factor A and C (RFA and RFC), proliferation cell nuclear antigen (PCNA) and minichromosome maintenance complex (MCM) facilitate the displacement of the strand. This permits a balance between AAV genome replication and packaging. The double stranded AAV genome serves as a template for additional replication whereas the single stranded AAV genome is packaged (de Oliveira and Fraefel, 2010, Weitzman and Linden, 2011)

AAV-derived vectors can be created through removing the *rep* and *cap* genes and providing them *in trans* during vector production while retaining only the ITRs, which flank the transgene (Xiao et al., 1998). Also, the viral integration element, the *cis* - p5IEE responsible for AAV integration is discarded to limit chromosomal integration.

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Particles generated in this manner can carry any type of transgene cassette that surpass the size of the genome of the wild-type virus, which is usually around 4.7 kb (Knipe et al., 2013). The properties that make AAVs useful include easy manipulation of the genome using recombinant DNA technologies, minimal immunogenicity, long-term transgene expression and efficient transduction of a wide range of dividing and non-dividing cell types (Chen et al., 2009).

Two commonly used recombinant AAVs (rAAVs) are self-complementary AAVs (scAAVs) and single stranded AAV (ssAAVs). These vectors differ according to their genomic DNA conformations, transgene capacity and expression kinetics (McCarty et al., 2001b). Whereas the ssAAV genome structure is similar to the wild-type virus, the scAAV has been designed by mutating trs at one ITR, preventing terminal hairpin resolution. During replication, this results in the formation of one closed hairpin loop end with complementary duplex strands and twin T-shaped loops on the opposite end [(Fig 1.5), (MacCarty.,2008)]. Since AAV transgene expression only occurs when the genome adopts double stranded conformation, as such, transgene expressions of scAAVs is quicker than ssAAVs, because ssAAVs genome require the conversion to dsDNA before transcription (McCarty et al., 2001a, McCarty et al., 2003). However, scAAVs are limited by the fact that they have about a maximum of 2.5 kb transgene capacity, which is half the size of wild type AAVs or ssAAVs (Fig 1.5).

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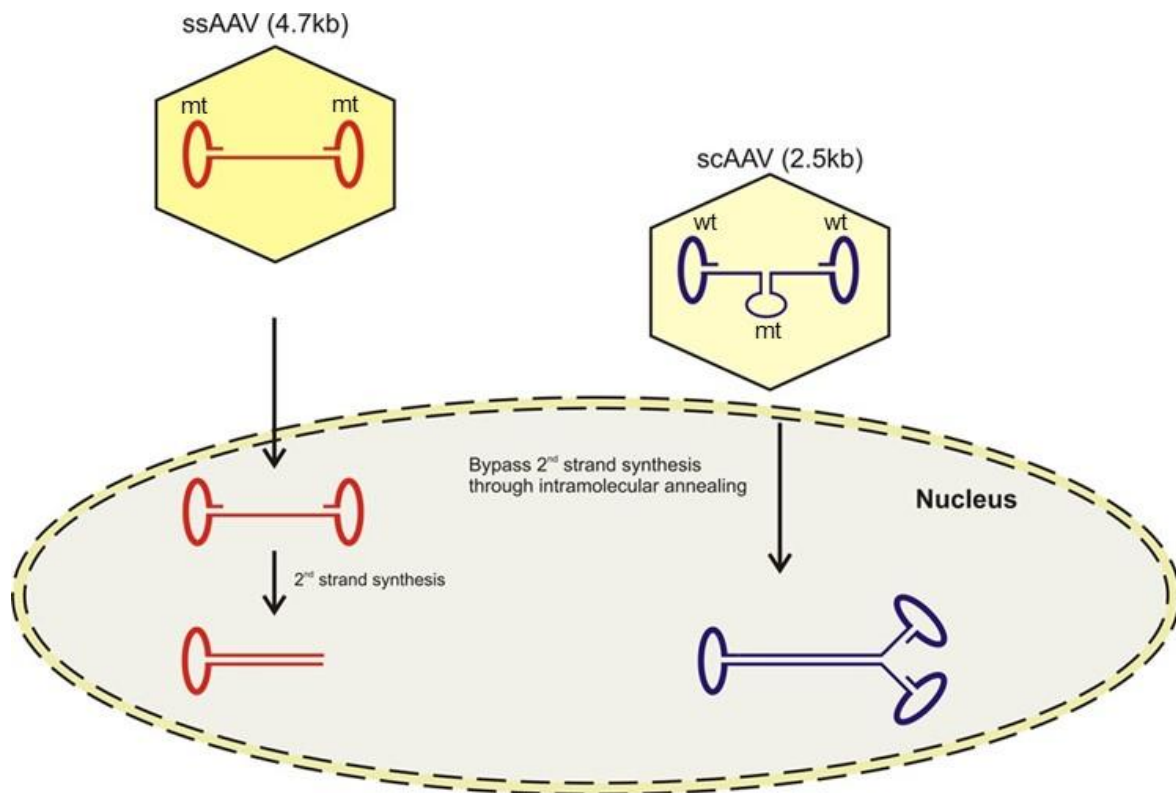


Figure 1.5: ssAAV and scAAV genome structure comparison.

ssAAV vector has a 4.7 kb ssDNA genome with two wildtype ITRs (wt) framing the expression cassette, once in the host, the ssDNA is converted to a dsDNA required for transcription. The scAAV vector contains a self-complementary genome with one internal mutant ITR (mt) and two terminal wildtype (wt) ITRs. Once in the host the genome fold back to form a molecule that can be directly used for transcription. This reduces the packaging capacity of scAAVs to about 2.5 kb. [adapted from (Le Bec and Douar, 2006)].

AAVs have been widely used to deliver anti-HBV shRNAs and artificial miRs (Maepa et al., 2017, Michler et al., 2016, Ye et al., 2017). HBV infection appears to enhance AAV transduction of hepatocytes making it more suitable for anti-HBV gene therapy development (Hosel et al., 2014). Hence, delivery of anti-HBV sequences with scAAV8 resulted in persistent inhibition of HBV replication that lasted over a period of 10 months (Maepa et al., 2017).

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The use of AAV to deliver replication-competent greater-than-genome-length of HBV genotype D in mice was previously explored. In these mice, high levels of HBsAg and HBcAg expression was observed. The replicative DNA intermediates of HBV (rcDNA and double stranded linear DNA) were detected. This HBV gene expression and replication persisted over a period of 6 months. Although the mouse model resulted in no liver inflammation, liver fibrosis was observed (Ye et al., 2015).

Given that the efficacy of novel anti-HBV therapeutics differ between HBV genotypes (Sun et al., 2010, Zhang et al., 2010), it is critical that anti-HBV therapeutic regimens be tested in the panel of described HBV genotype replication models to be able to attain broad efficacy in most chronic carriers of HBV. Hence the development of small animal models for each genotype is important. Subgenotype A1 is particularly common in sub-Saharan Africa, east and south-east, where complicating cirrhosis and hepatocellular carcinoma occur with high frequency. The use of AAV to model HBV subgenotype A1 has never been explored. The AAV system will be able to address difficulties experienced when using transgenic mice and adenoviral vectors as it will be possible to regulate the dose of AAV to influence the amount of replicating HBV without inducing a strong vector-specific immune response. The use of AAVs to model HBV A1 replication in vivo will also allow a safe long-term expression of HBV genes, closely mimicking the chronic HBV infection.

1.7. AIM AND OBJECTIVES

The purpose of the study was to use adeno-associated viral vectors to model HBV subgenotype A1 replication in vitro and in vivo. The focus was on the generation of HBV genome-bearing AAV vectors, delivering HBV genome sequence to

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hepatocytes and subsequently assessing HBV replication and exploring the use of the AAV-HBV model in evaluating anti-HBV therapeutics efficacy.

The objectives of the study were:

- Generating recombinant AAV plasmids carrying greater-than-genome-length HBV sequences from subgenotypes A1 and D3
- Evaluation of HBV gene expression from AAV plasmids carrying greater-than-genome-length HBV in cultured liver-derived cells
- Producing the AAV vectors carrying greater-than-genome-length HBV sequence subgenotype A1 and subgenotype D3
- Assessment of HBV gene expression following transduction of cultured cells with recombinant AAV-HBV vectors.
- Determination of the long-term HBV gene expression, HBV replication, inflammatory response and hepatotoxicity in mice transduced with recombinant AAV-HBV vectors.
- Assessment of the long-term suppression of HBV gene expression in mice co-transduced with AAV-HBV and an AAV vector expressing anti-HBV RNAi activators.

1.8 Significance of the study

Chronic HBV is a major global medical problem especially within sub-Saharan Africa, including South Africa. According to the South African guideline for the management of chronic hepatitis B, emphasis has been focused on developing treatment that would achieve viral eradication with sustained HBsAg loss. Development of HBV subgenotype A1 animal model will be useful to screen anti-HBV therapeutics, hence

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adding value in the development of innovative solutions to an aggressive viral infection of public health importance to South Africa.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

NEB® 10-beta Competent *Escherichia coli* [(*E. coli*, $\Delta(ara-leu)$ 7697 *araD139 fhuA* $\Delta lacX74$ *galK16 galE15 e14- ϕ 80dlacZ Δ M15* *recA1 relA1 endA 1 nupG rpsL* (Str^R) *rph spoT1 $\Delta(mrr-hsdRMS-mcrBC)$*] and competent DH5-alpha *Escherichia coli* [*supE44 $\Delta lacU169$ (F80 *lacZ Δ M15*) hsdR17 recA1 endA1 yrA96 thi-1 relA1*] were purchased from New England Biolabs (Madison, USA). The *E. coli* cells carrying plasmid were grown in Luria-Bertani (LB) medium and Luria agar (LA) [Appendix, 5.1.1 (a)] supplemented with ampicillin (100 μ g/mL) at 37 °C and shaking at 150 rpm.

2.2 Preparation of *E. coli* competent cells and transformation

Competent *E. coli* were prepared using the rubidium chloride method (Hanahan, 1983). A single colony was used to inoculate 5 millilitres (mL) Luria bertani (LB) then incubated overnight at 37 °C while shaking at 150 rpm. The pre-inoculum was diluted 1:1000 in LB Psi media [Appendix, 5.1.1(b)], followed by incubation at 37 °C until OD at 600 nm was between 0.48-0.6. The cells were cooled on ice for 15 minutes (min) before centrifugation at 6000 $\times$ g for 5 minutes, then washed with 40 mL transformation buffer (Tfb) I, [Appendix, 5.1.2(a)]. The pelleted cells were re-suspended in 4 mL of transformation buffer [(Tfb) II, [Appendix, 5.1.2(a)], and 100 μ L aliquots were stored at -80 °C. For transformation, 100 μ L of competent *Escherichia coli* cells were mixed with the appropriate amount of the plasmid or ligation mixture then incubated on ice for 30 minutes. The transformation mixture was heat-shocked at 42 °C for 1 minute and 30 seconds (sec), then it was incubated

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on ice for 5 minutes. Six hundred microlitres of LB was immediately added and the mixture was incubated at 37 °C for 1 hour with shaking at 150 rpm. The transformants were selected by plating the mixture on LB agar plates containing ampicillin (100 µg/mL) [**Appendix**, 5.1.1 (c)] and incubating the plates overnight at 37 °C.

2.3 Plasmid DNA extraction and purification from bacteria

2.3.1 Small scale plasmid DNA isolation

Five millilitres of LB medium containing ampicillin (100 µg/mL) was inoculated with a single bacterial colony carrying the plasmid of interest and incubated at 37 °C overnight with shaking at 150 rpm. The resultant culture was centrifuged at 10 000 ×g for 30 minutes to collect the cells. Plasmid DNA was extracted and purified from *E. coli* using Econospin® All-in-One Mini Spin Columns according to the manufacturer's instructions (Epoch Life Science, TX, USA). Briefly, the cells were re-suspended in 250 µL of buffer 1 [**Appendix**, 5.1.2(c)]. The cells were lysed by adding 250 µL of buffer 2 [**Appendix**, 5.1.2(c)] to the cell suspension, mixed through inverting and incubated at room temperature for 5 minutes. Followed by addition of 300 µL Buffer 3 and mixing by inversion [**Appendix**, 5.1.2(c)]. The lysate was placed on ice for 10 minutes followed by centrifugation at 14 000 ×g for 20 minutes to separate the cell debris from the supernatant. The supernatant was transferred to a new Econospin® All-in-One Mini Spin Column and centrifuged at 10 000 ×g for 1 minute. Wash buffer [**Appendix**, 5.1.2(c)] was added to the column then centrifuged at 14000 ×g for 1 minute. The flow-through was discarded and the column was spun for 30 seconds to remove the wash buffer remained. Subsequent to adding 100 µL of dH₂O, the DNA elution was carried out through centrifugation at 14 000 ×g for 1

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minute. The concentration of DNA was quantified using N80 Nanophotometer Spectrophotometer (Implen, Munich, Germany).

2.3.2 Large scale plasmid DNA isolation

Large scale plasmid DNA isolation was carried out using the QIAGEN® Plasmid Maxi Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, a single bacterial colony carrying the plasmid was inoculated in 200 mL LB containing ampicillin (100 µg/mL), followed by an overnight incubation at 37 °C with shaking at 150 rpm. The obtained bacterial cell culture was centrifuged at 6 000 ×g for 10 minutes. The harvested cells were resuspended in 10 mL buffer P1 then 10 mL buffer P2 was added. The mixture was inverted 4-5 times followed by incubation at room temperature (rtm) for 5 minutes. Subsequent to the addition of 10 mL buffer P3, the mixture was inverted 4-5 times, incubated on ice for 20 minutes. The lysates were then centrifuged at 14 000 ×g for 30 minutes at 4 °C. The supernatant was applied to QIAGEN-tip 500 column, followed by three washes with 30 mL of Wash buffer QC. The plasmid DNA was eluted with 10 mL Elution buffer QF, after which 15 mL ice-cold isopropanol was added to desalt the plasmid DNA. Thereafter, the mixture was centrifuged at 14 000 ×g for 30 minutes at 4 °C. The pellet was then washed with 10 mL 70 % ethanol, and subjected to centrifugation at 14 000 ×g for 20 minutes at 4 °C. The pellet was allowed to air dry at room temperature and re-suspended in 300 µL water. Finally, the DNA concentration was quantified using N80 Nanophotometer spectrophotometer (Implen, Munich, Germany).

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2.4 DNA manipulations

2.4.1 Restriction digestion and ligation

Restriction enzymes and T4 DNA ligase were obtained from (Thermo Fisher Scientific, CA, USA). Restriction digests were carried out by digesting 1 µg DNA with 1 unit of enzyme in a 1 × buffer and total reaction volume was brought up to 20 µL with distilled water. Samples were incubated at 37 °C for 3 hours.

DNA ligations were performed using 1-5 U T4 DNA ligase (Thermo Fisher Scientific, CA, USA) according to the manufacturer's instructions. The ligation reaction contained: 1 × ligation buffer, 1-5 U of T4 DNA ligase, 40 ng of vector plasmid DNA, insert DNA at a 3:1 molar ratio to plasmid vector and nuclease free water to a final volume of 20 µL. This was followed by an overnight incubation at 4 °C overnight, and the ligation reactions were used to transform NEB® 10-beta Competent *E. coli* as described in **Section 2.2**.

2.4.2 Agarose gel electrophoresis and DNA fragment purification

DNA was separated on 1% agarose gel which was prepared by dissolving 1 g in 100 mL of 1× TAE buffer [**Appendix**, 5.1.2 (d)] and ethidium bromide was added to a final concentration of 0.5 µg/mL. Prior to electrophoresis of DNA samples, 10 % bromophenol blue tracking dye in 50 % glycerol solution was added to a 1× dilution. DNA was then loaded and subjected to agarose gel electrophoresis at 120 volts until the DNA has resolved sufficiently and imaged using a Syngene G:BOX transilluminator (Syngene, Cambridge, UK).

To purify restricted DNA fragments from the gel, the QIAquick® gel extraction kit was used according to manufacturer's instructions (Qiagen, Hilden, Germany). Gel portions containing DNA fragments of interest were cut out and QG buffer

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added at a 3:1 ratio to the gel weight. This was followed by incubation at 50 °C for 10 minutes to permit the gel piece to melt. Addition of isopropanol to the sample in a 1:1 ratio was carried out. The mixture was loaded to the QIAquick mini column and centrifuged at 2 000 ×g for 1 minute at 4 °C. The flow-through was discarded and buffer QG (500 µL) was added to the spin column and centrifuged at 14 000 ×g for 1 minute at 4 °C. Then the flow through was discarded followed by addition of 750 µL buffer PE was into the column. The column was then centrifuged at 14 000 ×g for 1 minute at 4 °C. Afterwards, the flow through was discarded and the QIAquick mini column was centrifuged for an extra 1 minute at 14 000 ×g. DNA was eluted through addition of 50 µL dH₂O and centrifuged at 14 000 ×g for 1 minute. Thereafter, the concentration of the plasmid DNA was quantified using N80 Nanophotometer Spectrophotometer (Implen, Munich, Germany).

2.5 Construction of AAV plasmids carrying greater-than-genome-length HBV sequences

The plasmids containing greater-than-genome-length HBV subgenotype A1 (pcDNA-1.28 ×A1, Accession number KM519452) and D3 sequences (pcDNA-1.28 ×D3, Accession number KM519455.1) were kindly donated by Prof Anna Kramvis, Wits Hepatitis Virus Diversity Research Unit [(HVDRU, (Bhoola et al., 2014))]. The HBV genome sequence of subgenotype D3 was used as the positive control. To allow insertion of the HBV sequences into an AAV genome bearing plasmid (pAAV-CAG-GFP, **Table 2.1**), the CAG promoter and the GFP-encoding sequences were first replaced with a 32 bp DNA sequence containing unique restriction sites (**Fig 2.1**). To achieve this, two oligonucleotides containing *NotI*, *Sall*, *HindIII* and *EcoRV* sites

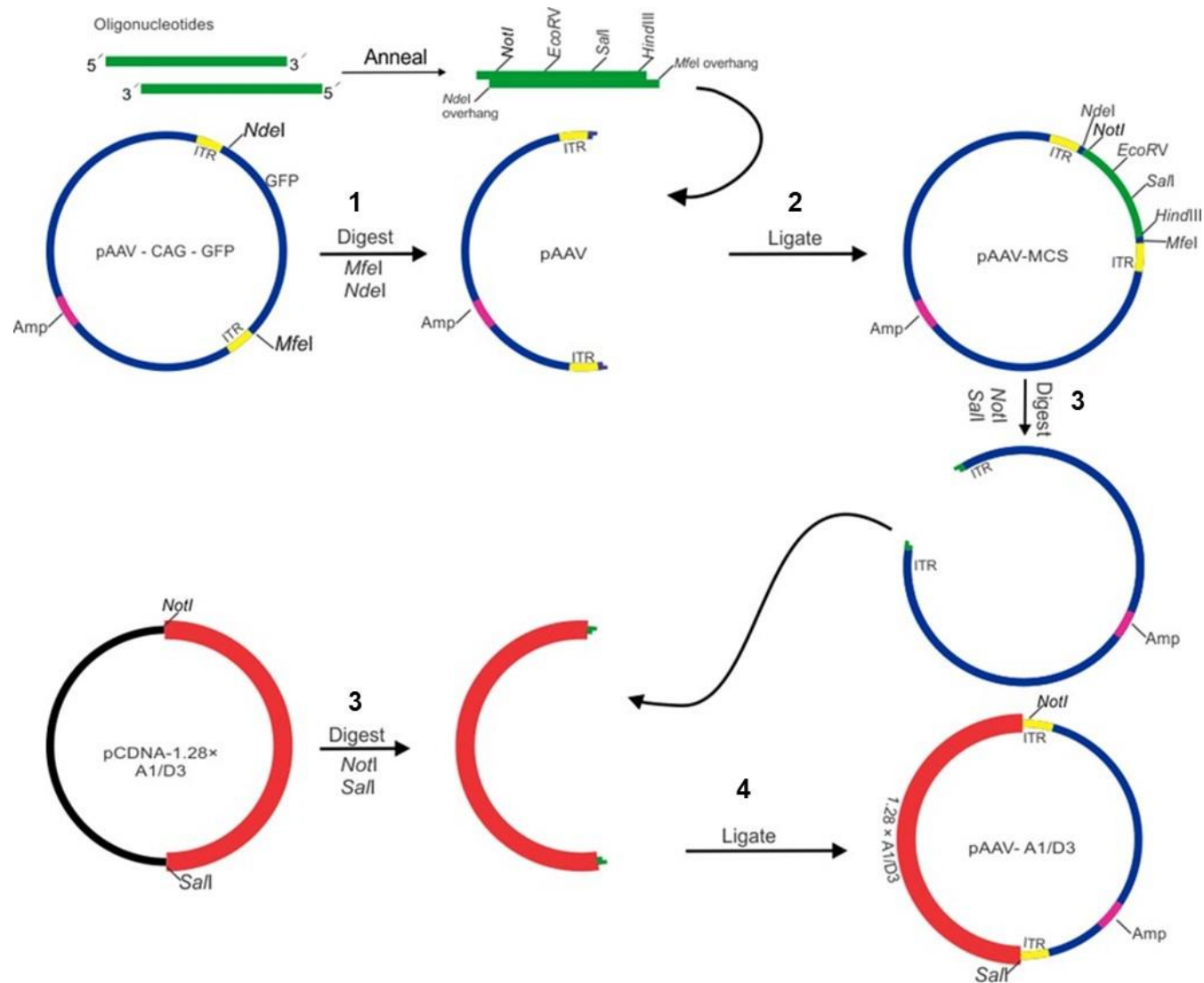
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were designed and synthesized such that when annealed they form a 32 bp double-stranded DNA sequence with *NdeI* and *MfeI* overhangs.

The two oligonucleotides (5'TATGCGGCCGCGATTCTGTCGACAAGCTTC-3' and (5'AATTGAAAGCTTGTCGACGATTCTGCGGCCGC-3') at amount of 100 picomoles were mixed in water and denatured at 95 °C for 5 minutes using thermal cycler C1000 (Bio-Rad, CA, USA). To enable annealing, they were allowed to cool down at room temperature for 2 hours. The resulting double-stranded DNA was ligated into pAAV-CAG-GFP digested with *NdeI* and *MfeI* to form pAAV-MCS. Successful cloning was confirmed through restriction analysis using the single digest of incorporated restriction sites, *NotI* or *SalI* or *HindIII* or *EcoRV*.

To construct the AAV plasmids carrying greater-than-genome-length HBV sequences, the pcDNA-1.28 × A1 or pcDNA-1.28 × D3 (**Table 2.1**) and pAAV-MCS were digested with *NotI* and *SalI*. The greater-than-genome-length HBV sequences (A1 or D3) were ligated into pAAV-MCS to generate pAAV-A1 and pAAV-D3. Subsequently, presence of the greater-than-genome-length fragment in the pAAV-A1 or pAAV-D3 constructs was confirmed by restriction analysis using *DraI* and *SmaI* single digests and a double digest with *NdeI* and *SalI* (**Fig 2.1**). Thereafter, *Eco88I* was used to assess the integrity of the inverted terminal repeats (ITR) sequences.

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Figure 2.1: Diagrammatic illustration of cloning strategy for construction of AAV plasmids carrying greater-than-genome-length HBV sequences.

1.The oligonucleotides consisting of unique restriction sites were annealed to form a double-stranded DNA with *NdeI* and *MfeI* overhangs. pAAV-CAG-GFP was digested with *NdeI* and *MfeI* to remove the GFP cassette. **2.**The dsDNA fragment was ligated into pAAV-CAG-GFP to form pAAV-MCS **3.** To construct the HBV genome-bearing AAV plasmids, the pAAV-MCS and pcDNA-A1 or pcDNA-D3 plasmids were digested with *NotI* and *Sall*. **4.**The greater-than-genome-length HBV sequences (A1 or D3) were individually ligated into pAAV-MCS to generate pAAV-A1 and pAAV-D3.

Table 2.1: Plasmids used in this study

Plasmid name	Description	Source or reference
pAAV2	An AAV plasmid carrying AAV2 <i>cap</i> and <i>rep</i> ORFs. It carries ampicillin resistance (Amp ^R) selection marker	(Grimm et al., 2008)
pAAV8	An AAV plasmid carrying AAV8 <i>cap</i> and <i>rep</i> ORFs. It carries ampicillin resistance (Amp ^R) selection marker.	(Grimm et al., 2008)
pCH-9/3091	Plasmid bearing a greater- than-genome length HBV genotype D sequence.	(Nassal et al., 1990)
pAd-helper	A plasmid bearing Ad genes <i>E1A</i> , <i>E1B</i> , <i>E2</i> and <i>E4</i> , it functions as a helper.	(Grimm et al., 2008)
pcDNA-1.28 ×A1	Plasmid containing greater-than-genome-length HBV subgenotype A1 sequence	(Bhoola et al., 2014)
pcDNA-1.28× D3	Plasmid containing greater-than-genome-length HBV subgenotype D3 sequence	(Bhoola et al., 2014)
pAAV-CAG-GFP	A plasmid containing AAV ITR sequences that flanks a <i>GFP</i> gene under the control of a CAG (CMV enhancer, chicken beta-Actin promoter and rabbit beta-Globin splice acceptor site) promoter.	(Mao et al., 2011))
pAAV-empty	AAV plasmid carrying mTTR promoter, FokI endonuclease and poly A	(Jacobs)
pAAV-MCS	A pAAV-CAG-GFP derived plasmid with CAG-GFP cassette replaced by a 32 bp multiple cloning sequence.	This study
pAAV-A1	A pAAV-MCS derived plasmid carrying greater-than-genome-length HBV subgenotype A1 sequence flanked by AAV ITR sequences	This study
pAAV-D3	A pAAV-MCS derived plasmid carrying greater- than-genome-length HBV subgenotype D3 sequence flanked by AAV ITR sequences	This study
pAAVMTTR31/5,8,9	A plasmid containing an MTTR promoter driving the expression of an anti-HBV sequence targeting X ORF of HBV (pri.miR 31/5,8,9) flanked by the AAV ITRs.	(Maepa et al., 2017)
pBS-mTTR-BCDE-pA	A plasmid containing an MTTR promoter driving the expression of anti-HCV pre-miR mimics flanked by AAV ITR sequence	(Bourhill et al., 2016)

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2.6 Tissue culture Methods

2.6.1 Tissue culture growth conditions

Liver-derived Huh 7, HepG 2.2.15 cells and Kidney-derived Hek293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fischer Scientific, CA, USA) supplemented with penicillin (50 IU/mL), streptomycin (50 µg/mL) and 10 % foetal bovine serum (FBS) [Appendix, 5.13 (a),(b) and (g)]. Cells were grown in a humidified incubator set at 37 °C and 5 % CO₂.

In order to culture the cells from freezer, the frozen cells stocks maintained at cryogenic conditions with liquid nitrogen were thawed in a 37 °C water bath and diluted in 10 mL DMEM (Thermo Scientific, CA, USA) and harvested through centrifugation at 200 ×g for 3 minutes. The pellet obtained was resuspended in 1 mL DMEM and the suspension mixture was transferred to a T25 flask (Sigma-Aldrich, MO, USA). This was followed by addition of 4 mL of DMEM and the flask was incubated at 37 °C, 5% CO₂ humidified incubator overnight. Post microscopic examination for growth of culture, medium was aspirated and replaced with fresh 5 mL pre-warmed DMEM. The cell culture was then re-incubated until it reached the confluency between 70 – 80% confluency before passaging.

2.6.2 Transfection of pAAV constructs and HBV gene expression assessment

Huh 7 cells were used to assess HBV gene expression from pAAV-A1 and pAAV-D3 plasmids. The cells were grown to 80 % confluency in a 48-well plate and transfected using Polyethyleneimine Max (PEI-MAX) (Polyscience Inc, PA, USA) as per the manufacturer's instructions. Briefly, 100 ng of pAAV-A1; pAAV-D3; pAAV-empty (negative control) or pCH-9/3091 (positive control) and 50 ng of pAAV-CAG-

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GFP (to assess transfection efficiency) were mixed and adjusted to final volume of 125 μL /well with OptiMEM (Thermo Scientific, CA, USA). In a separate tube, PEI-Max and OptiMEM (Thermo Scientific, CA, USA) were mixed to a final volume of 125 μL /well such that the PEI-Max:DNA mass ratio was 3:1. This was followed by incubation at room temperature for 15 minutes. The DNA:OptiMEM and the PEI-Max:OptiMEM mixes were then mixed and incubated for 15 minutes at room temperature (rtm). The PEI-Max:DNA mix was subsequently added dropwise into the wells containing cells and some wells did not receive transfection mix. Cells were then incubated at 37 °C, 5 % CO₂ in a humidified incubator.

At 24 hours post transfection, the transfection efficiency was assessed by analysing green fluorescent protein (GFP) expression using the EVOS FL fluorescence microscope (Thermo Fischer Scientific, CA, USA). After the detection of GFP, the culture medium was harvested from cells and the HBsAg concentration was evaluated by Enzyme-linked Immunosorbent Assay (ELISA) using the Monolisa® Ag HBs Plus immunoassay kit (Bio-Rad, CA, USA) according to manufacturer's instructions.

Briefly, 100 μL of the supernatant collected was added to each well of the provided antibody-coated plates. Additionally, 100 μL of the ELISA kit positive (R4) and negative controls (R3) were included as experimental controls. Fifty microlitres of conjugate working solution (R6+ R7) was added to each sample well in a microplate well containing supernatant. The mixture was thoroughly mixed by pipetting and the plate was incubated for 90 minutes at 37 °C. After incubation, the plate was washed using ImmunoWash™ 1575 Microplate washer (Bio-Rad, CA, USA). This was followed by addition of 100 μL of development solution (R8 + R9) per well and the reaction was placed in the dark for 30 minutes before adding a stopping solution.

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Four minutes post adding the stop solution, optical density at 490/655 nm was measured using the iMARK™ Microplate reader (Bio-Rad, CA, USA).

2.7 Large scale AAV production

2.7.1 Transfection for viral production and harvesting

To produce all recombinant AAV vectors, triple transfection was carried out using PEI-max (Polyscience Inc, PA, USA) according to the manufacturer's instructions. A total of 319 µg of DNA consisting of pAAV-A1, pAAV-D3, pAAV-empty or pAAVMTTR31/5,8,9, pAd helper as well as pAAV2 or pAAV8 was transfected into HEK293T cells at 24.6 µg or 98.4 µg or 196 µg, respectively. For each construct, four 150 cm² plates of about 80 % confluent HEK293T cells in 20 mL DMEM media each were transfected with 2 mL/plate transfection mix as described in **Section 2.6.2**. Cells were incubated for 120 hours to allow production and propagation of AAV vectors. At 24, 48, 96 and 120 hours post transfection the spent media was collected, stored at 4 °C and fresh 20 mL DMEM added to each plate before incubation. At 120 hours, cells were harvested by centrifugation at 200 ×g for 3 minutes, followed by resuspension in 5 mL 1 × Phosphate buffered saline (PBS) [**Appendix**, 5.1.3(e)]. The cells were then collected by centrifugation at 200 ×g for 8 minutes. The cell pellets were re-suspended in lysate buffer [**Appendix**, 5.1.4(a)] followed by storage at -80 °C until cell lysis was performed. The cell lysis was performed through carrying out the five freeze thaw cycles (alternating 37 °C and -196 °C), followed by sonication for 80 seconds in a sonication water bath. To degrade DNA and RNA, the sample was treated with 50 units of Benzonase (Sigma, MO, USA) per mL of sample. The benzonase reactions were incubated at 37 °C, removed only to vortex for 15 seconds at 10 minutes intervals over the 1 hour period.

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The cell-free extract was centrifuged at 600 ×g and 4 °C for 15 minutes to remove cell debris.

Precipitation of virions from the collected supernatants was carried out by first filtering the media containing AAV using 500 mL Stericup® (0.22 µm, Merck, MA, USA) into sterile schott bottles. Subsequently, 25 mL of 5 × polyethylene glycol (PEG) 8000 (Sigma, MO, USA) was added into 100 mL of filtrate, mixed and stored at 4 °C overnight. This was followed by centrifugation at 600 ×g for 30 minutes, thereafter, the pellet was resuspended with AAV-containing supernatant previously collected from cell lysates. The suspension mixture was then subjected to iodixanol gradient purification.

2.7.2 AAV purification

The purification of AAV was performed by transferring the viral suspension and iodixanol solution through Pasteur pipettes into 16 × 76 mm centrifuge tubes (Beckman Coulter Inc., CA, and USA). The viral suspension was added first, followed by sequential addition of 15%, 25%, 40% and 60% each iodixanol solution at concentrations [Appendix, 5.1.4 (f)] respectively. This was followed by ultracentrifugation using Beckman Optima-L80XP centrifuge and 70.1 Ti fixed angle Titanium rotor (Beckman, CA, USA) at 195650 ×g at 4 °C for 2 hours. Extraction of the fraction containing pure virus was achieved by extracting the 40% iodixanol fraction.

2.7.3 AAV quantification by qPCR

Viral genomic DNA (gDNA) was first extracted from the 50 µL of purified AAV using QIAamp DNA blood mini kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, 20 µL of 20 mg/mL of the proteinase K enzyme was added into a

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1.5 mL microcentrifuge tube. This was followed by addition of 50 μ L of pure viral sample and 150 μ L of 1 $\times$ PBS. Two hundred microlitres of AL buffer was added into the sample followed by incubation at 55 $^{\circ}$ C for 10 minutes. Two hundred microlitres of 100 % ethanol was added into the mix and pulse-vortexed for 15 seconds. The mix was applied into the spin column followed by centrifugation 6000 $\times$ g for 1 minute. The spin column was washed with 500 μ L of buffer AW1 and centrifuged at 6000 $\times$ g for 1 minute. This was followed with a second wash step by adding 200 μ L of buffer AW2 and centrifuging at 6000 $\times$ g for 3 minutes. A centrifugation step at 6000 $\times$ g was carried out to completely remove the wash buffer. Viral DNA was eluted by adding 50 μ L of distilled water to the column and centrifugation at 6000 $\times$ g for 1 minute.

The viral vectors produced were quantified using qPCR. The PCR was prepared by addition of 1 μ L of extracted DNA, 10 μ L of 2 $\times$ FastStart essential DNA Green master (Roche, Indianapolis, US), 0.4 μ M of Forward primer (HBVs-F, **Table 2.2**) and 0.4 μ M of reverse primer (HBVs-R, **Table 2.2**). For quantification of AAV-mTTR-BCDE or AAV-empty, or scAAV miR-31/5,8,9 the MTTR forward and MTTR reverse primers were used (**Table 2.2**). The volume was adjusted to 20 μ L using Nuclease-free water. The AcroMetrix[®] HBV panel of HBV standards (Thermofisher Scientific, MA, USA) were run concurrently with the experimental samples for quantification of AAV carrying HBV greater-than-genome sequence. The qPCR standards for quantification of AAV carrying anti-HBV pri-miR; anti-HCV pri-miR and AAV-empty were prepared for qPCR data analysis by serially diluting plasmid DNA (pAAV-mTTR-BCDE-pA, **Table 2.1**). The starting concentration before 10-fold serial dilutions was set at 10^{10} copies/ μ L, and molar mass per base pair was assumed to be 650 (g/mol)/bp. The PCRs were subjected to the following qPCR conditions: denaturation at 95 $^{\circ}$ C for 30 seconds, annealing at 62 $^{\circ}$ C for 1 minute, and an

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extension step at 72 °C for 1 minute. The cycles were repeated 40 times and final extension step was done at 72 °C for 1 minute using the Bio-Rad CFX96™ Real Time System (Bio-Rad, Hercules, CA). The melting curve analysis was used to confirm the specificity of the qPCR and the quantification of absolute copies of AAV vectors produced were determined from the standard curve generated using HBV standards or AAV standards. All AAV vectors produced are listed in **Table 2.3**.

Table 2.2: Primers used in this study

Primer Name	Primer sequence (5' - 3')	Target sequence or purpose	Source: Reference
HBVs-F	GATAAGCCTCCCAAGGTGCTTTTGA	HBV <i>surface</i> gene, quantification of VPes	(Crowther et al., 2016)
HBVs-R	TAGGAATTCCATATGCCGCTGTCTTTT	HBV <i>surface</i> gene, quantification of VPes	(Crowther et al., 2016)
MTTR-Reverse	CCCCTGTTCAAACATGTCCT	MTTR promoter, used for quantification of AAV-mTTR-BCDE vectors	(Maepa et al., 2017)
MTTR-Forward	GCACTGGGAGGATGTTGAGT	MTTR promoter, , used for quantification of AAV-mTTR-BCDE vectors	(Maepa et al., 2017)
mIFIT1-F	TAAAAGTTATTTGGCCGACAAGGCTG GGATCGAAACC	mouse Interferon induced protein with <i>tetratricopeptide repeats 1</i> , mRNA gene expression of IFIT1	(Song et al., 2005)
mIFIT1-R	CGATCCCAGCCTTGTCGGCCAAATAA CTTTTAATAGA	mouse Interferon induced protein with <i>tetratricopeptide repeats 1</i> , mRNA gene expression of IFIT1	(Song et al., 2005)
mIFN- β -F	CCACAGCAGCTTCTGACACTGAAAA	mouse <i>Interferon-beta</i> , mRNA gene expression of IFN- β	(Song et al., 2005)
mIFN- β -R	CGATCCCAGCCTTGTAACCAAATAA CTTTTAATCGAA	mouse <i>Interferon-beta</i> , mRNA gene expression of IFN- β	(Song et al., 2005)
mGAPDH-F	TAAAAGTTATTTGGCCTCACAAGGCTG GGATCGAAACC	mouse <i>glyceraldehyde-3-phosphate dehydrogenase</i> , mRNA gene expression of GAPDH	(Song et al., 2005)
mGAPDH-R	CGATCCCAGCCTTGAGGCCAAATAA CTTTTAATAGA	mouse <i>glyceraldehyde-3-phosphate dehydrogenase</i> , mRNA gene expression of GAPDH	(Song et al., 2005)
mOAS1-F	CGATTAAAAGTTATTTGGTTTCACAAGG CTGGGATCGAAAC	mouse <i>2'-5'-oligoadenylate synthetase 1</i> mRNA gene expression of mouse OAS1	(Song et al., 2005)
mOAS1-R	CGATCCCAGCCTTGTAACCAAATAA CTTTTAATCGAAGCA	mouse <i>2'-5'-oligoadenylate</i> , mRNA gene expression of mouse OAS1synthetase 1	(Song et al., 2005)
AAV-F	GCCATGCTCTAGGAAGATCGTACC	AAV genome specific sequence between the ITR and the HBVs sequence, quantification of AAV vectors	This study
AAV-R	CCGTAAATAGTCCACCCATTGACGT	AAV genome specific sequence between the ITR and the HBVs sequence , quantification of AAV vectors	This study

Table 2.3: Recombinant AAV vectors used in this study

Vector name	Description	Source or reference
AAV2-empty	Recombinant AAV2 bearing murine transthyretin receptor (MTTR) promoter sequence and no transgene.	This study
AAV8-empty	Recombinant AAV8 bearing the promoter sequence (mTTR) and no transgene.	This study
AAV2-A1	Recombinant AAV2 vector bearing a greater than-genome -length HBV sequence subgenotype A1.	This study
AAV8-A1	Recombinant AAV8 vector bearing a greater-than- genome- length HBV sequence subgenotype A1.	This study
AAV2-D3	Recombinant AAV2 vector bearing a greater-- than-genome-length HBV sequence subgenotype D3.	This study
AAV8-D3	Recombinant AAV8 vector bearing a greater-than-genome-length HBV sequence subgenotype D3.	This study
sc AAV2-miR 31/5,8,9	Self-complimentary (sc) Recombinant AAV2 vector expressing anti-HBV primary microRNA 31/5,8,9 from MTTR promoter.	(Maepa et al., 2017)
sc AAV8-miR 31/5,8,9	Self-complimentary recombinant (sc) AAV8 vector expressing anti-HBV primary microRNA 31/5,8,9 under MTTR promoter.	(Maepa et al., 2017)
AAV2-mTTR-BCDE	Recombinant AAV2 vector expressing primary microRNA against HCV from the murine transthyretin receptor (MTTR) promoter.	(Bourhill et al., 2016)
AAV8-mTTR-BCDE	Recombinant AAV8 vector expressing primary microRNA against HCV from the murine transthyretin receptor (MTTR) promoter.	(Bourhill et al., 2016)

2.8 Characterisation of AAV-HBV model in cultured mammalian cell line

The use of AAV to model subgenotype A1 replication has been explored both in liver-derived cell line and in vivo through infection with AAV carrying HBV greater-than-genome-length HBV sequence.

2.8.1 Infection of Huh 7 cells with AAV bearing HBV to evaluate HBV gene expression and HBV particle production

To assess if Huh 7 cells infected with AAV vectors carrying HBV genome will result in HBV gene expression, cells were allowed to grow until they reach confluency of ~ 70% in 24-well plates. The cells were then infected at a multiplicity of infection (MOI) of 1×10^4 with AAV2-empty or AAV2-A1 or AAV2-D3. For optimal transduction, the media was removed, and viral vector diluted in 100 μ L of media was added dropwise onto the cells. Cells were then incubated for 1 hour at 5% CO₂ and 37 °C with gentle rocking every 10 minutes. The medium was subsequently adjusted to a final volume of 500 μ L through adding DMEM. One hundred microlitres of supernatant was collected at 48 and 72 hours post transduction and used for HBsAg ELISA as described in **Section 2.6.2**.

The immunofluorescence staining for HBcAg expression was carried out using cells transduced with AAV vectors as above. Forty-eight hours post infection, medium was removed, and cells were fixed by gently adding ice cold 100 % methanol to each well, followed by incubation at -20 °C for 10 minutes. The cells were washed 3 times with 0.5 % BSA in PBS [**Appendix, 5.1.5 (b)**] followed by permeabilisation through adding 200 μ L 0.1% Triton X [**Appendix, 5.1.5 (a)**] and incubated for 10 minutes. The cells were washed with 200 μ L 1 $\times$ PBS followed by incubation with mouse anti-

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HBcAg antibody (Abcam, Cambridge, UK) for 1 hour at room temperature. This was followed by a wash with $1 \times$ PBS, addition of 1:200 dilution in 1% BSA of Alexa flour 488-labelled secondary mouse Ab (Life Technologies, CA, USA) and incubation in a humidity chamber for 40 minutes. The cells were then washed twice with 200 μ L of $1 \times$ PBS. The PBS was removed and 100 μ L of $1 \times$ 4',6 -diamidino-2-phenylindole (DAPI) [**Appendix**, 5.1.5 (c)] nuclear stain was added followed by incubation at rtm for 2 minutes. The cells were washed twice with 200 μ L of $1 \times$ PBS and imaged using an EVOS FL microscope (Thermo Fisher Scientific, CA, and USA).

To quantify HBV viral particles produced in vitro, the supernatant was collected on days 1, 3, and 5 from the cells infected with AAV vectors as above. The total genomic DNA (gDNA) was extracted using QIAamp DNA mini kit (Qiagen, Germany) and subjected to qPCR as described in **Section 2.7.3**. Quantification of total viral particles was achieved using HBV specific primers (HBVs-F and HBVs-R, **Table 2.2**) for q-PCR. The AcroMetrix® HBV panel of HBV standards (Thermofisher Scientific, MA, USA) were used to quantify VPEs. The quantification of AVV vectors was also carried out concurrently using the AAV specific primers (AAV-F and AAV-R, **Table 2.2**). The standards for quantification of AAV were prepared using pAAV-A1. The amount of HBV particles was presented as the difference between the copy numbers of AAV detected and total viral particles.

2.8.2 Application of AAV-HBV in screening anti-HBV therapeutics in cell culture

The feasibility of using the AAV-HBV model for screening antiviral drugs was assessed using a well characterised AAV expressing anti-HBV primary microRNA 31/5,8,9 [(scAAV pri-miR 31/5,8,9, (Maepa et al., 2017)]. Huh 7 cells were co-infected with AAV carrying RNAi activator and either AAV2-A1 or AAV2-D3 at an

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equal MOI of 1×10^4 . The other wells were co-infected with either AAV carrying non-specific pri-miR (AAV2-mTTR-BCDE) and AAV2-A1 or AAV2-D3, and incubated for 48 hours in 5 % CO₂ and at 37 °C. The supernatant from the cells co-infected with AAV vectors was used to measure the concentration of HBsAg ELISA using the Monolisa® Ag HBs Plus immunoassay kit (Bio-Rad, CA, USA) according to manufacturer's instructions (**Section 2.6.2**).

2.9 Generation and characterisation of AAV-HBV mouse model in vivo

All experiments on animals were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee (**Appendix, 5.5**). A total of 224 Naval Medical Research Institute (NMRI) female mice were kept in groups of a maximum of 5 per cage and given food and water ad libitum.. The mice were monitored and weighed weekly. Tail vein injection of vectors was used to infect the mice. Blood was collected using retro orbital puncture at different time points while mice were anaesthetised with isoflurane (**Appendix, Fig 5.4**). To assess the safety of delivering high and low AAV vectors in mice, four mice per group were injected and bled 6 hours post infection. For long-term HBV gene expression, eight mice per group were bled at 2, 4, 8, 12 and 24 weeks post infection. For histological staining 3 mice per group were sacrificed and tissue harvested at 8 and 24 weeks post infection. At termination, mice were transferred to a chamber connected to carbon dioxide and oxygen cylinders. The chamber was filled with O₂, followed by CO₂ and mice were left to stand until they are dead before dissection, tissue collection and disposal.

2.9.1 Assessment of immune induction post administration of AAVs carrying HBV sequence

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(i) Immunological analysis by cytometric bead array and RT-qPCR

To determine the safety of delivering AAV8 vectors at low and high dose in mice, both 5×10^{11} and 9×10^{11} AAV were tested per mouse. Four mice per group were each injected with 300 μ L containing 5×10^{11} or 9×10^{11} particles of AAV8-A1, AAV8-D3 or AAV8-empty. One group of mice was injected with saline to serve as a negative control. As an inflammation positive control, one group of mice was hydrodynamically injected with 3 mL of 100 μ g polyinosinic: polycytidylic acid (Poly I:C) per mouse. Six hours post injection, a terminal bleed was performed, and serum was collected and used to quantify levels of inflammatory cytokines by a cytometric bead array (CBA).

The cytometric bead array mouse inflammation kit (BD Biosciences, CA, USA) was used to assess induction of interleukin 6 (IL-6), interleukin 10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon gamma (IFN- γ), tumour necrosis factor alpha (TNF- α) and interleukin 12p70 (IL-12p70) according to manufacturer's protocol. Briefly, 50 μ L of serially diluted standards and undiluted serum samples were added into sample tubes containing 50 μ L of mouse inflammation capture bead mix. Additionally, 50 μ L of the mouse inflammation PE detection reagent was added to all assays. This was followed by an incubation for 2 hours in the dark, followed by addition of 1 mL of 1 $\times$ Wash buffer to each assay tube then centrifuged at 1600 $\times$ g for 5 minutes to pellet the capture beads. The supernatant was discarded and capture bead pellets re-suspended in 300 μ L of Wash buffer. CBA reactions were processed by Fortessa FSR flow cytometer utilising the BD FACSDiva software and data analysed using FCAP Array software 3.0 (BD Biosciences, CA, USA).

After terminal bleeding as described in **Section 2.9**, mice were euthanised, and livers harvested. The livers were homogenised in 1 mL Trizol (Thermo Fisher

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Scientific, CA, USA) and incubated at room temperature for 10 minutes. The lysates were then centrifuged at 10 000 ×g for 10 minutes at 4 °C to separate cell debris and the supernatant was transferred into a clean microcentrifuge tube. Two hundred microlitres of chloroform was added to the supernatant, the samples were mixed through inversion followed by incubation at rtm for 5-10 minutes. The samples were centrifuged at 12 000 ×g for 15 minutes at 4 °C. The top aqueous layer was transferred to a clean tube and five hundred microlitres of isopropanol added. The precipitated RNA was centrifuged at 12 000 ×g for 10 minutes at 4 °C and the RNA pellet washed with 1mL of 75 % ethanol prepared in diethylpyrocarbonate (DEPC) water. The RNA was air dried, re-suspended in DEPC water and quantified using the N80 Nanophotometer Spectrophotometre (Implen, Munich, Germany) and was stored at -70 °C.

The RNA extracted was used to carry out RT-qPCR using Luna® Universal One-Step RT-qPCR master mix kit (New England Biolabs Inc., MA, US). The reaction was carried out by addition of 1 µg of RNA, 5 µL of 2× Luna Universal One-step reaction mix, 1 µL of Luna WarmStart RT enzyme mix, 0.4 µM of Forward primer and 0.4 µM of reverse primer. The volume was adjusted to 10 µL using nuclease-free water. The *IFN-β*, *IFIT1* and *OAS1* transcripts were quantified relative to murine *glyceraldehyde-3-phosphate dehydrogenase* (*mGAPDH*) using *mGAPDH-F* and *mGAPDH-R*, *IFN-β* (*mIFN-β-F* and *mIFN-β-R*), *IFIT1* (*mFIT1-F* and *mFIT1-R*) and *OAS1* (*mOAS1-1F* and *mOAS1-1R*) specific primers (**Table 2.2**). The following conditions were used for PCR amplification: reverse transcription at 55 °C for 10 minutes and initial denaturation cycle at 94 °C for 1 minute, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 60 °C for 30 seconds. The RT-qPCR was carried out using CFX96™ Real-Time system (Bio-rad, CA,USA).

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The efficiency of the PCR and primer performance was assessed using CFX manager 2.1 software and the efficiency (E), Slope, coefficient of correlation (R^2), and y intercept values of 90 to 110%, - 3.58 to -3.10, > 0.99 and ≤ 40 were acceptable. Relative gene expression was determined using *GAPDH* as a reference gene in the comparative C_T method (Livak and Schmittgen, 2001).

(ii) Assessment of hepatotoxicity by serum alanine transaminase activity assay

The measurement of AAV hepatotoxicity in mice was carried out by measuring the level of Alanine aminotransferase (ALT) marker produced as a result of liver damage. The serum from the mice injected with AAV8-A1; AAV8-D3; AAV8-empty at 5×10^{11} or 9×10^{11} VPEs/mouse or saline was collected and used to carry out the ALT assay. ALT activity was measured in serum samples collected at weeks 2, 4, 8 post AAV injection. The assay was carried out using an Advia 1800 Chemistry System (Siemens) at the accredited facilities of the South African National Health Laboratory Service (NHLS, Johannesburg, South Africa).

2.9.2 Evaluation of HBV gene expression through detection of HBsAg and HBcAg

To determine the optimal AAV dose required to establish HBV gene expression in mice, eight mice per group were used. Each mouse was injected via tail vein with either AAV8-A1; AAV8-D3; AAV8-Empty at 5×10^{11} or 9×10^{11} VPEs/mouse or Saline. Blood was collected through retro-orbital puncture at different time points (2, 4, 8, 12 and 24 weeks post injection). The serum was harvested by incubation of blood at 4 °C for 2 hours. This was followed by centrifugation at $6000 \times g$ for 10 minutes, serum was transferred into a new tube and stored at -80 °C for future use.

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To assess HBsAg expression, the serum was diluted in a ratio of 1:4 and used for ELISA using Monolisa® Ag HBs Plus immunoassay kit (Bio-Rad, CA, USA) as described in **Section 2.6.2**. For immunohistochemical staining of HBcAg in mouse livers, three mice per group were sacrificed at 8 and 24 weeks post infection, livers collected and fixed in formalin. The livers were sent to AMPATH National laboratories, Fourways, JHB for embedding in paraffin wax and sectioning. The HBcAg staining was carried out at National Health Laboratory service (NHLS), Johannesburg, South Africa using standard procedures.

2.9.3 Assessment of liver injury by histological analysis

To investigate whether liver fibrosis and chronic liver injury were present after AAV injection, livers harvested from mice sacrificed at 8 and 24 weeks post injection with AAV8-A1; AAV8-D3; AAV8-empty at 5×10^{11} or 9×10^{11} VPEs/mouse or Saline were used.

To detect inflammatory cells infiltration and liver fibrosis, the 10 % formalin fixed tissues were sent to AMPATH laboratories for embedding, sectioning, haematoxylin and eosin (H&E) and Picrosirious red staining according to standard procedures (Ampath laboratories, JHB, South Africa).

2.10 Assessment of HBV replication post AAV injection in mice

2.10.1 Detection of HBV DNA replicative intermediates by qPCR and Southern blotting

(i) Genomic DNA extraction

Genomic DNA was extracted from the livers of mice injected with recombinant AAV vectors. Each liver sample was weighed, an equal volume of saline added,

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and the samples were mechanically homogenised. One millilitre of lysis buffer [Appendix,5.1.6(a)] was added to the homogenate and incubated at 55 °C for 3 hours. This was followed by a dropwise addition of 250 µL 5 M sodium chloride (NaCl) and incubation overnight at 4 °C. The mixture was centrifuged at 16 000 ×g for 60 minutes, followed by collection of supernatants. After addition of 700 µL of phenol/chloroform and mixing, the samples were centrifuged at 16 000 ×g for 15 minutes. The supernatant was collected, DNA was precipitated by addition of 450 µL isopropanol and centrifuged at 16000 ×g for 30 minutes. The pellet was washed with 500 µL of 70% ethanol, centrifuged at 7500 ×g for 5 minutes and allowed to air dry at room temperature and re-suspended in 100 µL water.

(ii) Running gel, blotting, UV crosslinking

A 1.5 % agarose gel was prepared as described in **Section 2.4.2**. Ten micrograms of DNA separated on agarose gel was photographed using Syngene G BOX Gel Documentation and Analysis system (Syngene, UK). The DNA was de-purinated by immersing the gels in 0.25 M HCl for 30 minutes followed by denaturation of DNA by incubation in denaturation buffer [Appendix, 5.1.6(c)] for 15-20 minutes. The gels were then briefly equilibrated in neutralising solution [Appendix,5.1.6(d)]. To transfer DNA on to the membrane, four 3 MM Whatman blotting papers and the Hybond™-N⁺nylon membrane (Sigma,MO,US) cut to the size of the gel were soaked in 20× SSC buffer [Appendix, 2(d)]. The gel was sandwiched by placing the two Whatman blotting papers on a glass plate, followed by the gel, then the Hybond™-N⁺nylon membrane and lastly the two Whatman blotting papers. The sandwich prepared above was flooded with 20×SSC buffer and a stack of towels put on top of it. Another glass plate was laid on top of the stack of paper towel, and this was left overnight.

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To cross-link the DNA on to the membrane, the nitrocellulose membranes were removed from the sandwich and UV irradiated in a UV Stratalinker1800 (Stratagene, USA) at 1200 mJ/cm² and baked for 3 hours at 80 °C.

(iii) Radioactive labelling and purification of the probe

To detect HBV DNA intermediates, probe with sequences 5' TAGGAATTCCATATGCCGCTGTCTTTT 3' was radioactively labelled. Labelling was done by setting up a reaction mixture catalyzing the transfer of radiolabelled Easytides® Adenosine 5' Triphosphate, [γ -³²P] (ATP, γ -³²P), to 5' OH of oligonucleotide. The reaction mixture was prepared through adding: T4 Polynucleotide Kinase (PNK) 1 × buffer A, 2 μ L of 10 μ M probe, 1 – 5 μ L ATP, γ -³²P and 1 μ L of PNK (Thermofischer scientific, CA, USA), the volume was adjusted to a 20 μ L with distilled water. The reaction was incubated at 37 °C for 45 minutes. The labelled probe was loaded onto a size exclusion Sephadex G75 column. To collect the purified probe, the column was centrifuged at 750 ×g for 2 minutes.

(IV) Hybridisation

To hybridise the probe to the blot, 10 mL hybridisation buffer was added, and the blot inserted into a roller bottle before pre-warming to 42 °C in a hybridisation oven for 10 minutes. Thirty microlitres of the probe prepared as described in **Section 2.10.1 (iii)**, was added into the roller bottle and the blot was allowed to incubate at 42 °C overnight rotating. This was followed by membrane wash with 50 mL 5 × SSC (Appendix ,5.1.1 (g)) containing 0.1% SDS at rtm for 20 minutes then two washes with 50 mL 1 × SSC containing 0.1% SDS at 42 °C for 15 minutes. The nylon membrane was wrapped with plastic, placed to the imaging plates while kept in the

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dark for 2 days. The images were developed using a Phosphoimager FLA-700 (Fujifilm, Tokyo, Japan) and processed using Multi gauge software.

2.10.2 Detection of HBV RNA transcripts by Northern blotting

(i) RNA extraction

To detect the HBV RNA transcripts, the liver tissue sections harvested from injected mice **Section 2.9.2** were used. Liver tissue in 50 mL centrifuge tube was homogenised immersed in 1 mL Trizol reagent (Sigma, MI, US) to lyse cells. Five hundred microlitres of cell lysates were transferred to a 2 mL microcentrifuge and to further lyse cells, the sample was pipetted up and down and incubated for 5 minutes at room temperature. Two hundred microlitres of chloroform was added, the sample was mixed vigorously by inverting for 15 seconds and then allowed to stand at rtm for 10 minutes. The sample was centrifuged at 12 000 ×g for 15 minutes and 400 µL of the upper aqueous solution was transferred to new tube, followed by addition of an equal volume of isopropanol.

The mixture was centrifuged at 12 000 ×g for 10 minutes at 4 °C and the RNA pellet washed with 500 µL of 75 % ethanol prepared in DEPC water [**Appendix**, 5.16 (d)]. The RNA pellet was allowed to dry then re-suspended in 100 µL of DEPC water and quantified using N80 Nanophotometer (Implen, Munich, Germany). The RNA was stored at -70 °C.

(ii) Running gel, blotting, UV crosslinking and hybridisation

One percent Formaldehyde agarose gel was prepared by adding 72 mL DEPC water, 10 mL of 10 × MOPS buffer and allowed to dissolve through heating. The gel was placed in a fume hood followed by addition of 18 mL of 37 % formaldehyde and

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0.5 µg/mL of ethidium bromide. The gel was poured into a tray and allowed to solidify. Prior to electrophoresis, 2 × RNA loading dye (ThermoFischer Scientific, CA, USA) was added into equal volume of 30 µg RNA and heated at 75°C for 5 minutes. The samples were placed on ice for 5 minutes, loaded into the formaldehyde agarose gel and resolved at 80 volts.

The gel was washed with DEPC water for 30 minutes followed by 10× SSC buffer for 30 minutes. The transfer of RNA to the nylon membrane was carried out as described in **Section 2.10**. Hybridisation of the RNA to the probe was carried out using a probe prepared in **Section 2.10.1(iii)** and performed as described in **Section 2.10.1**.

2.10.3 Quantification of HBV particles equivalents by qPCR

To quantify the HBV genome copy numbers from the mice injected with AAV vectors bearing HBV genome sequence, the serum from mice injected with AAV vectors under **Section 2.9.3** was used. Total DNA extraction was carried out using QIAamp DNA blood mini kit (Qiagen, Germany) as described in **section 2.7.3**. The qPCR as described previously **Section 2.7.3** was performed. To ascertain viral particles estimate count, HBV primers (HBsF and HBsR) were used for DNA amplification. HBV standards used for data analysis were acquired commercially (AcroMetrix® HBV Panel (Life Technologies, CA, US), and were stored at -80 °C. The AAV quantification was carried out using primer (AAV-F and AAV-R). The AAV standards used for data analysis were prepared for qPCR data analysis by serially diluting plasmid DNA (pAAV-A1, **Table 2.1**). The starting concentration before 10-fold serial dilutions were done was set at 10¹⁰ copies/µL, and molar mass per base pair. Quantification of total viral particles was done as described in **Section 2.8.1**

2.11 Application of the AAV-HBV mouse model for screening anti-HBV therapeutics in vivo

2.11.1 In vivo assessment of AAV-HBV and scAAV8 miR3-1/5,8,9-mediated toxicity

To assess the use of the AAV-HBV mouse model for screening of anti-HBV therapeutics, the AAV expressing an anti-HBV RNAi activator (scAAV8 pri-miR-31/5,8,9), which has proven to be effective in the inhibition of HBV replication, was used (Maepa et al., 2017). The safety of co-delivering AAV vectors containing HBV and scAAV8 miR-31/5, 8, 9 in mice was first evaluated.

Five mice per group were co-infected with AAV8-A1 or AAV8-D3 at 5×10^{11} or 9×10^{11} and scAAV8 miR-31/5,8,9 at 1×10^{11} . The other group was injected with saline only to serve as a negative control whereas the mice hydrodynamically injected with 100 μ g Poly I:C served as a positive control for inflammation. Six hours post injection a terminal bleed was performed, the serum was collected and used to quantify levels of interleukin 6 (IL-6), interleukin 10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon gamma (IFN- γ), tumour necrosis factor alpha (TNF- α) and interleukin 12p70 (IL-12p70) using CBA as in **Section 2.9.1,(i)**. Liver samples were harvested, and RNA was isolated for assessment of induction of *IFN- β* , *OAS1*, and *IFIT1* by RT-qPCR as in **Section 2.9.1,(i)**.

2.11.2 Evaluation of HBV knockdown in AAV-HBV mouse model

To assess the use of AAV-HBV animal model for screening of anti-HBV therapeutics, the antiviral efficacy of scAAV pri-miR-31/5,8,9 in mice infected with AAV8-A1 or AAV8-D3 was assessed. The mice were co-injected with 1×10^{11} of scAAV8 miR-

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31/5,8,9 or AAV8 expressing anti-HCV pri-miR (AAV8-mTTR-BCDE) and 5×10^{11} or 9×10^{11} of AAV8-A1 or AAV8-D3. The inhibition of HBV replication was measured by HBsAg ELISA (**Section 2.9.2**) and qPCR to quantify circulating viral particles as described in **Section 2.8.1**.

CHAPTER 3: RESULTS

3.1 Successful generation of AAV plasmids carrying greater-than-genome-length HBV

To enable cloning of the HBV genome fragment into the pAAV plasmid, two 32 bp oligonucleotide sequences with multiple restriction sites were annealed to form a double stranded DNA followed by cloning into a pAAV plasmid to generate pAAV-MCS. The presence of the 32 bp fragment in pAAV-MCS plasmid was confirmed through restriction analysis using the unique enzyme sites incorporated in the DNA fragment. The expected single fragment of 3200 bp was obtained when the plasmid was digested with *Sall*, *NotI*, *EcoRV* or *HindIII* (**Appendix, Fig 5.1 A**). The pAAV-MCS was subsequently used to clone the greater-than-genome-length DNA of HBV A1 and D3 sequences. The successful cloning of greater-than-HBV-genome length sequence into pAAV-MCS plasmid was confirmed through restriction analysis and the plasmids constructed were denoted as pAAV-A1 or pAAV-D3 for HBV A1 and HBV D3 genome-containing plasmid respectively (**Appendix, Fig 5.1 B**). The integrity of the inverted terminal repeats is essential for AAV genome packaging. Hence, their presence in pAAV-A1 and pAAV-D3, and successful cloning of the HBV genome was confirmed, by restriction analysis using *Eco88I* enzyme. Digestion of pAAV-A1 with *Eco88I* resulted in 4 fragments of sizes 2680 bp, 2121 bp, 1673 bp and 909 bp. Whereas the pAAV-D3 resulted in 6 fragments of sizes 2680 bp, 1336 bp, 970 bp, 876 bp, 785 bp, 697 bp, which is a positive indication of successful cloning of the greater-than-genome-length HBV. The 2680 bp fragment shows the integrity of the ITRs in both pAAV-A1 and pAAV-D3 plasmids (**Appendix, Fig 5.1 B**).

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The plasmids were further digested with multiple cutter restriction enzymes and resulted in expected number of bands and base pair sizes (**Appendix, Fig 5.1 B**). Digestion of pAAV-A1 with either *Dral* or *SmaI* or *MfeI* and *NdeI* resulted in the following fragments sizes, 2330 bp, 1933 bp, 1202 bp, 891 bp, 692 bp, 338 bp and 19 bp or 4664 bp and 2680 bp or 4194 bp and 3211 bp respectively. Restricting pAAV-D3 with either *Dral* or *SmaI* or *MfeI* and *NdeI* enzymes yielded the following base pair sizes, 2291 bp, 1933 bp, 1202 bp, 891 bp, 692 bp, 338 bp and 19 bp or 4664 bp and 2680 bp or 4143 bp and 3223 bp, respectively. The pAAV-MCS which was used as the backbone was also digested with either *Dral* or *SmaI* or *MfeI* and *NdeI* and yielded the following fragments, 2680 bp and 520 bp or 2680 bp and 3200 bp respectively. The fragments obtained from digestion of pAAV-A1 and pAAV-D3 showed that the greater-than-genome-length HBV sequence of genotype A1 and D3 were successfully cloned.

To assess if pAAV-A1 and pAAV-D3 plasmids constructed can mediate HBV gene expression under HBV basal core promoter and enhancers, HBsAg expression was measured in cultured liver-derived Huh 7 cells. Huh 7 cells were co-transfected with plasmids carrying HBV genome sequences (pAAV-D3, pAAV-A1 or pCH-9/3091) or pAAV-empty and pAAV-CAG-GFP. pCH-9/3091 (**Table 2.1 /Section 2.6.2**), pAAV-empty (**Table 2.1/Section 2.6.2**) and pAAV-CAG-GFP (**Table 2.1/Section 2.6.2**) were used as a positive control, negative control and to assess transfection efficiency, respectively. At 24 hours post transfection, expression of GFP was detected in all transfected wells, indicating a successful and efficient transfection (**Appendix, Fig 5.2**). Assessment of HBV gene expression by HBsAg ELISA at 48 and 72 hours post transfection showed significant levels of HBsAg in supernatants of Huh 7 cells transfected with pAAV-A1, pAAV-D3 or pCH-9/3091 as compared to

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levels obtained after transfection with pAAV-empty plasmid (**Fig 3.1**). These results demonstrate that the plasmids carrying the genomes of subgenotype A1 or D3 were successfully cloned and resulted in HBV gene expression.

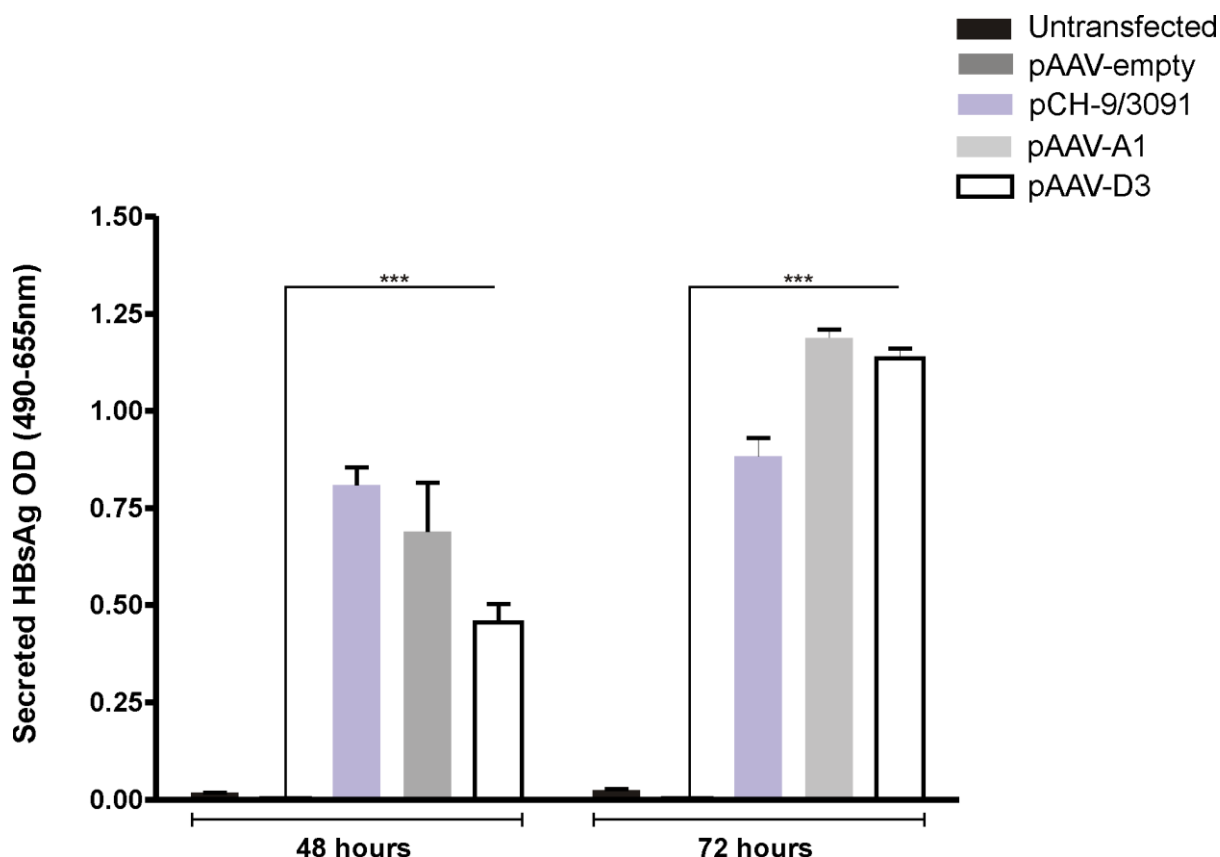


Figure 3.1: Assessment of HBV gene expression from pAAV-A1 and pAAV-D3.

Huh 7 cells were transfected with pAAV-A1, pAAV-D3, pCH-9/3091 or pAAV-empty plasmid. The quantification of HBsAg was performed using ELISA at 48 and 72 hours post transfection. Values represent mean and $\pm$ standard error of mean (SEM) of triplicate transfections. Statistically significant differences between samples were calculated using a Student's two tailed paired test. $***P \leq 0.001$ was considered statistically significant.

3.2 Large-scale production of AAV2 and AAV8 vectors

Recombinant AAV vectors produced in this study were pseudo-typed with serotype 2 (AAV2) or serotype 8 (AAV8) capsids for use in vitro and in vivo studies, respectively. To model HBV replication in vitro and in vivo, vectors of serotype AAV2 and AAV8 carrying the subgenotype A1 sequence (AAV2-A1 and AAV8-A1), the

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genotype D3 sequence (AAV2-D3 and AAV8-D3) or empty vector (AAV2-empty and AAV8-empty) were produced in three large scale batches and viral particle equivalents (VPEs) quantified by qPCR and the quantities of the vector obtained were within $2.2\text{E}+11$ - $1.8\text{E}+13$ VPEs/ml. The vectors produced were sufficient for in vitro and in vivo studies (**Table 3.1, Appendix, Table 5.1 and 5.2**). For evaluation of the application of AAV-based HBV replication model in screening the anti-HBV novel therapies, vectors of serotype AAV2 and AAV8 carrying a self-complementary (sc) genome encoding anti-HBV pri-miR (scAAV2 miR-31/5,8,9 and scAAV8 miR-31/5,8,9) or anti-HCV pri-miR (AAV2-mTTR-BCDE and AAV8-mTTR-BCDE) were produced in three large scale batches and quantified by qPCR. The titre ranges of $5.43\text{E}+10$ - $4.72\text{E}+12$ VPEs/ml were obtained (**Table 3.1, Appendix, Table 5.1 and 5.2**).

Table 3.1: Recombinant AAV vector first batch yields quantified by qPCR

First batch			
AAV vectors	VPEs/mL	Volume of prep (mL)	Total VPEs
AAV2-A1	4.42E+12	1.0	4.42E+12
AAV2-D3	1.8E+13	1.2	2.16E+13
AAV-mTTR-BCDE	2.21E+12	1.3	2.86E+12
scAAV2miR-31/5,8,9	2.160E+12	1.2	2.59E+12
AAV2-empty	2.2E+12	1.0	2.2E+12
AAV8-A1	9.58E+12	1.5	1.16E+13
AAV8-D3	1.18E+13	1.2	1.4E+13
scAAV8miR-31/5,8,9	5.43E+10	1.5	8.145E+11
AAV8-mTTR-BCDE	4.7E+12	1.0	4.7E+12
AAV8-Empty	2.6E+12	1.2	3.12E+12

3.3 Evaluation of AAV-HBV model in vitro

3.3.1 AAV2 carrying HBV genome sequence mediate HBV gene expression in liver-derived cell line

HBV gene expression from AAV vectors bearing HBV genome sequences (A1 or D3) was determined in Huh 7 cells. The recombinant AAV vectors (AAV2-A1, AAV2-D3 or AAV2-empty) were used to infect Huh 7 cells in quadruplicate, followed by determination of HBV gene expression through detection of HBsAg. Following transduction with AAV2-A1, AAV2-D3 or AAV2-empty, an HBsAg ELISA was carried out at 48 hours post infection. The supernatant of cells infected with AAV2-A1 or

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AAV2-D3 resulted in significant levels of HBsAg secretion, whereas the non-transduced cells and cells infected with AAV2-empty did not (**Fig 3.2**).

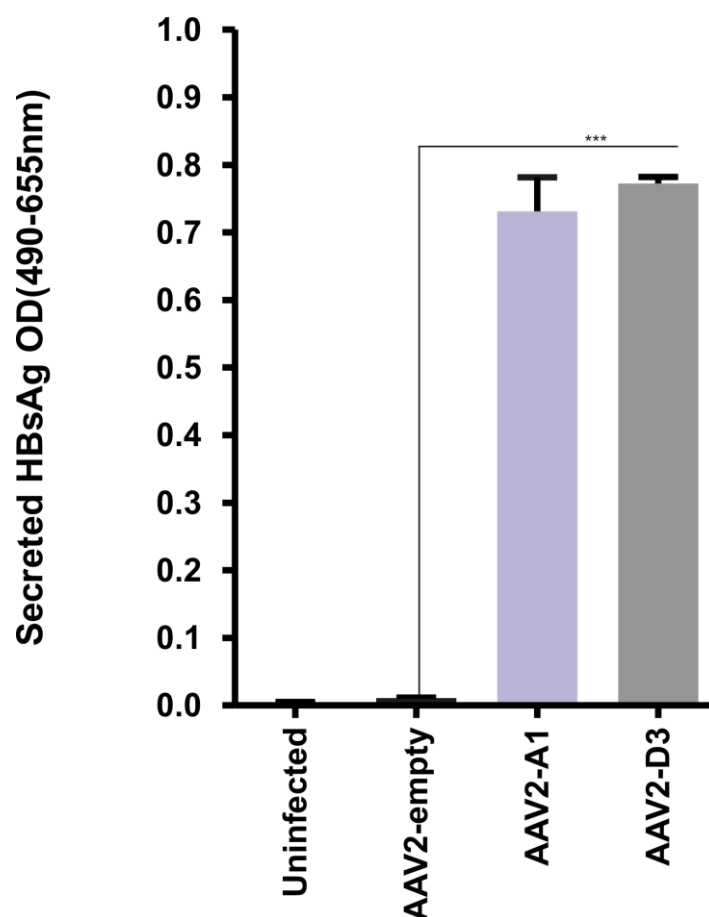


Figure 3.2: HBsAg expression in Huh 7 cells transduced with HBV genome bearing AAVs.

Huh 7 cells were left uninfected or infected with AAV2-A1, AAV2-D3 or AAV2-empty. The quantification of HBsAg was performed by ELISA using the supernatant of the cell culture at 48 hours post infection. Values represent mean and standard error of mean ($\pm$ SEM) of quadruplicate infections. Statistically significant differences between samples were calculated using a Student's two tailed paired t-test. *** $p \leq 0.001$ were considered statistically significant.

To further confirm that recombinant AAV vectors carrying HBV genome sequence result in HBV gene expression, Immunofluorescence staining was conducted to

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detect HBcAg expression in Huh 7 cells infected with AAV vectors (**Fig 3.3**). The nucleus was stained with DAPI. The cells transduced with AAV vectors carrying HBV genome sequence resulted in both blue signal from DAPI and green signal from HBcAg expression which was not observed from non-transduced cells. Significant expression of HBcAg was detected in cells transduced with AAV2-A1 or AAV2-D3. Taken together, this data suggests that the AAV vectors produced are able to transduce liver-derived cell line and results in HBV gene expression.

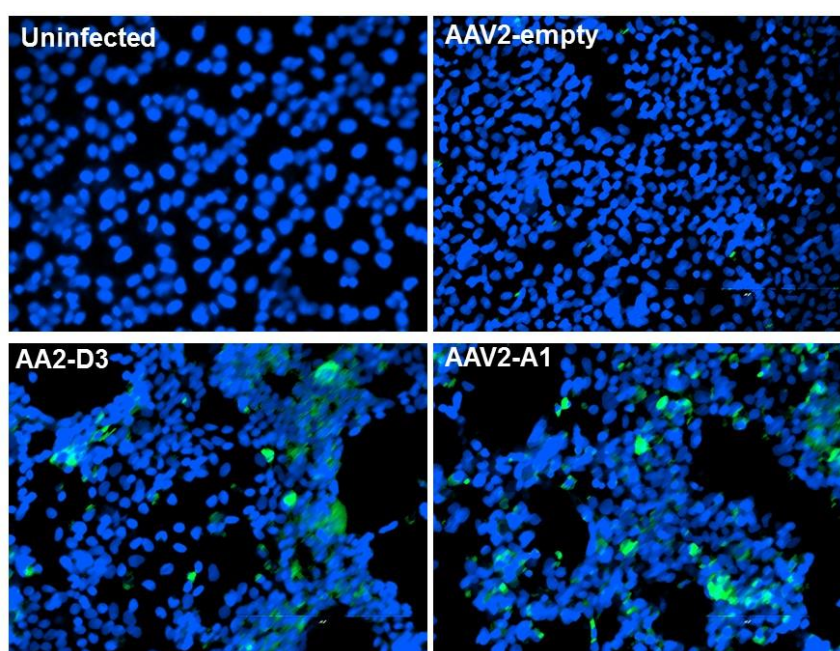


Figure 3.3: Detection of HBcAg in Huh7 cells infected with AAV-HBV vectors using immunofluorescence.

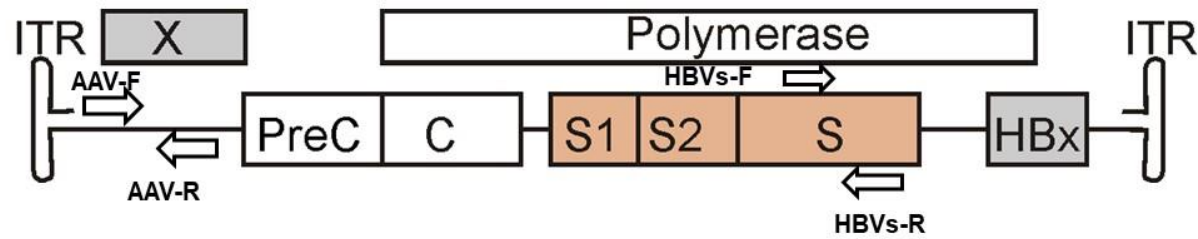
Huh 7 cells were infected with AAV vectors carrying HBV genome sequence of A1 or D3 or the AAV vector without HBV genome sequence (AAV2-empty) and some wells left uninfected. An anti-HBcAg primary antibody followed by fluorescently labelled secondary antibody was used to detect the HBcAg and imaged using fluorescence microscopy. The nuclei were stained with DAPI and cells were imaged at 20× magnification. The HBcAg is shown in green.

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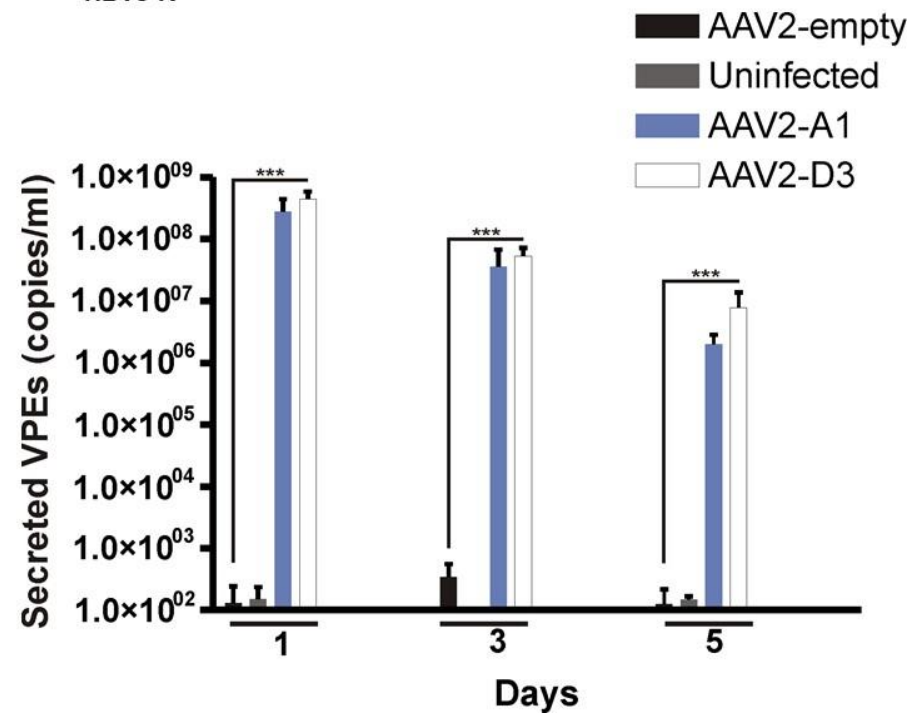
3.3.2 HBV genome carrying AAV2 mediates viral replication in liver-derived cell lines

To assess whether the AAV vectors carrying HBV genomes will result in HBV production and replication in vitro, secreted VPEs were quantified in AAV transduced Huh 7 cells. DNA was extracted from supernatant collected on day 1, 3 and 5 and subjected to qPCR. The primers designed to bind the S region of HBV (HBVs-F and HBVs-R **Table 2.2**) were used to determine total VPEs and another set binding the AAV sequence (AAV-F and AAV-R **Table 2.2**) were used to quantify AAV genome sequences (**Fig 3.4A**). The amount of VPEs was determined as the difference between the total VPEs and the amount of AAV obtained. Compared to uninfected cells or cells infected with AAV-empty, significant numbers of VPEs in supernatant of cells infected with AAV2-A1 and AAV2-D3 were detected (**Fig 3.4 B**). The observed data indicate that the AAVs bearing HBV genome sequence are able to mediate HBV production and replication in cultured cells.

A



B



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Figure 3.4: Liver-derived cells transduced with HBV genome bearing AAV vectors result in HBV replication.

A: The HBV greater-than-genome length of subgenotype A1 or D3 was generated through subcloning it into pAAV vector containing ITR of AAV serotype 2 at both ends. The PCR primers for quantification of AAV (AAV-F and AAV-R) and HBV (HBs-F and HBs-R) are indicated. **B:** Huh 7 cells were infected with AAV2-A1 or AAV2-D3 or AAV2-empty at a MOI of 1×10^4 . The supernatant concentration of VPEs were quantified by q-PCR. The data represent the mean difference between total VPEs and AAV VPEs. The Standard error of mean ($\pm$ SEM) was derived from n=4. Statistically significant differences between samples were calculated using student's two tailed paired test. *** $P \leq 0.001$.

3.4 In vivo generation and characterisation of AAV-HBV A1 model

Given that the in vitro results demonstrated that AAVs carrying an HBV genome sequence can support HBV gene expression and replication, an in vivo study for characterisation of the AAV-HBV model was carried out. To characterise the AAV-HBV A1 replication model, the AAV vectors bearing HBV genome sequence were delivered in mice through tail vein injection.

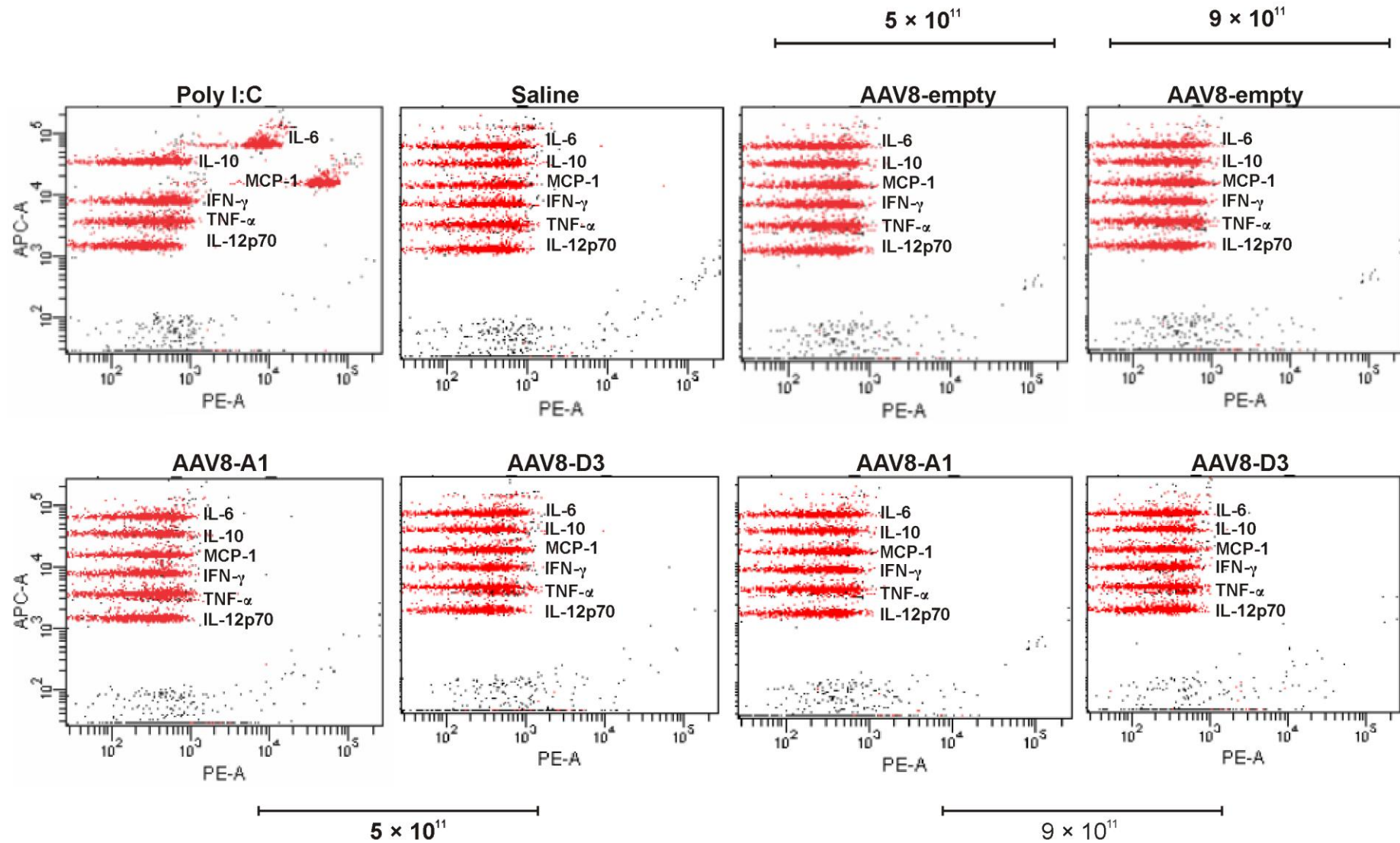
3.4.1 Administration of AAV-HBV vectors at low (5×10^{11}) and high (9×10^{11}) dose in NMRI mice showed no toxic immunostimulatory effects

To determine the ideal dose of HBV genome-bearing AAVs to use, a pilot study was performed first. The following doses were evaluated for the expression of HBV proteins: 1×10^9 , 1×10^{10} and 1×10^{11} VPEs/mouse. Only at a dose of 1×10^{11} VPEs/mouse was significant expression of HBsAg from AAV8-D3 detected (**Appendix, Fig 5.3**). However, this requires 100 μ l of undiluted serum to be used. Therefore, a high volume of the blood (~ 500 μ l minimum, which gives ~ 250 μ l of serum) is required to have sufficient serum for HBsAg assays (100 μ l), toxicity assays (100 μ l) and circulating HBV particles quantification (≥ 50 μ l). Bleeding 500 μ l of blood from a 30-35 g (average weights of the NMRI mice at injection) is $\sim 3\times$ over the allowed 7.5 % of circulating blood volume and it poses the risk of distress, pain and death to the mice. Based on the results obtained from the pilot study, it was therefore decided to inject the mice with higher doses of 5×10^{11} VPEs/mouse or 9×10^{11} VPEs/mouse. The safety of these vector doses in mice was determined by measuring the induction of pro-inflammatory markers using a CBA. The concentrations of interleukin 10 (IL-10), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α), monocyte chemoattractant protein-1 (MCP-1), interferon gamma

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(INF- γ) and interleukin 12p70 (IL-12p70) were measured in serum samples of mice 6 hours post injection (**Fig 3.5 and 3.6**). Mice injected with saline served as a negative control whereas poly I:C injected mice were used as positive control for innate immune response. The concentration of all pro-inflammatory markers in all AAV-injected mice were not statistically different from mice treated with saline (**Fig 3.6**). As expected, the levels of all the cytokines in mice injected with poly I:C were significantly higher than levels in saline and AAV injected mice.

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Figure 3.5: Assessment of pro-inflammatory response in NMRI mice post injection of AAV vectors.

Representative dot blots of cytometric bead array to quantify interleukin 10 (IL-10), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1), interferon gamma (IFN- γ) and interleukin 12p70 (IL-12p70). Measurements were carried out 6 h post-injection of AAV vectors at 5×10^{11} or 9×10^{11} VPEs/mouse. Poly I: C and Saline served as positive and negative controls respectively. APC-A: Allophycocyanin and PE: Phycoerythrin.

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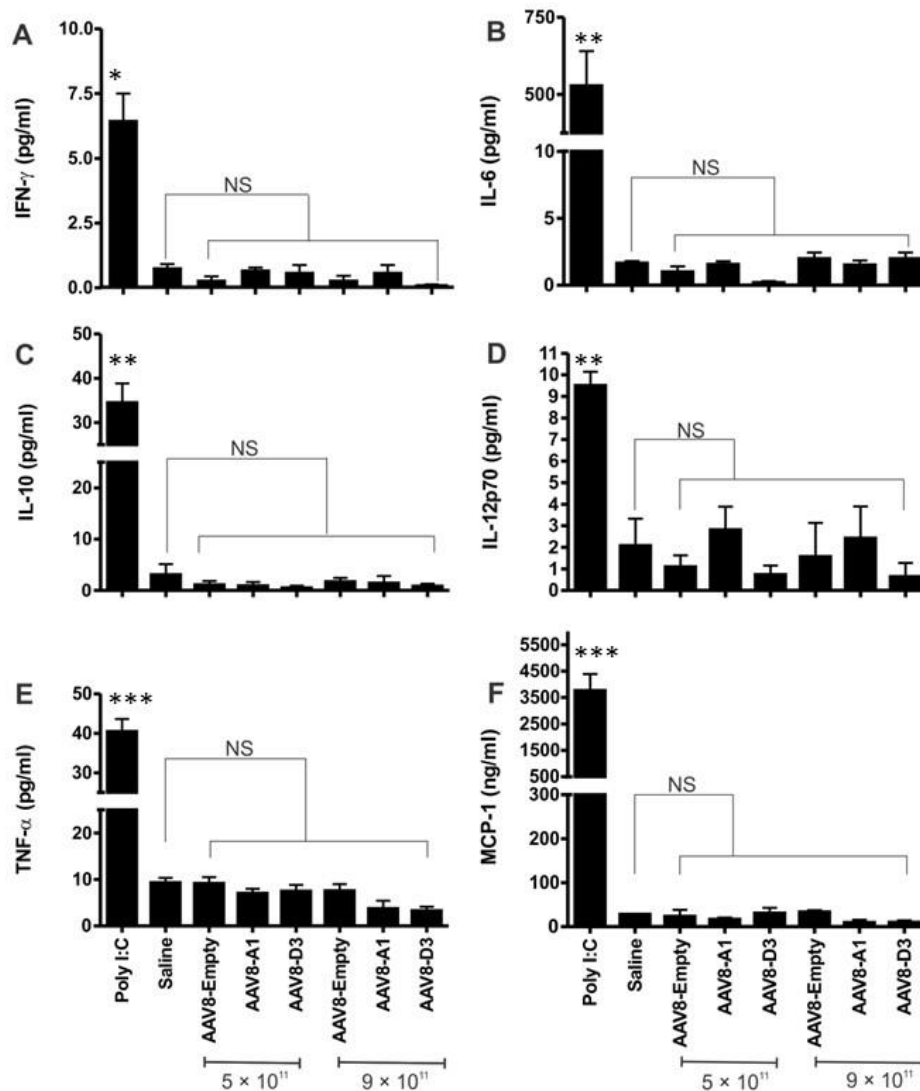


Figure 3.6: Quantification of cytokine levels in NMRI mice post injection with AAV vectors.

Cytometric bead array assay was performed to quantify interleukin 10 (IL-10), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α), chemoattractant protein 1 (MCP-1), interferon gamma (IFN- γ) and interleukin 12p70 (IL-12p70). Measurements were carried out 6 h post injection of AAV vectors at 5×10^{11} or 9×10^{11} VPEs/mouse, Poly I: C or saline. Statistically significant differences between samples were calculated using Student's two tailed paired t-test. Mean concentrations $\pm$ SEM were calculated from injection of four mice per group. * $P \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$ was considered statistically significant NS: non-significant.

The safety of delivering high (9×10^{11} VPEs/mouse) and low (5×10^{11} VPEs/mouse) AAV vector doses was further confirmed by carrying out an RT-qPCR for quantification of additional innate immune stimulation markers. The RNA extracted

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from the livers of mice injected with AAV vectors was used to measure the level of mRNA derived from *oligoadenylate synthase (OAS-1)*, *interferon beta (IFN- β)* and *interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)* gene relative to Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA. The RNA from the mice injected with poly I: C was used as a positive control. Levels of *OAS-1*, *IFN- β* and *IFIT1* mRNA from the mice injected with a high or low dose of AAV vector were similar to the mice treated with saline. The level of RNA from mice injected with AAVs and negative control were significantly lower than the levels in mice injected with poly I:C (**Fig 3.7**). The data thus suggest that neither high nor low AAV vector doses induced the innate immune response.

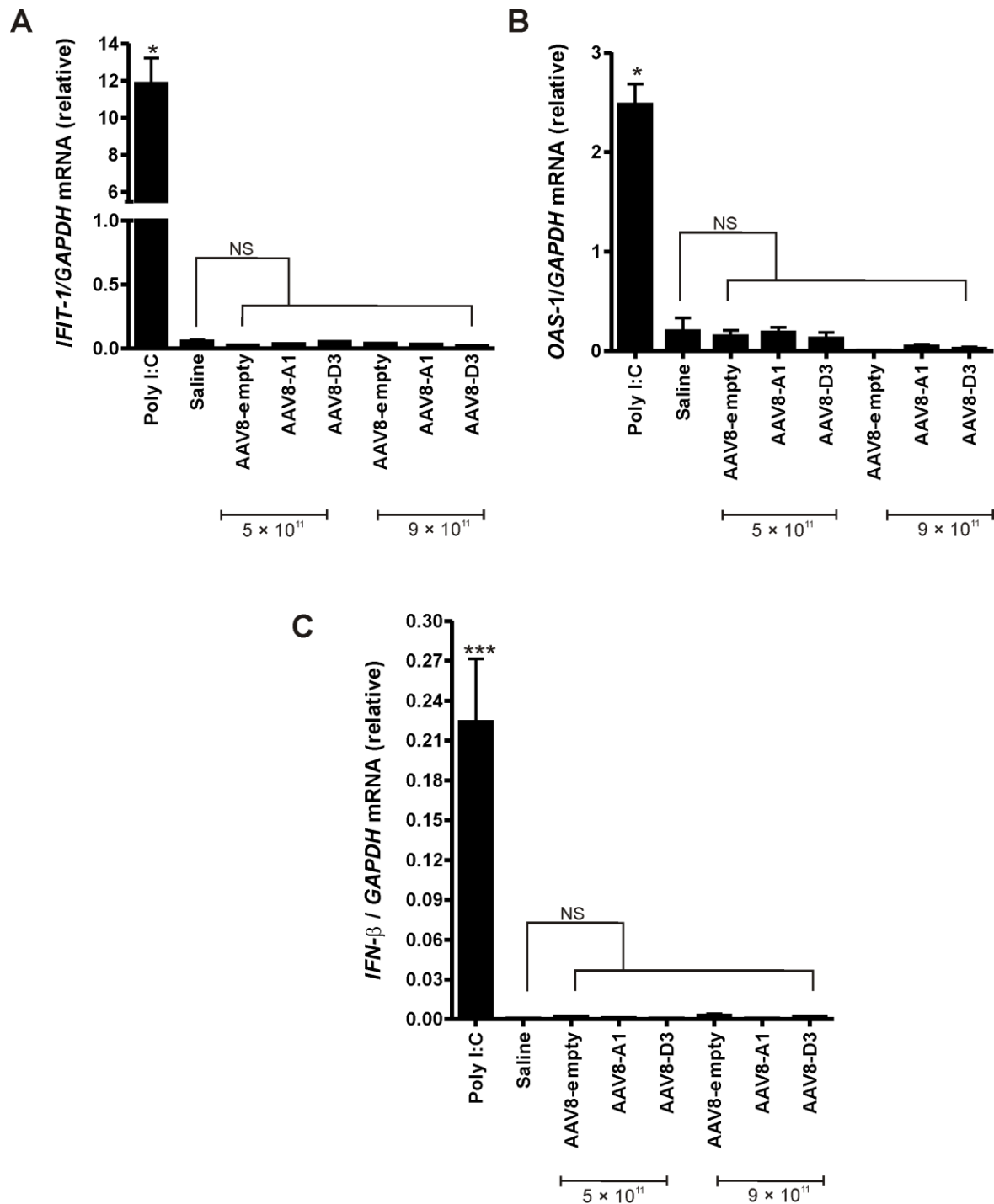


Figure 3.7: Assessment of interferon response stimulation in NMRI mice by RT-qPCR following intravenous administration of AAV vectors.

Liver RNA was prepared from livers collected 6 hours post-injection of mice with saline or poly I:C or AAV8. The RNA samples were subjected to RT-qPCR to measure level of mRNA from *OAS-1*, *IFN-β*, *IFIT-1* relative to *GAPDH* genes. Data shown is presented as the ratio of mRNA from interferon response genes to *GAPDH*. Values represent mean and $\pm$ SEM from four mice per group. Statistically significant differences between samples were determined using Student's two tailed paired t-test, * $P \leq 0.05$; *** $P \leq 0.001$ was considered statistically significant, NS: non-significant.

3.4.2 AAVs carrying HBV genome do not result in liver toxicity

Alanine transaminase (ALT) enzyme levels from the serum of mice with AAV vectors at 5×10^{11} or 9×10^{11} VPEs/mouse collected at 2, 4 and 8 weeks post injection was used to assess liver damage. The normal range of ALT is from 10-40 IU/L and the level above 100 IU/L is indicative of toxicity as per the NHLS guidelines (Wang et al., 2012). ALT levels in all the groups were well below the threshold of 100 IU/L. However, mice injected with either high or low dose of AAV8-A1 or AAV8-D3 resulted in ALT levels higher than in mice injected with Saline or AAV-empty and were also above 40 IU/L at two weeks post injection. However, these levels return to control levels over 8 week's period (**Fig 3.8**). This indicates little hepatocellular damage or toxicity associated with the effects of HBV gene expression from AAV8-A1 or AAV8-D3 which is reversed over time.

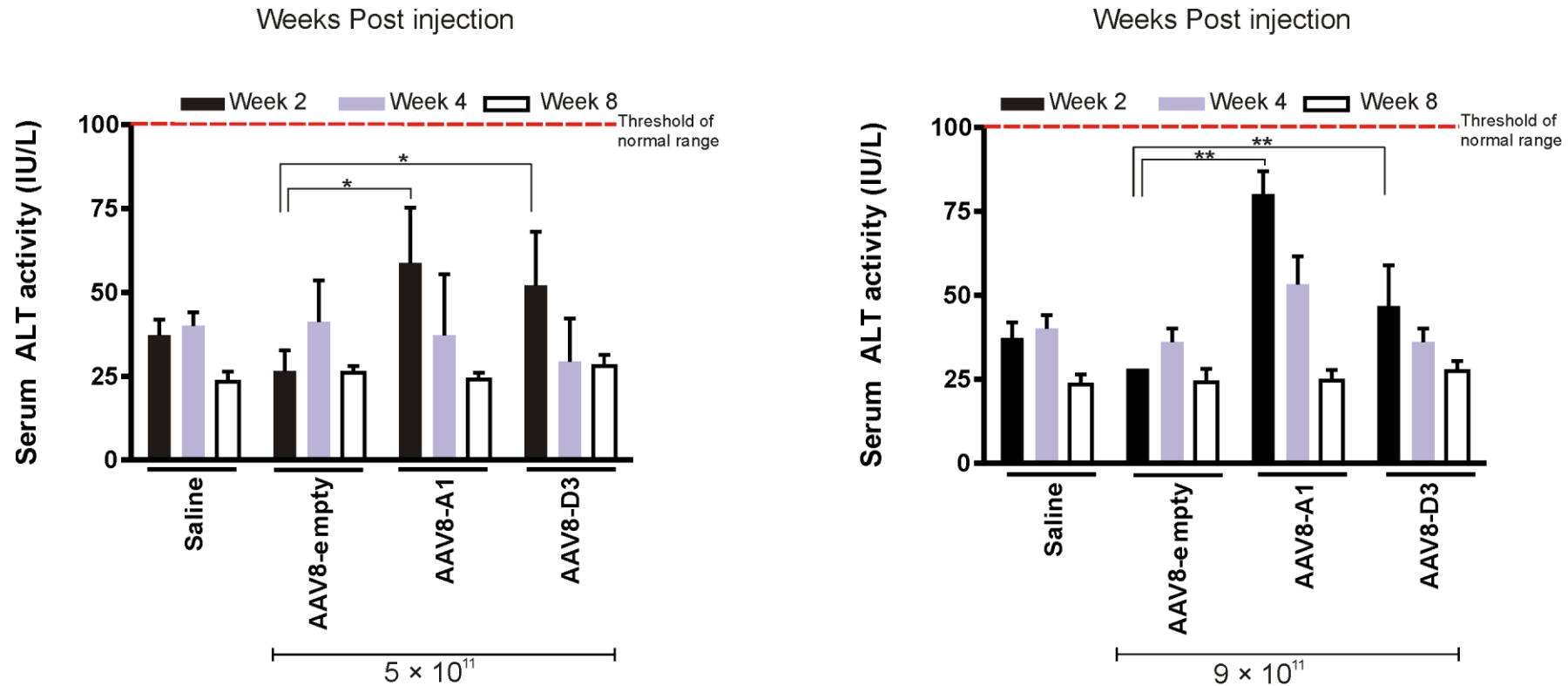


Figure 3.8: ALT activity in mice following administration of high or low dose AAV vectors.

Serum collected from mice treated with saline or AAV-empty or AAV8-A1 or AAV8-D3 at weeks 2, 4 and 8 post injection was assayed for ALT activity. Means and $\pm$ SEM were calculated from data obtained from four mice. Statistically significant differences between samples were calculated using Student's two tailed paired t-test. *P $\leq$ 0.05 and **P $\leq$ 0.01 were considered statistically significant. The red dotted line indicates threshold of accepted normal range < 100 IU/L.

3.4.3 Transduction of mice liver with AAV8-HBV vectors results in HBV gene expression

To assess the dose-dependent response of AAV vector mediated HBV gene expression in mice injected with 9×10^{11} or 5×10^{11} VPEs/mouse of AAV8-A1 or AAV8-D3, the level of HBsAg secreted was measured in serum collected at 2, 4, 8, 12 and 24 weeks post injection using ELISA. The mice administered with Saline or AAV8-empty served as negative controls. Sustained expression of HBsAg was detected in mice injected with AAV vectors carrying the HBV genome (AAV8-A1 or AAV8-D3). The mice injected with 5×10^{11} VPEs/mouse of AAV8-A1 resulted in low level expression of HBsAg as compared to the mice injected with 9×10^{11} VPEs/mouse at all-time points. The mice injected with AAV8-D3 resulted in similar HBsAg expression at both high and low dose infection. Although decreasing over time, significant HBsAg expression was sustained over the experiment period of six months when using both doses of either AAV-A1 or AAV-D3 (**Fig 3.9**).

HBV gene expression in mice was further confirmed by detection of HBcAg in the liver. Sectioned liver samples collected at 8 and 24 weeks post AAV infection were subjected to immunohistochemical staining of HBcAg. Successful expression of HBcAg was observed in mice injected with AAV carrying the HBV genome. The HBcAg was mainly located in the nuclei of the livers of mice injected with AAV8-A1. Interestingly, the HBcAg from livers of mice injected with AAV8-D3 was mostly found in the cytoplasm. As expected, the sections of the liver collected from mice injected with saline or AAV-empty did not show any HBcAg staining (**Fig 3.10**). Collectively, these data confirm that the AAV vectors used can efficiently transduce hepatocytes of mice and promote HBV gene expression over a period of 6 months.

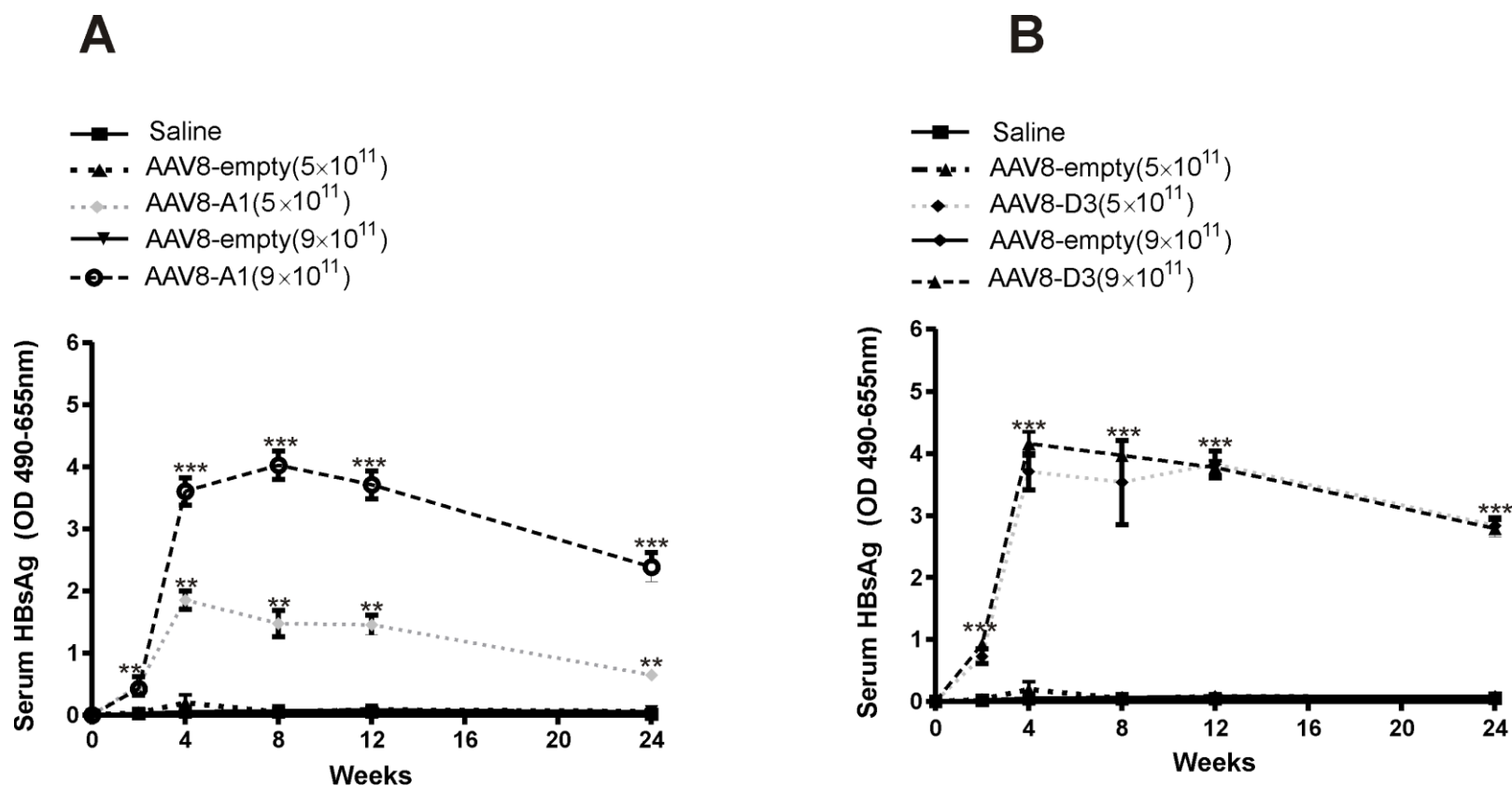


Figure 3.9: HBsAg expression in NMRI mice post injection of AAV vectors.

Serum collected from mice treated with saline or empty AAV–empty or AAV8-A1 or AAV8-D3 from 2 to 24 weeks was used to measure secretion of HBsAg using ELISA. Means and $\pm$ SEM were calculated from data obtained from 8 mice per group. Statistically significant differences between samples were calculated using Student's two tailed paired (were n is equal between the groups) or unpaired t-test (when n is not equal between the groups). *** $P \leq 0.001$.

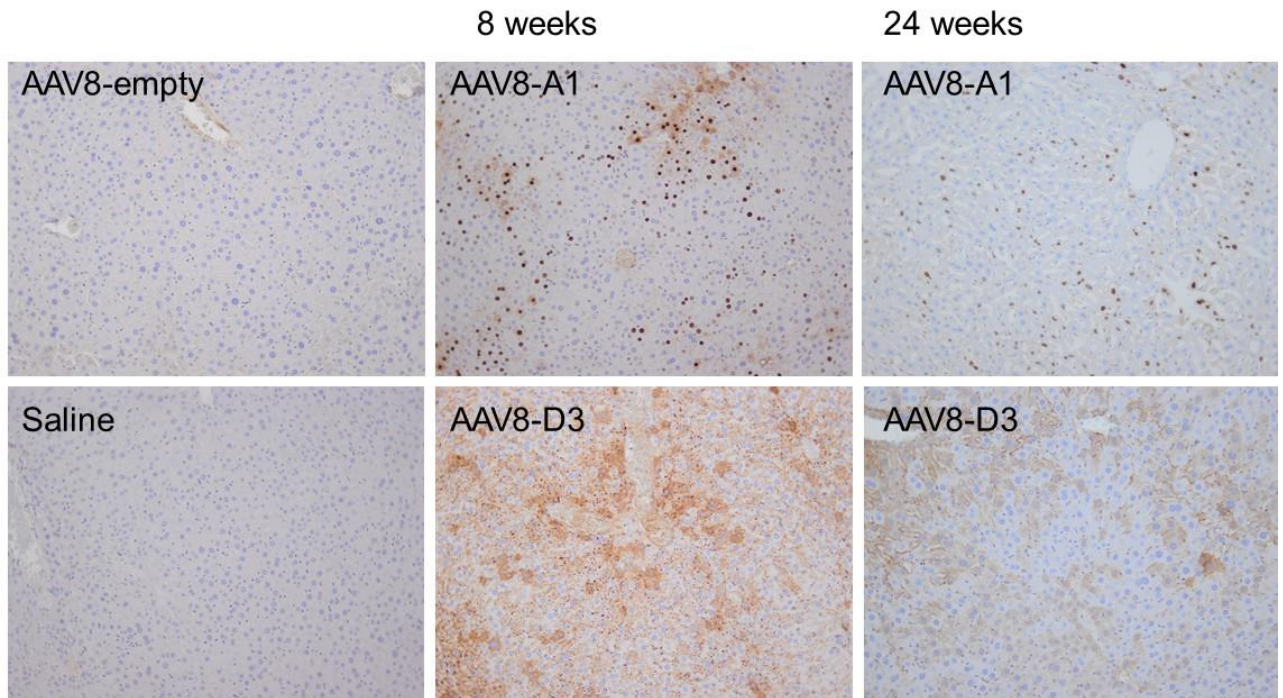


Figure 3.10: Immunohistochemical staining of HBcAg in liver sections of mice injected with AAV vectors.

Representative images showing HBcAg-immunopositive hepatocytes were taken from the liver sections of mice at 8 and 24 weeks post treatment with saline or (AAV8-empty or AAV8-A1 (9×10^{11}) or AAV8-D3 (9×10^{11}). Staining was performed with tissue sections from three mice per group. Brown stain shows HBcAg positive cells.

3.4.4 AAV vector-mediated HBV replication in vivo

HBV production and replication following transduction with AAV-HBV vectors was assessed by measuring circulating viral particle equivalents (VPEs), replicative DNA intermediates and RNA transcripts using qPCR, Southern blotting and Northern blotting respectively. Following injection of AAV vectors, assessment of circulating VPEs was carried out using serum collected at 2, 4, 8, 12 and 24 weeks post

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infection as positive indicators of HBV production and replication. Administration of either low or high dose of AAV vectors carrying HBV genomes resulted in significant levels of VPEs circulating in mice. The circulating VPEs appeared to be decreasing over time, however, levels stabilised from week 12 until termination of the experiment at 6 months (**Fig 3.11**). These results correlate with the trend of HBsAg levels (**Fig 3.9**) and suggest that HBV production and replication is sustained over a long period. The Southern blot analysis of the total genomic DNA from the livers of mice injected with AAV vector bearing HBV genome successfully revealed HBV replicative DNA intermediates, however, this was not observed from the DNA of mice liver harvested 8 weeks post injection with AAV vectors (**Appendix, Fig 5.7**). The relaxed circular DNA (RC) intermediates and double stranded linear (DL) were detected (**Fig 3.12 A**). However, the covalently closed circular (ccc) DNA was not detected. These might be a result of the fact that mice produce little or no cccDNA (Guidotti et al., 1994). Further confirmation of HBV replication was carried out using Northern blot analysis of RNA extracted from mice liver. The Northern blot analysis revealed the HBV RNA transcripts (3.5, 2.4 and 2.1 kb) produced during replication cycle (**Fig 3.12 B**). All together these results suggest that the HBV DNA production and transcription in the mice liver occur.

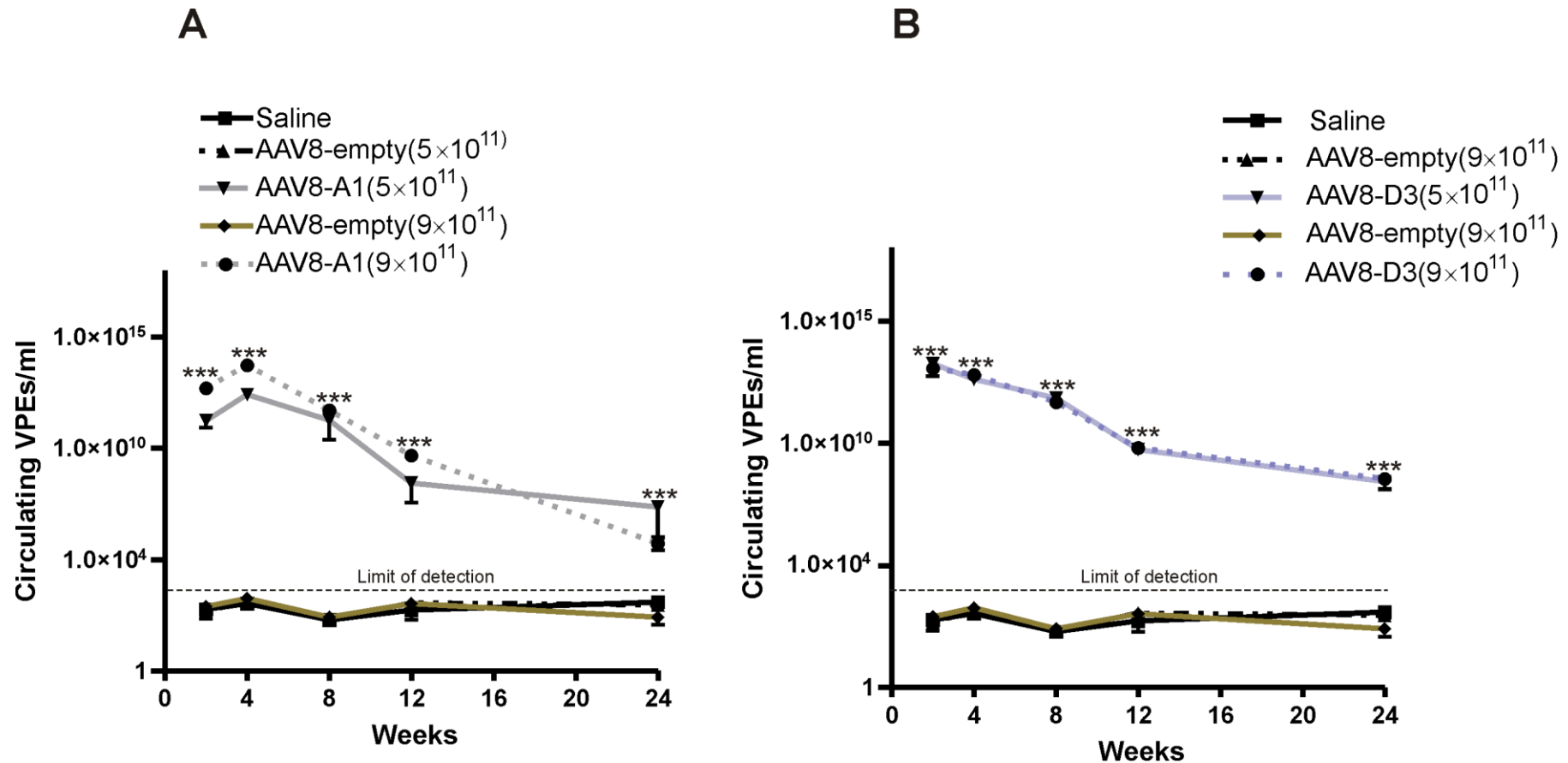


Figure 3.11: Measurement of HBV VPEs in NMRI mice injected with AAV-HBV.

Blood samples were collected at 2, 4, 8, 12 and 24 weeks post-injection of NMRI mice with saline or AAV vectors. The serum concentrations of HBV VPEs were detected by qPCR. Values represent the means and SEM calculated from data obtained from 4 mice per group. Statistically significant differences between samples were calculated using Student's two tailed paired test. *** $P \leq 0.001$ was considered statistically significant. The values above the assay background are considered positive detection.

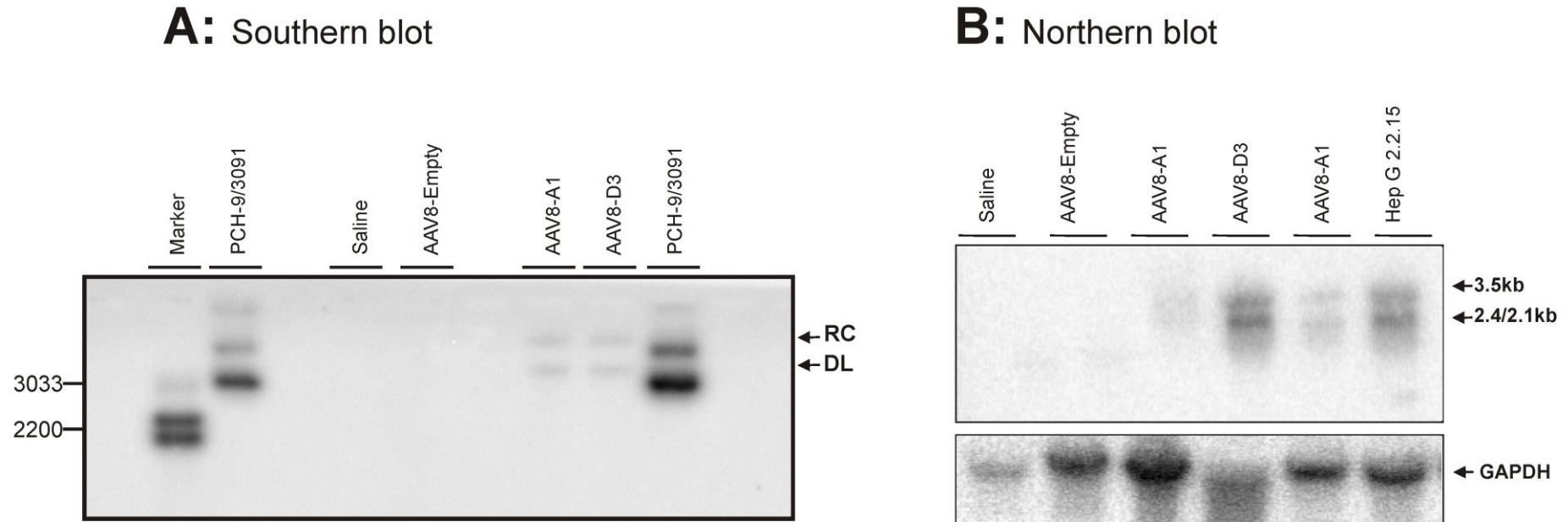


Figure 3.12: Southern and Northern blot Analysis of HBV replication and transcription in vivo.

A. Detection of HBV intermediates. Genomic DNA from livers of mice injected with saline or AAV8-empty, or AAV8-A1 or AAV8-D3 was extracted 24 weeks post injection. The pCH-9/3091 plasmid carrying HBV sequence was also included on agarose gel as the control to show probe hybridisation. The relaxed circular (RC) and double stranded linear (DL) are indicated. **B.** RNA extraction from the liver of mice injected with saline or AAV8-empty, or AAV8-A1 or AAV8-D3. RNA extracted from Hep G 2.2.15 cells was included as positive control to show the transcripts produced. Northern blot analysis for detection of HBV transcripts, pre-genomic RNA (3,5 kb) and, 2,4 kb and 2,1 kb subgenomic RNAs in mice of the livers 6 months post injection are indicated. Blot was probed with *GAPDH* specific probe as the loading control.

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3.4.5 Long term HBV gene expression from AAV-HBV vectors mediate minimal liver pathology

To assess whether prolonged HBV gene expression in mice results in liver pathology, haematoxylin and eosin (H&E), and Sirius Red staining were performed on tissue sections prepared at 6 months post infection with 9×10^{11} AAV VPEs/mouse. Haematoxylin and Eosin stained tissue sections from mice were assessed using criteria published by Liang and colleagues (Liang et al., 2014). Presence of the following variables were evaluated: macrovesicular steatosis, microvesicular steatosis, cell hypertrophy, inflammation, and fibrosis. After H&E staining no macro/microvesicular steatosis or cellular hypertrophy in mice injected with saline or AAV8-empty was observed. Very minimal inflammation was noted within hepatic lobules from mice injected with AAV8-D3 and was considered within normal limits (**Fig 3.13**).

A Sirius red stain of collagen was used to detect fibrous connective tissue. Following Sirius red staining, collagen deposition surrounding vessels was considered normal and hence none of the mice injected with AAV vectors demonstrated pathological fibrosis (**Fig 3.13**).

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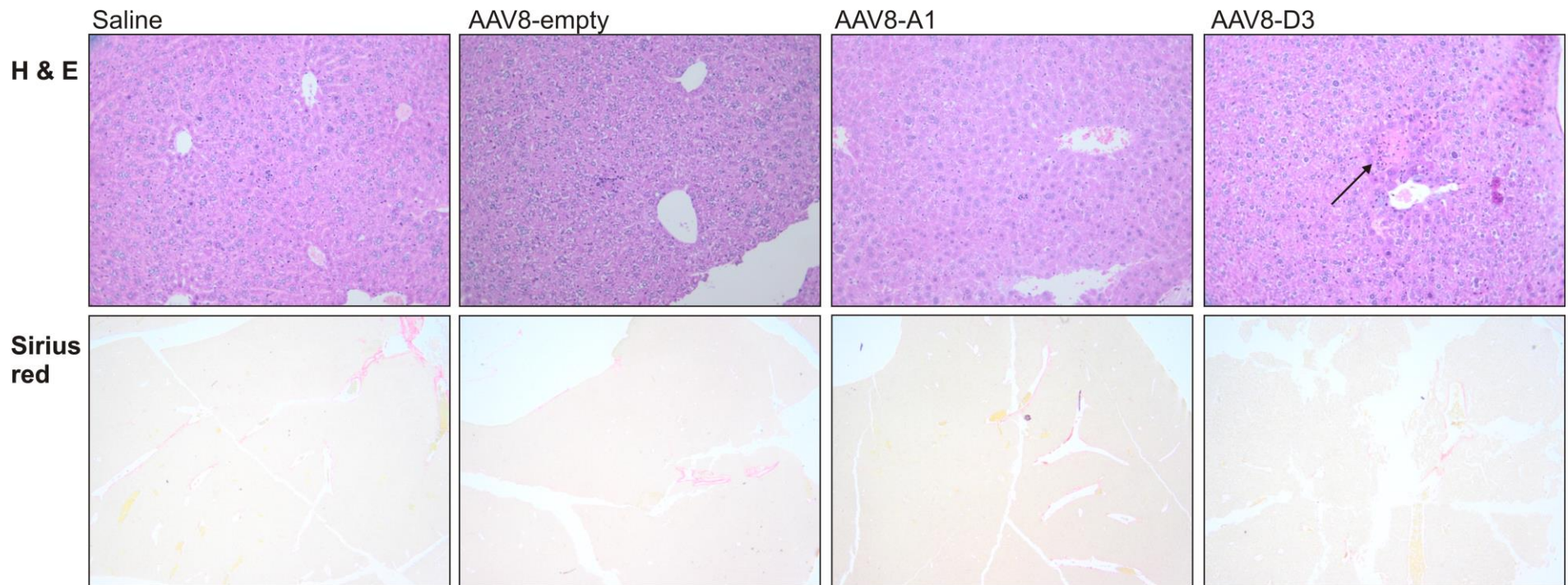


Figure 3.13: Injection of mice with AAV vectors results in minimal inflammation and no liver damage.

Liver sections were collected from the mice 24 weeks post injection with AAV8-A1 or AAV8-D3 or AAV8-empty or saline and stained with H&E or Sirius red. The black arrow indicates minimal inflammation with blue dots representing the nuclei of infiltrating mononuclear lymphocytes. The red colour indicates collagen fibres.

3.5 Successful application of AAV-HBV model to validate anti-HBV gene silencing

The application of AAV-HBV model in assessing the efficacy of anti-HBV therapeutics was explored through co-infecting the mice or cells with AAV vectors carrying HBV genome and AAV vectors carrying anti HBV RNAi activators. The HBV gene expression knockdown was evaluated, and the results are presented below:

3.5.1 HBsAg expression from AAV-HBV vectors in a liver-derived cell line can be suppressed by anti-HBV pri-miR

The application of the AAV-HBV model to screening anti-HBV therapeutics was assessed in cultured cells by co-infection of the Huh 7 liver-derived line with AAV2-A1 or AAV2-D3 and AAVs expressing artificial primary-microRNAs that target the HBV X region [(scAAV2 miR-31/5,8,9, **Appendix, Fig 5.5**] (Maepa et al., 2017)]. Cells co-infected with AAV2-A1 or AAV2-D3 and AAV2 vector expressing anti-HCV pri-miR [AAV-mTTR-BCDE, (Bourhill et al., 2016)] was used as a negative control. Suppression of HBV replication was evaluated by detecting the HBsAg levels from supernatant of the cultures. In comparison to cells co-infected with AAV8-mTTR-BCDE, AAVs encoding anti-HBV sequences effected significant HBsAg knockdown, with about 97% silencing observed within 48 hrs for both subgenotype A1 and D3 (**Fig 3.14**).

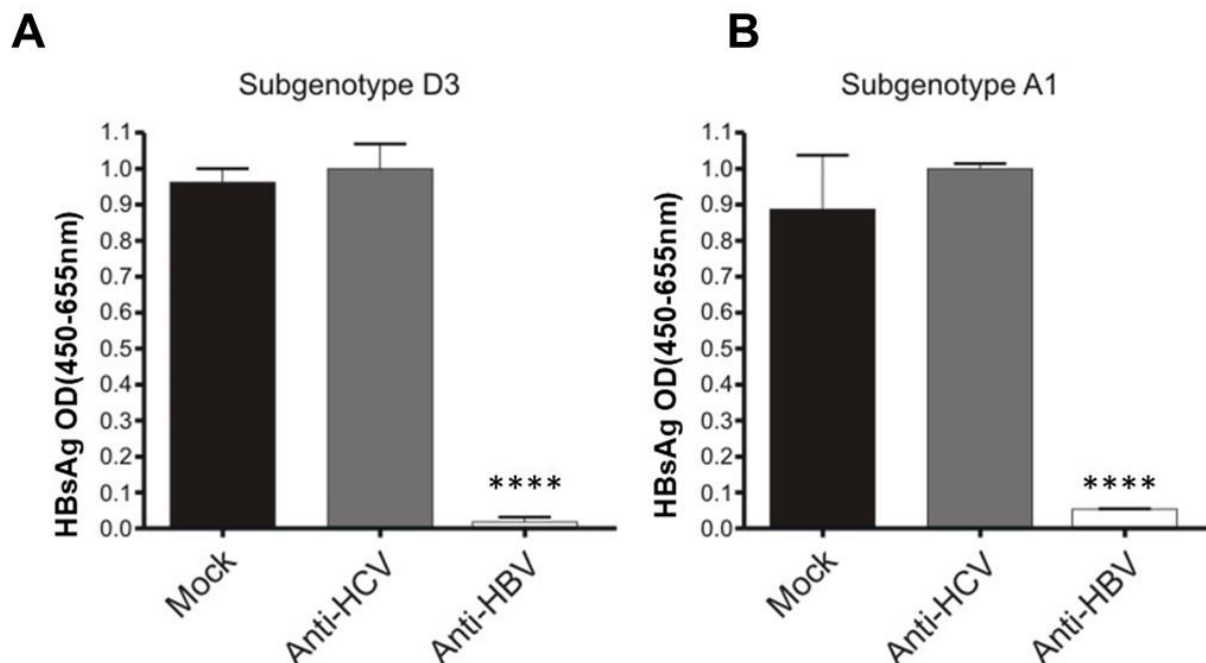


Figure 3.14: Inhibition of HBV gene expression by scAAV2-miR31/5,8,9 in cultured cells.

A. Huh 7 cells were co-infected with AAV2-D3 and AAV2-mTTR-BCDE (anti-HCV) or scAAV2-miR31/5,8,9 (anti-HBV) or infected with AAV2-D3 only (mock). **B.** Huh 7 cells were co-infected with AAV2-A1 and AAV2-mTTR-BCDE (anti-HCV) or scAAV2-miR31/5,8,9 (anti-HBV) or infected with AAV2-A1 only (Mock). A MOI of 1×10^5 was used for all the vectors. The quantification was normalised to data from AAV-mTTR-BCDE (anti-HCV) co-infected cells. ELISA was performed at 48 hours post co-infection with AAV vectors. The error bars indicate $\pm$ SEM derived from triplicate values. Statistically significant differences between samples were calculated using Student's two tailed paired t-test **** $P \leq 0.0001$.

3.5.2 Assessment of toxic and inflammatory effects following co-injection of AAV containing anti-HBV pri-miR and AAV-HBV vectors in mice

A previously reported observation demonstrated that injection of mice with 1×10^{12} VPEs/Mouse resulted in induction of immune response (Chen et al., 2009). Safety of delivering both AAV-HBV and scAAV2 miR-31/5,8,9 vectors in the mice was therefore assessed by co-injection of five mice per group with 5×10^{11} or 9×10^{11}

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VPEs/mouse of AAV8-A1 or AAV8-D3 and 1×10^{11} VPEs/mouse of scAAV2 miR-31/5,8,9. The serum collected at 6 hours post injection was used for a CBA assay to assess induction of inflammatory markers. The concentration of pro-inflammatory markers IL-12p70, IFN- γ , TNF- α , IL-10 and IL-6 and MCP-1 in AAV injected mice were not statistically different from those in mice treated with saline. As expected, the response from mice injected with poly I:C was higher than that observed in mice injected with saline. **(Fig 3.15 and Fig 3.16).**

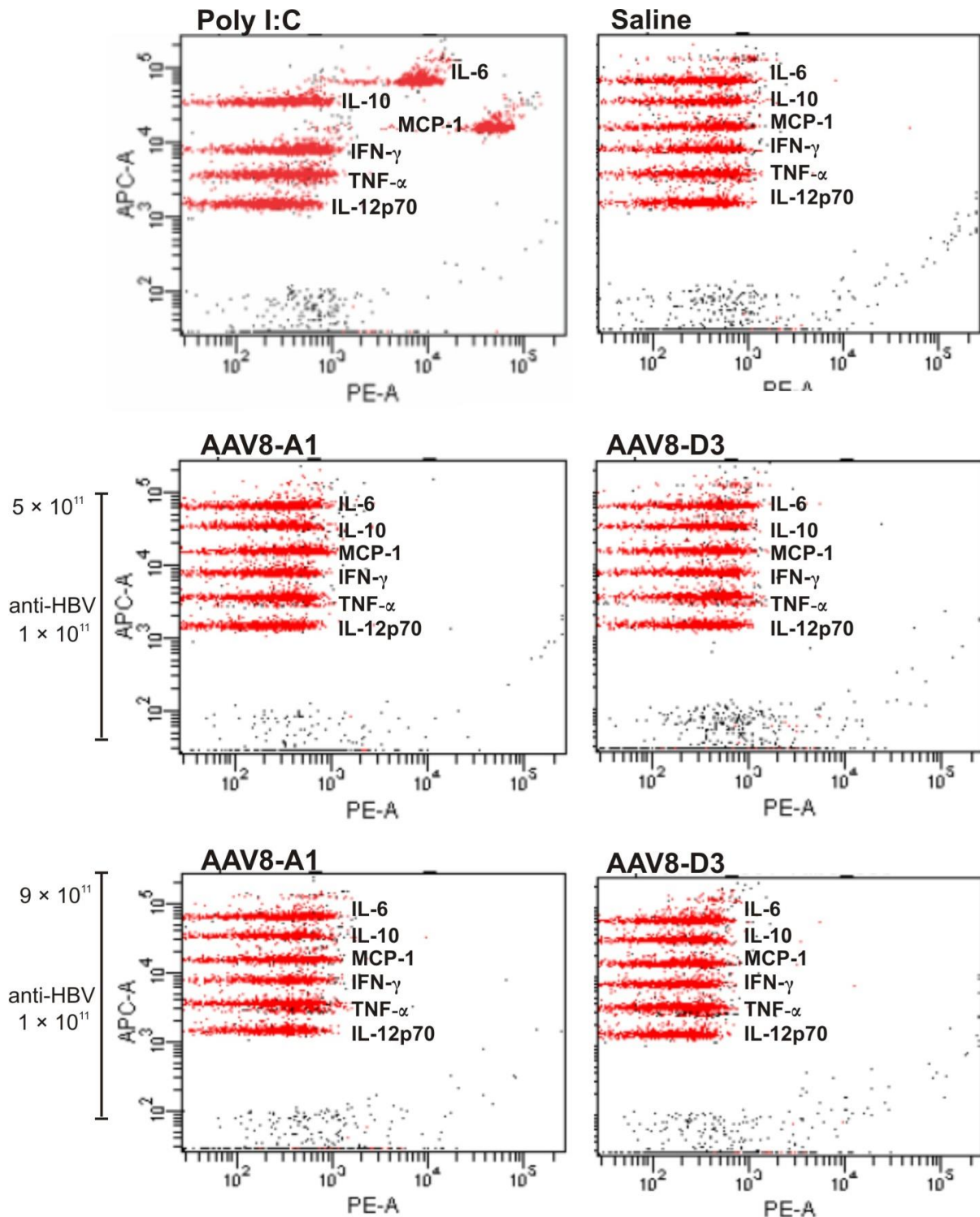


Figure 3.15: Assessment of cytokine induction in NMRI mice post co-injection of AAV-HBV and anti-HBV pri-miR.

Representative dot blots of cytometric bead array assay to quantify interleukin 10 (IL-10), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), chemoattractant protein 1 (MCP-1), interferon gamma (IFN-γ) and interleukin 12p70 (IL-12p70). Measurements were carried out 6 h post injection of AAV vectors at 5×10^{11} or 9×10^{11} VPEs/mouse and scAAV8-miR31/5,8,9 at 1×10^{11} VPEs/mouse. Poly I: C and saline served as positive and negative controls, respectively. APC-A: Allophycocyanin, PE-A: Phycoerythrin.

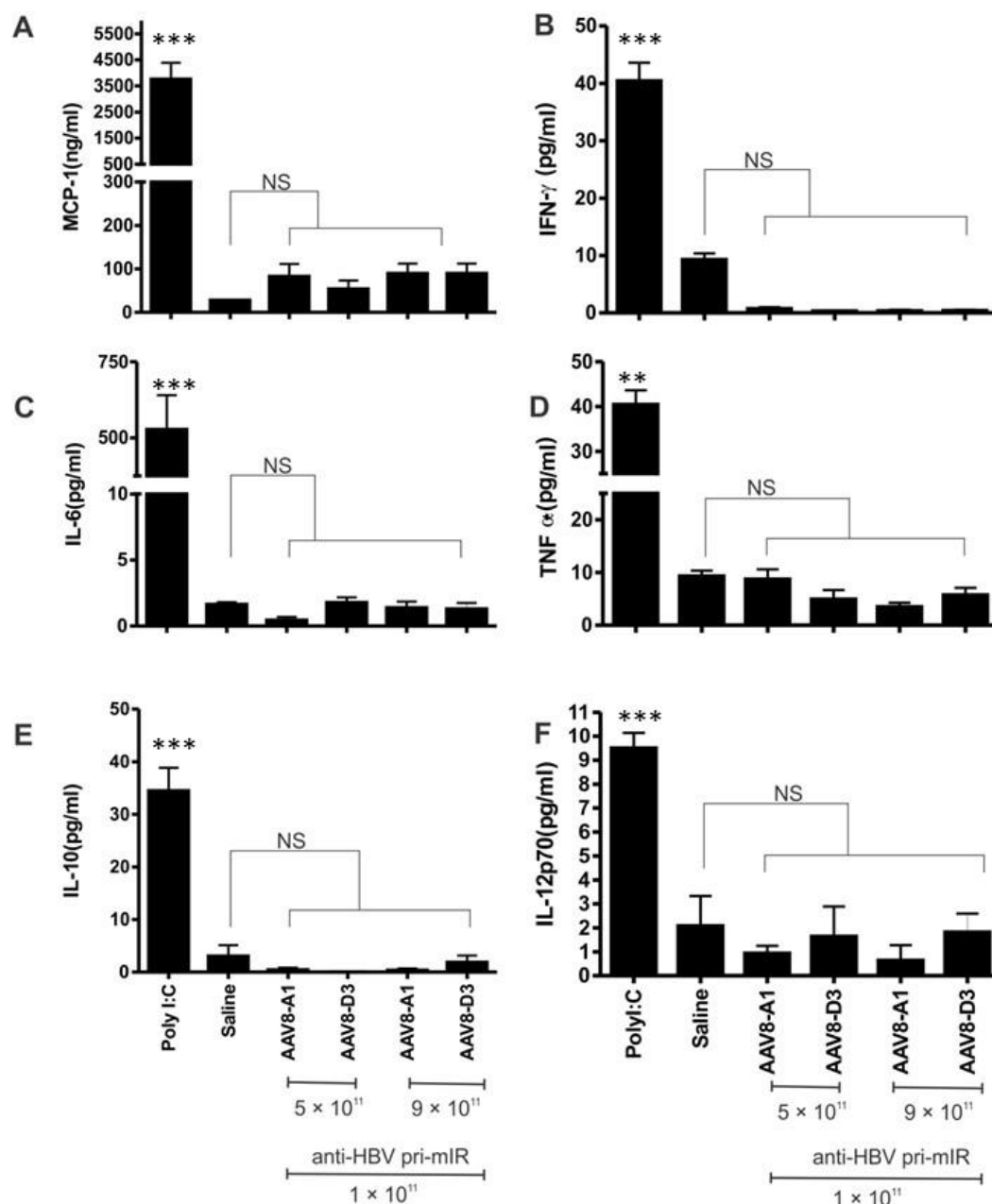


Figure 3.16: Assessment of cytokine induction in NMRI mice post co-injection of AAV8-A1 or AAV8-D3 vector and scAAV8 miR-31/5,8,9.

Cytometric bead array assay was performed to quantify **A.** chemoattractant protein 1 (MCP-1), **B.** interferon gamma (IFN- γ), **C.** interleukin 6 (IL-6) **D.** tumour (TNF- α) **E.** Interleukin 10 (IL-10), and **F.** interleukin 12p70 (IL-12p70). Measurements were carried out 6 h post co-injection of AAV vectors or Poly I: C or saline. Mean concentrations ($\pm$ SEM) were calculated from data obtained from four mice per group. Statistically significant differences between samples were calculated using the Student's two tailed paired test, ** $P \leq 0.01$; *** $P \leq 0.001$ and NS: non-significant.

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To further assess whether administration of AAV-HBV and scAAV8 miR-31/5,8,9 stimulates the interferon response, reverse transcriptase quantitative PCR was carried out to quantify mRNA of interferon response genes *IFIT1*, *OAS-1* and *IFN- β* . Both *IFIT1*, *OAS-1* and *IFN- β* mRNA levels in AAV injected mice were similar to those in the saline-injected mice. The mRNAs of the *IFIT1*, *OAS-1* and *IFN- β* in livers of mice injected with poly I:C were significantly higher when compared to the mice injected with saline or AAV vectors (**Fig 3.17**). These results suggest that treatment of mice with both AAV-HBV and anti-HBV vectors did not induce an inflammatory response.

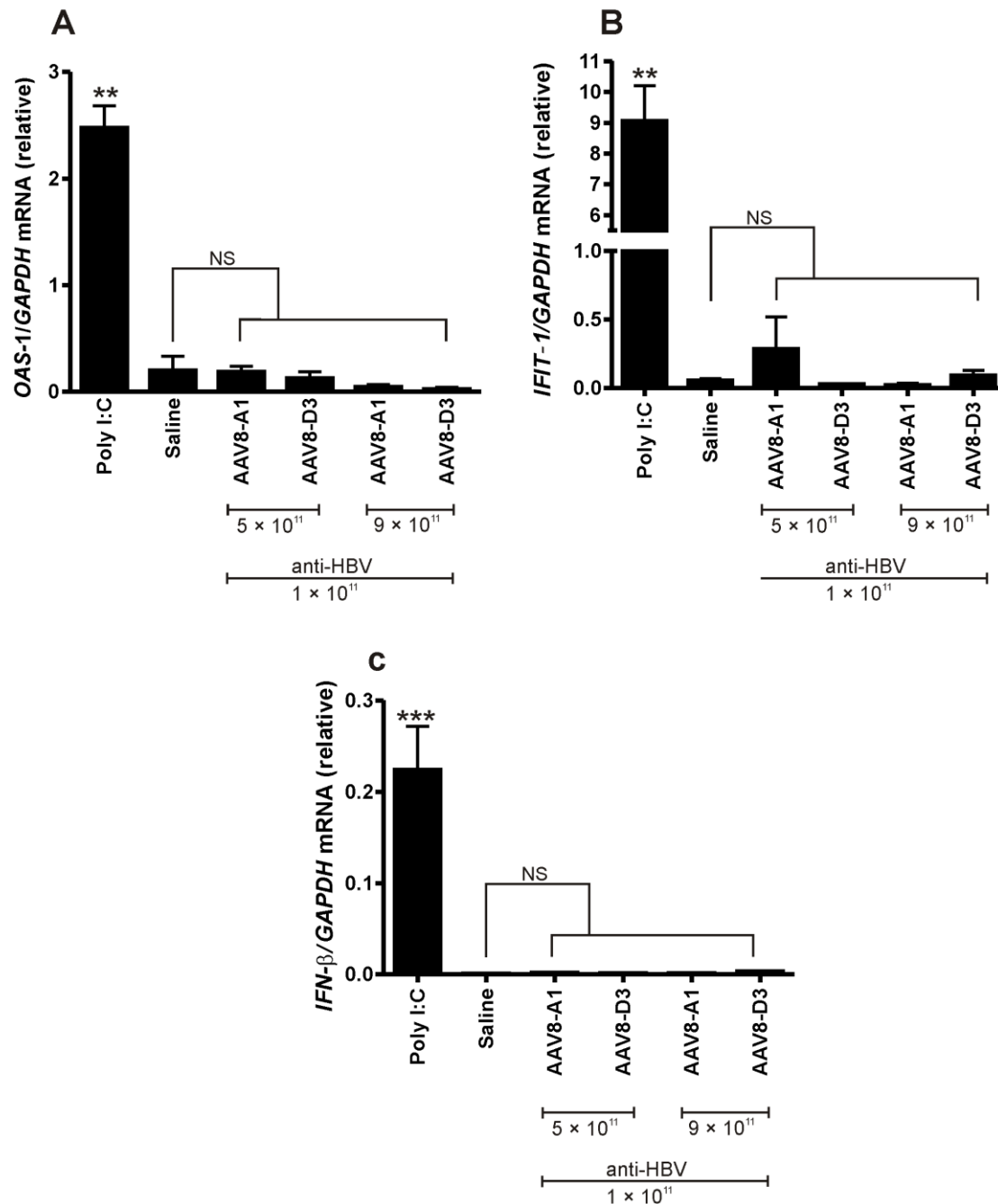


Figure 3.17: Assessment of stimulation of the IFN response in NMRI mice following intravenous co-administration of the AAV-HBV and anti-HBV pri-miR-containing AAV.

Hepatic mRNA was extracted 6 hours post injection of mice with high or low dose AAV containing HBV genome A1 or D3 and AAV containing anti-HBV-pri-miR or poly (I:C) or saline. After reverse transcription, samples were subjected to quantitative PCR to measure the concentrations of mRNA from **A.** *OAS-1*, **B.** *IFIT1*, **C.** *IFN-β*, and *GAPDH* genes. Values represent the means and standard error of the means $\pm$ SEM following injection of four different animals. Statistically significant differences between the samples were determined using the Student's two tailed paired t-test where $**P \leq 0.01$ and $***P \leq 0.001$ were considered statistically significant. NS: not statistically significant.

3.5.3 Injection of AAV-HBV and scAAV8 miR-31/5,8,9 vectors result in no hepatotoxicity in vivo

Toxicity as a result of overexpression of RNAi activator may be significant and potentially lethal. The ALT studies carried out in transgenic mice injected with scAAV8 miR-31/5,8,9 resulted in no liver toxicity (Maepa et al., 2017). The delivery of both AAV-HBV and scAAV8-miR-31/5,8,9 was assessed for its toxicity through an assay for ALT. The serum collected at 2, 4 and 8 weeks post injection from mice co-injected with AAV vectors carrying HBV genome and scAAV8 miR-31/5,8,9 were assayed for ALT levels. The ALT assay revealed concentrations within the normal range in all mice (**Fig 3.18**). These results indicate that there is no liver toxicity arising from co-injection of AAV vectors carrying HBV and scAAV8 miR-31/5,8,9.

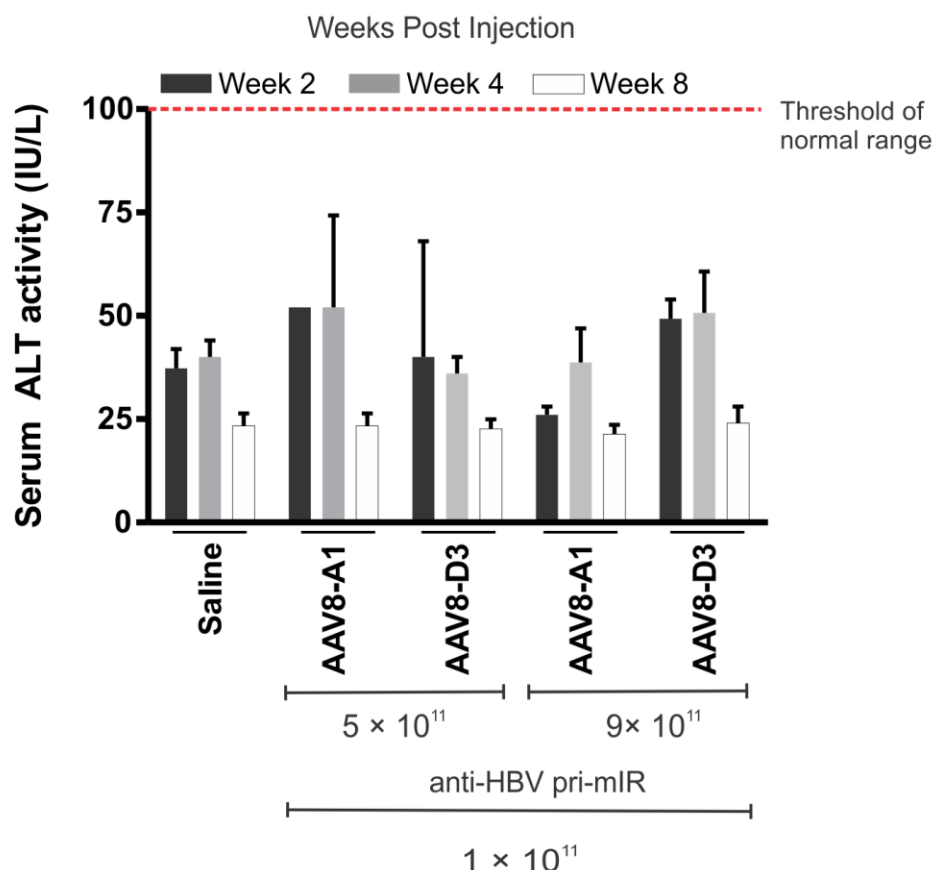


Figure 3.18: Assessment of hepatotoxicity following co-administration of high or low dose AAV-HBV vectors and scAAV8-miR31/5,8,9.

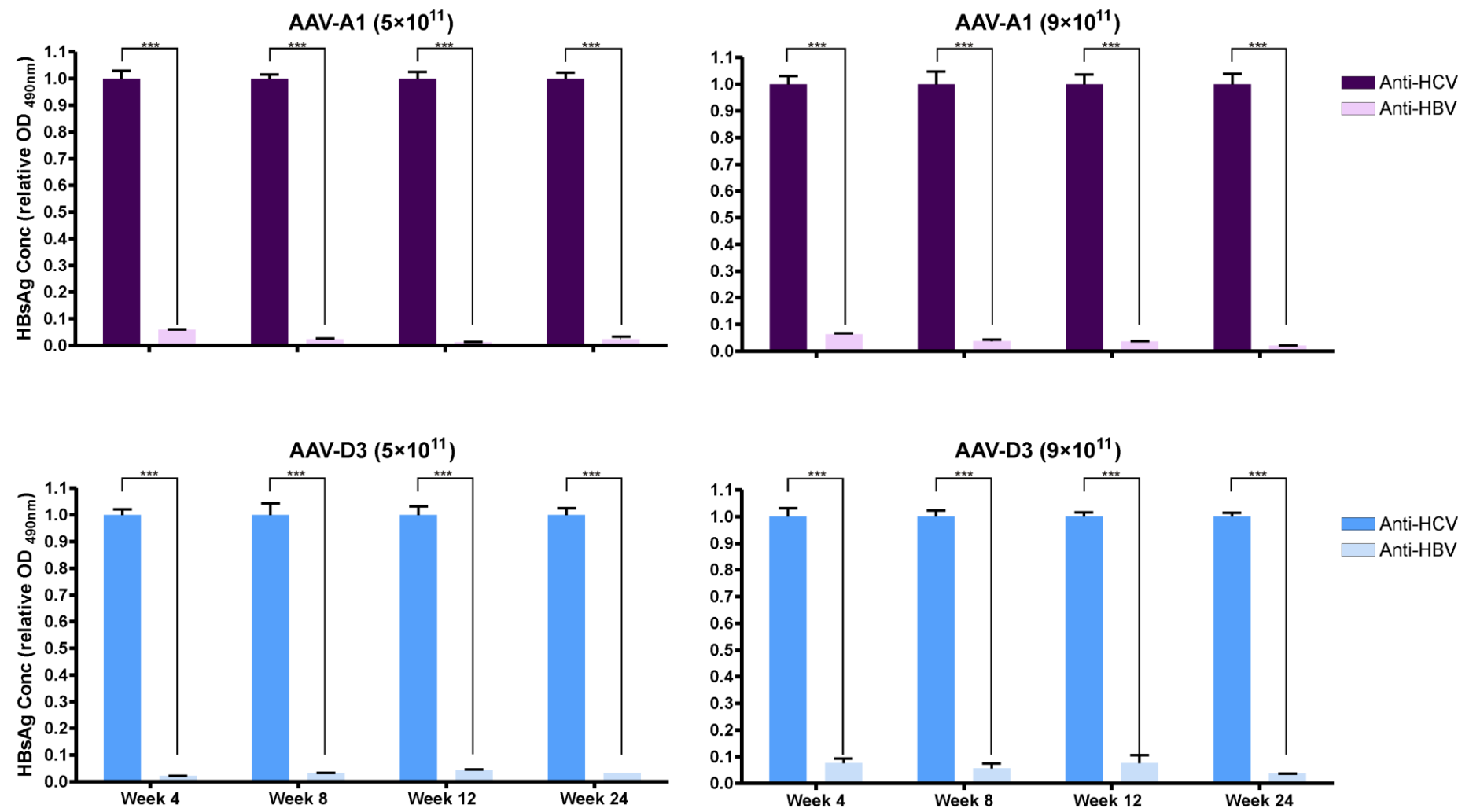
NMRI mice were injected with saline as a negative control, or co-injected with low or high dose of AAV vectors containing HBV genome and 1×10^{11} VPEs/mouse AAV bearing anti-HBV pri-miR. Serum collected at weeks 2, 4, and 8 were assayed for ALT activity. The dotted line indicates threshold of accepted normal range (<100 IU/L). Means $\pm$ SEM were calculated from four mice per group.

3.5.4 In vivo anti-HBV pri-miR-mediated gene silencing in the AAV-HBV mouse model.

To assess the use of the AAV-HBV A1 mouse model in screening of anti-HBV gene silencing therapeutics, mice were co-injected with AAV8-A1 or AAV8-D3 and AAV8-mTTR-BCDE or scAAV8 miR-31/5,8,9. The mice co-injected with AAV8-mTTR-BCDE were used as a negative control. Subsequent to the co-injection of AAV vectors, HBsAg from the serum of mice was used to assess the effect of scAAV8

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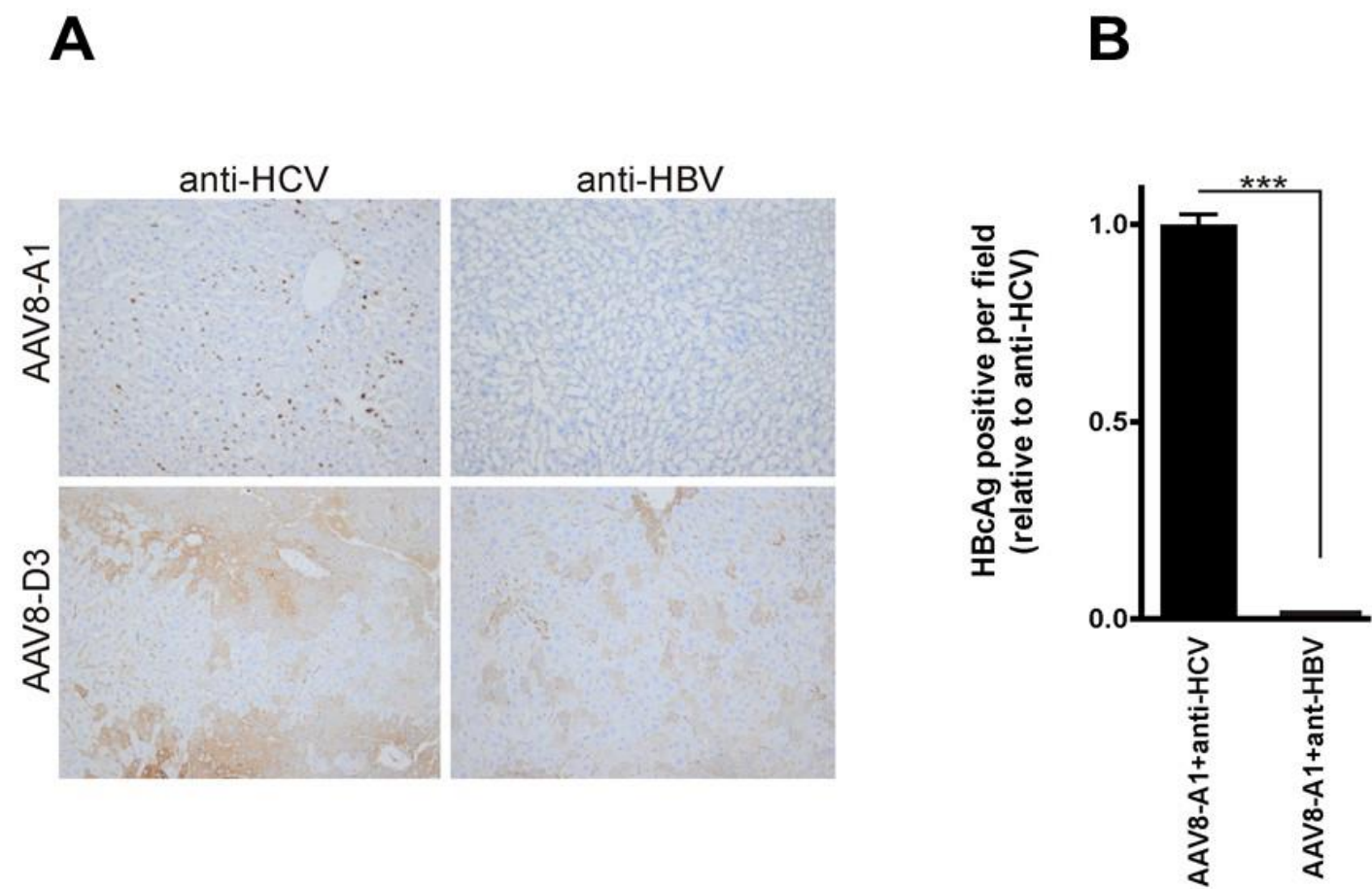
miR-31/5,8,9. In comparison with the mice co-injected with AAV8-mTTR-BCDE, scAAV8 miR-31/5,8,9 effected a significant HBsAg expression knockdown of about 98 % and 97 % in mice co-injected with AAV vectors carrying HBV subgenotype A1 and D3 genome respectively (**Fig 3.19**). This knockdown was observed from 2 weeks post co-injection in mice injected with either low or high dose AAV-HBV and was sustained over a period of 24 weeks (**Fig 3.19**). Similar results were obtained when immunohistochemical analysis of HBcAg was carried out, whereby mice co-injected with AAV8-A1 or AAV8-D3 with scAAV8 miR-31/5,8,9 had significantly reduced HBcAg positive hepatocytes (**Fig 3.20A**). Quantification of HBcAg positive cells showed 99 % AAV8-A1 gene silencing (**Fig 3.20B**). However, HBcAg levels in AAV8-D3 infected mice could not be accurately quantified as a result of cytoplasmic localisation and diffused staining (**Fig 3.20A**). The efficacy of trimeric cassette observed corroborates the previous study that was carried out (Maepa et al., 2017). The cytoplasmic localisation of HBcAg in the livers of mice co-injected with AAV-D3 and pri-miR expressing AAV corroborate the data presented in **Fig 3.10**. Together, these data suggest that the AAV-HBV mouse model is a good model for anti-HBV therapeutics screening.



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Figure 3.19: Assessment scAAV8-miR31/5,8,9-mediated knockdown of HBsAg in the AAV-HBV mouse model.

NMRI mice were co-injected with AAV vectors carrying an HBV genome and scAAV8 miR-31/5,8,9 or AAV8-mTTR-BCDE. Blood samples were collected at different time points (4, 8, 12 and 24 weeks). The level of HBsAg was measured using ELISA. The error bars represent the SEM. Statistically significant differences between the samples were determined using the Student's two tailed paired (were n is equal between the groups) or unpaired t-test (when n is not equal between the groups). *** $P \leq 0.001$ was regarded as statistically significant.



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Figure 3.20: Immunohistochemical staining of HBcAg in liver sections of mice injected with AAV vectors.

A. Representative images showing HBcAg-immunopositive hepatocytes from the liver sections of mice co-injected with AAV8-A1 or AAV8-D3 and scAAV8 miR-31/5, 8,9 or AAV8-mTTR-BCDE. Staining was performed in tissue section from three mice per group. **B.** The data are represented relative to number of nuclei detected in the fields from mice co-injected with AAV8-A1 and scAAV8 miR-31/5,8,9. Statistically significant differences between the samples were determined using the Student's two tailed paired t test. *** $P \leq 0.001$ was regarded as statistically significant.

The inhibition of HBV replication was further assessed through quantification of circulating VPEs. Following co-injection of AAV vectors carrying HBV genome and AAV vectors bearing anti-HBV pri-miR, serum collected at 2, 4, 8, 12 and 24 weeks post injection was used to quantify VPEs by qPCR. Administration of mice with AAV carrying HBV genome and AAV carrying anti-HBV-pri-miR resulted in a significant reduction of VPEs at all the time points when compared to control mice co-injected with AAV carrying HBV genome sequence and AAV carrying pri-miR against HCV (**Fig 3.21**). The inhibition of HBV replication was sustained over a period of six months as compared to the control. The fact that ALT levels remained normal in these mice (**Fig 3.18**), proves that the reduction of HBV replication observed here is not as a result of liver toxicity, rather the effect of scAAV8-miR-31/5,8,8,9 in AAV-HBV mouse model. Taken together, these results confirmed that the AAV-HBV A1 model is an excellent model for screening of expressed HBV gene silencers.

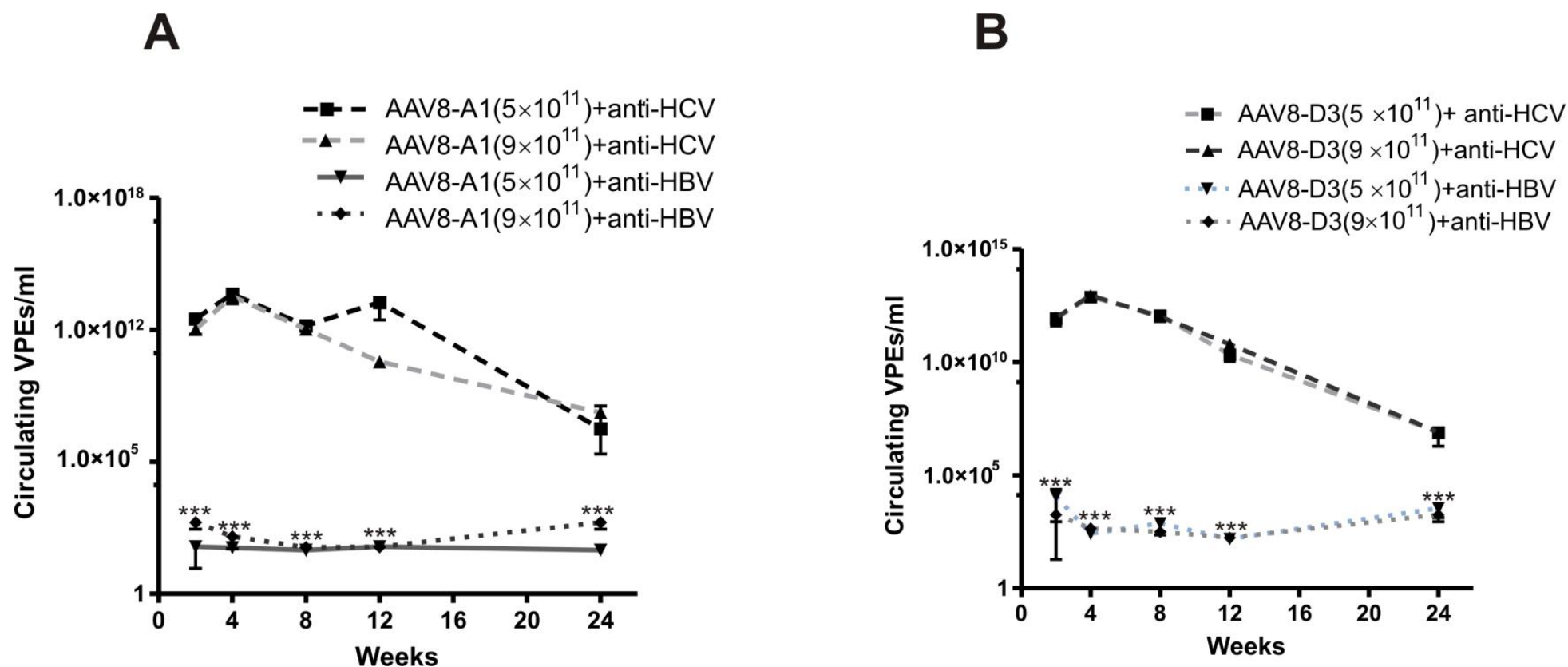


Figure 3.21: Anti-HBV effect of pri-miR in vivo.

A. NMRI mice were co-injected with AAV vectors-bearing the HBV genome sequence and scAAV8 miR-31/5,8,9 or AAV8-mTTR-BCDE. Blood samples were collected at 2, 4, 8, 12 and 24 weeks post-co-injection. The level of viral particle equivalents (VPEs) was measured using qPCR. The values represent mean and Standard error of mean $\pm$ SEM. Statistically significant differences between the samples were determined using the Student's two tailed paired t test. *** P ≤ 0.001 were regarded as statistically significant

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 DISCUSSION

Chronic HBV infection is a medical problem worldwide, with serious consequences resulting to cirrhosis and liver cancer. HBV-related deaths are estimated at approximately 800 000 annually (Al-Sadeq et al., 2019). Sub-Saharan Africa, including South Africa, bears a significant burden of the disease. South Africa, in particular, is hyper-endemic for the A1 subgenotype of HBV which is associated with particularly aggressive liver cancer (Spearman and Sonderup, 2014). Modelling the replication of this genotype is of critical importance as it will aid in understanding the disease caused. Importantly, the HBV subgenotype A1 animal replication model will be useful to assess efficacy of novel antiviral therapeutics including gene therapy.

Newly developed gene therapy tools such as RNAi (Maepa et al., 2017), clustered regularly interspaced short palindromic repeats (CRISPR/cas) (Scott et al., 2017), ZINC Finger nucleases and Transcription activator-like effector nucleases (TALENs) (Bloom et al., 2013, Li and Samulski, 2020) have shown potential in eradicating HBV infection.

However, good in vivo models are required to validate these novel therapeutic approaches before they can reach the clinic. The fact that HBV is a good target for potential treatment based on RNAi has been widely established (Maepa et al., 2017, Ely et al., 2009, Ely et al., 2008b, Dominguez II et al., 2016, Nelson et al., 2016). However, sequence variation among HBV genotypes and subgenotypes could influence silencing efficacy (Sun et al., 2010). The genotype variation has been demonstrated to have influence in clinical outcomes and response to treatment. It is

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therefore important that anti-HBV therapeutic regimens be tested in the panel of HBV genotype models to be able to achieve broad efficacy in most chronic carriers of HBV, hence a model for each genotype is important. Studies have shown that infection of mice with AAVs carrying HBV genotype D genome sequence mimicked chronic HBV infection (Ye et al., 2015, Yang et al., 2014). Given the fact that genotype D varies significantly to genotype A1 in terms of clinical manifestation and treatment response (Kramvis, 2014), it is important that a model for genotype A1 be developed. This study, therefore, used AAV to model HBV subgenotype A1 replication in cell culture and mice and assessed its potential in screening of novel anti-HBV therapies. This study is the first to demonstrate the use of AAV to model subgenotype A1 replication both in cell culture and in vivo.

The greater-than-genome length HBV sequence of subgenotype A1 and D3 were successfully cloned in the pAAV vector and transfection of Huh 7 cells with the plasmid resulted in HBV gene expression. The greater-than-genome length HBV sequence was successfully packaged in AAV2 and AAV8 serotype for in vitro and in vivo studies and AAV vectors carrying HBV sequence were produced in sufficient amounts. Infection of Huh 7 cells with AAV vectors carrying HBV resulted in HBsAg and HBcAg expression. The production of HBV virions (VPEs) was observed on day 1, 3 and 5. Overall, the results obtained demonstrated that AAV vectors carrying HBV transduced the liver-derived cell line and results in HBV gene expression and replication.

4.1.1 Safety of delivering AAV carrying HBV sequence in mice

Despite the endeavours devoted to the improvement of transduction efficacy, several challenges for the use of recombinant AAV vectors remain. Among them, vector immunogenicity, which reflects the interactions of rAAVs with the host immune

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system (Ronzitti et al., 2020). AAV has been shown to activate innate pattern recognition receptors (PRRs) such as toll-like receptor (TLR)-2 and TLR-9, resulting in the release of inflammatory cytokines and type I interferons. In mice, AAV genome configuration may influence the innate response; self-complementary AAV genomes increase the innate immune response to the transgene, whereas the response to single stranded vectors is less severe (Martino et al., 2011). When adenoviral vectors (Ad) were used to deliver greater-than-genome-length of HBV sequence in mice, it resulted in HBV gene expression and HBV replication. However, persistent infection resulted in mice developing immune response against the capsid (Sprinzl et al., 2001). In this study, the safety of delivering AAVs at 5×10^{11} or 9×10^{11} VPEs/mouse was assessed. Injection of mice with 5×10^{11} or 9×10^{11} VPEs/mouse of single stranded AAVs carrying HBV genome followed by CBA (**Fig 3.6**) and RT-qPCR (**Fig 3.7**) assay showed no inflammatory response at 6 hours post infection. Suggesting that the AAV safely delivered the HBV genome sequence in the mice livers.

Generally, infectious diseases are thought to be caused by the immune reaction against viral antigens (Yamada and Tsuji, 1993, Chisari, 1995). Previous studies have shown that harmful inflammatory response translate to liver injury which then results in increase in ALT. In a study by Yang et al, the levels of AST and ALT were elevated (± 443 IU/ml) in mice transduced with 3×10^{12} VPEs/mouse of AAV/HBV in C57BL/6 mice (Yang et al., 2014). This study evaluated the ALT level in mice post injection with 5×10^{11} or 9×10^{11} VPEs/mouse. Minimal elevation of ALT was noted in mice injected with AAV-HBV at 2 weeks but not in AAV8-empty vector injected mice, suggesting that this may be as a result of HBV gene expression. However, ALT levels reverted to saline levels at 4 weeks and remained within the normal range

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(below 100 IU/ml) over 8 weeks post-injection. Collectively, these data suggest that delivery of 5×10^{11} or 9×10^{11} dose results in no liver injury. Similar findings were observed when 2×10^{11} dose was used to deliver HBV genome sequence in C57BL/6 mice (Ye et al., 2015).

4.1.2 AAV transduces the mouse liver to mediate HBV gene expression and replication.

HBV gene expression was assessed through transducing mice liver with AAV vectors carrying HBV greater-than-genome sequence length. The dose-dependent gene expression from AAV-HBV was evaluated in a pilot study where mice were injected with 1×10^9 , 1×10^{10} and 1×10^{11} VPEs/mouse viral particles. Of the different doses tested in the study, only 1×10^{11} VPEs/mouse dose resulted in significant expression of HBV surface antigen (HBsAg) detected in NMRI mice serum injected with AAV-D3. From mice injected with 1×10^{11} VPEs/mouse of AAV carrying subgenotype A1 genome sequence, there was no detection of HBsAg. Increasing AAV dose to 5×10^{11} or 9×10^{11} VPEs/mouse resulted in significant detection of HBsAg as well as HBcAg and VPEs in mice infected with AAV carrying HBV sequence of A1 or D3 (**Fig 3.9, 3.10 and 3.11**). Earlier study showed that AAV doses of 2×10^{11} or 5×10^{10} VPEs/mouse were reported to successfully deliver greater-than-genome length HBV in C57BL/6 mice and resulted in significant HBV gene expression and replication over a period of six months to a year following a single systematic administration (Dion et al., 2013, Ye et al., 2015). Previously, different mice strains were proven to result in differential HBV gene expression. When AAV-HBV was administered in BALB/C, C57BL/6, FVB and ICR, it resulted in mouse strain specific differential HBV gene expression. The BALB/C and ICR showed higher HBV gene expression than C57BL/6 and FVB. In another study, $1 \times$

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10^{12} genome copies (GC) of AAV9 were used to deliver GFP encoding sequence in C57BL/6 and BALB/c mice. High transduction efficiency was observed throughout the brain and the spinal cord, as measured by detection of GFP fluorescence in C57BL/6 mice. However, this was not the case when the same experiment was performed in BALB/c mice, where the low transduction and gene transfer was obtained in the central nervous system (CNS) (Hordeaux et al., 2018, Deverman et al., 2016, Matsuzaki et al., 2018). We can therefore suppose that NMRI mice used in this study could contribute to the lower transduction efficiency and requirement of a higher dose to observe significant HBV gene expression.

In this study, mice injected with AAV8-A1 showed a dose dependent gene expression, while gene expression from AAV8-D3 showed similar levels at 5×10^{11} and 9×10^{11} VPEs/mouse. Importantly, the HBcAg was detected in 50 % of hepatocytes in our model as compared to the hydrodynamic injection of HBV genome sequence model which resulted in 5-10% of hepatocytes expressing HBcAg (Liu et al., 1999). Surprisingly, the HBcAg in liver of mice injected with AAV-D3 was more dominantly localised in the cytoplasm, unlike with AAV8-A1, which localised mainly in the nucleus (**Fig 3.10**). HBcAg bears a nuclear localisation signal, hence it is expected to predominately localise in the nucleus. The study by Jazayeri et al revealed that the basis for differential cellular distribution of the core was correlated to sequence variation within the core C-terminus. The replacement of amino acid residue within the C-terminus of HBcAg rendered a shift of HBcAg from the nucleus to the cytoplasm or vice versa (Jazayeri et al., 2004). Another study reported specific core promoter mutations that resulted in HBcAg localisation in the cytoplasm (Kawai et al., 2003). To determine whether any of these variations may account for cytoplasmic localisation of HBcAg from HBV subgenotype D3 sequence used in this

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study, the amino acid sequence was aligned to HBcAg sequence from pCH-9/3091. The pCH-9/3091 carries the HBV sequence and expressed HBcAg has been proven to be localised in the nucleus of mice hepatocytes (Bloom et al., 2013). The HBV subgenotype D3 greater-than-genome length sequence used was identical to the pCH-9/3091 sequence, except for a L148/V148 variation. Interestingly, this variation was within the C-terminus arginine rich region implicated in promoting cytoplasmic localisation upon mutation (**Appendix, Fig 5.5**). It is known that more predominant cytoplasmic HBcAg tends to be associated with more active HBV DNA replication (Chu et al., 1995). Interestingly, in this study, HBsAg gene expression was observed in mice injected with 1×10^{11} VPEs/mouse of AAV-D3 and not AAV-A1 suggesting a subgenotype A1 lower transduction efficiency than subgenotype D3 (**Fig 5.3**). Supporting this is the fact that 5×10^{11} VPEs/mouse dose resulted in higher HBsAg expression in AAV-D3 than AAV-A1 injected mice (**Fig 3.9**).

The HBV transgenic mice, initially developed by Chisari et al, comprised the HBV envelope, core, precore or X sequences (Chisari et al., 1985, Milich et al., 1990, Kim et al., 1991). The model was further improved by Guidotti et al and it comprised 1.3 $\times$ greater-than-genome length HBV DNA integrated into the host genome (Guidotti et al., 1994). This model successfully results in HBV replication which leads to a continuous secretion of new virions. However, the difficulties in using these mice is the variation in HBV gene expression between mice which complicates reliability of gene expression measurements. In this study, the use of AAV to deliver HBV sequences of subgenotype A1 and D3 resulted in HBV gene expression and VPEs production with less variability between mice over a period of six months. Detection of HBV replicative DNA intermediates and RNA transcripts in livers of mice injected with AAV-HBV validated HBV replication (**Fig 3.12**).

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Extensive research has shown that cccDNA is responsible for persistent HBV infection in chronically infected individuals. Ongoing research focuses on elimination of cccDNA which will potentially lead to the cure of HBV. Developing an animal model which will result in production of cccDNA is critically important. Similar to previous reports (Ye et al., 2015, Dion et al., 2013), cccDNA was not detected in this study (data not shown). However, the fact that mouse injected with AAV carrying HBV genome sequence resulted in a sustained production of several HBV replication markers makes it a good HBV replication model and can be applied in assessing the effect of novel anti-HBV therapeutics on HBV replication.

4.1.3 Prolonged HBV gene expression and replication from AAV-HBV results in minimal inflammation with no liver damage

Pronounced liver inflammation and damage was previously reported in mice infected with 2×10^{11} AAV carrying genotype D genome (Ye et al., 2015, Huang et al., 2011). However contradicting results were reported by Yang and colleagues using 2×10^{10} - 1×10^{11} VPEs/mouse of AAV vectors carrying similar HBV genotype D genome sequence in C57BL/6 mice. Supporting the latter is the fact that C57BL/6 male mice infected with 2×10^{10} - 1×10^{11} VPEs/mouse AAV vectors carrying HBV genotype D does not result in liver injury (Yang et al., 2014). In this study, the effect of long-term HBV gene expression and replication from AAV-HBV was assessed for induction of liver inflammation and fibrosis using H&E and Sirius red staining. Although higher doses were used, there was no significant liver inflammation or liver damage observed (**Fig 3.13**).

According to a study carried by Huang et al., the host genetic background plays a crucial role in HBV replication and the infection manifestation (Huang et al., 2011). The fact that different mice strains and sexes were used in the different studies might

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be a contributing factor to the contradicting observations made. This needs to be further investigated by carrying side-to-side comparisons between different strains and sexes. Nevertheless, it has been established that as one of the mechanisms of HBV persistence in chronically infected individuals, the virus paralyses the immune response by T cell exhaustion among many other mechanisms (Saeidi et al., 2018, Wherry and Kurachi, 2015). Hence, the fact that mice injected with AAV carrying HBV genome sequence resulted in no induction of immune response mimic the situation in chronically infected patients. In addition, this may simulate a chronic infection by avoiding clearance of AAV infected hepatocytes and prolonging HBV gene expression.

4.1.4 Successful application of AAV-HBV model for screening novel anti-HBV therapeutics

4.1.4.1 Co-administration of AAV-HBV and scAAV8miR-31/5, 8, 9 results in no liver inflammation and toxicity in mice liver

RNAi activators and viral infections have been previously shown to stimulate cytokine production in eukaryotic cells (Österlund et al., 2005, Sioud, 2005). Safety of exogenous RNAi activators is vitally important for their use in a therapeutic context. Previous study showed that the scAAVs pseudotyped with AAV8 capsid to deliver pri-miRs against HBV results in no evidence for stimulation of IFN response (Maepa et al., 2017). The study carried by Ye et al., showed that sequential injection of AAV-HBV (2×10^{11}) and AAV expressing a shRNA in mice induce pro-inflammatory cytokine IL-6 and cytotoxic T-cell differentiation factor (Ye et al., 2017). In the current study, the co-injection of AAV-HBV and scAAV8 miR-31/5,8,9 in mice resulted in no induction of IL-6, IL-10, TNF- α , MCP-1, INF- γ and IL-12p70 encoding genes as assessed by CBA. RT-qPCR analysis also showed no induction of IFN response

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genes encoding OAS-1, IFN- β , and IFIT1 (**Fig 3.16 & 3.17**). Earlier research confirms the detrimental inflammatory response in hepatocytes which leads to liver tissue harm, and causes increase of extracellular enzymes, in particular ALT. In addition, injection of 3×10^{12} VPEs/mouse of AAV-HBV resulted in a significant increase of ALT (Huang et al., 2011). This study evaluated the ALT level in mice co-transduced with AAV-HBV and scAAV8 miR-31/5,8,9 at a total dose of 6×10^{11} and 1×10^{12} (**Fig 3.18**). The level of ALT was within the normal range for both doses used. Together, these data indicate that delivering both scAAVs encoding pri-miR and AAV encoding HBV sequence are safe in mice.

4.1.4.2 Efficiency of RNAi activators in AAV-HBV mouse model

Developing new approaches to effect a cure from HBV infection has gained much interest recently. Having established the excellent HBV replication model which resulted in long term HBV gene expression and replication, the feasibility of using the model in screening novel anti-HBV therapeutics was assessed. The ability of scAAV2 miR-31/5,8,9 and scAAV8 miR-31/5,8,9 to silence HBV gene expression from AAV-HBV were assessed both in vitro and in vivo respectively. The liver-derived cell lines were co-transduced with scAAV2 miR-31/5,8,9 and AAV-HBV. The knockdown of HBsAg expression was observed in cell culture when ELISA assay was used (**Fig 3.14**). In vivo co-injection of mice with scAAV8 miR-31/5,8,9 and AAV8-A1 or AAV8-D3 resulted in silencing of HBV HBsAg level by 98 % and 97 % respectively (**Fig 3.19**). The production of VPEs was also reduced by 96 % (**Fig 3.21 A & B**). Similar results were previously observed when scAAV8 miR-31/5,8,9 was injected in transgenic mice, whereby HBsAg, HBcAg and HBV DNA levels were reduced by 95%, 94% and 94% respectively for a period of 10 months (Maepa et al., 2017).

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These facts are supported by the study carried out by Ye et al. (2017) which demonstrated good silencing of HBV gene expression in AAV-HBV mice following co-administration of 1×10^{11} VPEs/mouse of scAAVs expressing dual shRNA against different coding regions of HBV (Ye et al., 2017). The scAAV-shRNA resulted in 90% HBV replication inhibition. Previously, the use of the AAV-HBV model to screen for an anti-HBV vaccine was evaluated using vectors carrying HBV genotype D sequence. In addition to that, the AAV/HBV mouse model was tested for resistance of HBsAg vaccine through challenging the mice with aluminum adjuvant (Engerix-B). AAV infected mice failed to develop anti-HBsAg antibody responses, which indicated immune tolerance toward HBsAg. Furthermore, immunisations of AAV-HBV mice with conventional HBV vaccine induced clearance of HBV viremia and strong antibody production and T-cell response (Yang et al., 2014). Collectively, this data suggest that the AAV-HBV model can be successfully used in screening anti-HBV therapeutics and vaccine candidates.

4.2 Conclusion

Collectively the data obtained showed that the AAV-HBV subgenotype A1 replication model was successfully developed for the first time. The demonstration that AAVs can be used to safely deliver HBV subgenotype A1 or D3 genome sequence and mediate long-term HBV replication is advantageous over adenoviral mediated gene transfer. The possibility of exploiting the use of AAV-A1 replication model to assess the efficacy of novel anti-HBV-pri-miR was also demonstrated for the first time. This creates more options in screening anti-HBV therapeutics in different HBV genotypes, more especially screening of RNAi activators with inhibitory efficacy affected by minor sequence variability.

4.3 Future studies

Although the AAV-HBV A1 replication model described in this study resulted in long term HBV gene expression and replication, the cccDNA pools were not established. This is due to the fact that mice are not infectable by HBV. Hence future studies may use these AAV-HBV vectors to transduce previously established non-human primates expressing hNTCP. These primates are susceptible to HBV infection (Burwitz et al., 2017b), consequently, HBV viral particles produced from AAV-HBV vectors may infect the hepatocytes and deliver the rcDNA and initiate cccDNA synthesis.

Ye et al showed significant liver inflammation and fibrosis following infection of C57BL/6 mice with AAV-HBV vectors, mimicking a natural HBV infection closely. However, no liver damage was observed in this study, hence future evaluation of AAV-HBVA1 model in different mice strains like C57BL/6 need to be carried out.

HBV subgenotype A1 showed nuclear localisation of HBcAg as compared to D3, which showed nuclear localisation. Further investigation needs to be carried out using different HBV genotypes to access the difference observed on HBcAg localization.

The AAV-HBV model was proven to be useful in screening of anti-HBV RNAi activators through co-administration of AAV-HBV and scAAVS expressing miR-31/5,8,9. However, to mimic a natural setting, a future study may involve sequential administration of AAV-HBV followed by AAV expressing miR-31/5,8,9 pseudotyped with different capsids.

CHAPTER 5: APPENDIX

5.1 Buffers, Media and Solutions

5.1.1 Bacterial culture media and solutions

(a) Luria Bertani (LB)

Ten grams of Tryptone, with 5 g Yeast Extract (Oxoid, UK) and 5 g NaCl were dissolved in 1 L of distilled water. Thereafter, LB medium was autoclaved for 20 minutes at 121°C and 1kg/cm² and kept at room temperature.

(b) LB Psi

Five grams yeast extract, 10 g tryptone and 5 g magnesium sulphate were dissolved in distilled water and pH was adjusted to 7.6 using potassium hydroxide. The volume was adjusted to 1L with dH₂O. This was followed by autoclaving the LB Psi medium at 121°C and 1kg/cm².for 20 minutes.

(c) Luria-Agar (LA) plate

Bacterial agar plates were prepared by dissolving 10 g of Bacto-tryptone, 5 g yeast extract, 5 g NaCl and 10 g Agar (Oxoid Ltd, Hampsphire, England) in a litre of dH₂O. This was followed by autoclave at 121°C and 1kg/cm² for 20 minutes. It was allowed cool to approximately 55°C and the desired antibiotic (100 µg/mL ampicillin) was added. The medium was poured into petri dishes and allowed to set at room temperature and stored at 4 °C.

5.1.2. Buffers

(a) Transformation Buffers

- **TFBI**

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Transformation buffer I (TFBI) was prepared by dissolving 0.588 g of potassium acetate (30 mM), 2.42 g rubidium chloride (100 mM), 1.1 g calcium chloride (10 mM) and 2.0 g manganese chloride (50 mM) in dH₂O and 30 mL of glycerol (15% v/v) was added. The pH of the solution was adjusted to 5.8 with acetic acid. The volume was adjusted to 200 mL using dH₂O followed by filter sterilisation.

○ **TFBII**

A TFBII solution was prepared by dissolving 0.21 g of MOPS (10 mM), 0.121 g rubidium chloride (10 mM), 1.1 g calcium chloride (75 mM) in distilled water. Followed by addition of 15 mL of glycerol (15% v/v), and pH of the solution was adjusted to 6.5 using NaOH. Thereafter, the volume was adjusted to 100 mL using dH₂O followed by filter sterilization.

(b) 1000 × Ampicillin

One gram of Ampicillin (Roche, Germany) was dissolving 1 g in 10 mL of 50 % ethanol, and was stored at -20 °C.

(c) Plasmid isolation buffers

○ **Buffer 1**

Six grams of Tris-Base and 3.72 g of EDTA was dissolved in 800 ml distilled water. The pH was adjusted to 8 using NaOH and the volume adjusted to 1 L with dH₂O. The solution was autoclaved for 20 minutes at 121 °C and 1kg/cm². The solution was cooled to rtm, RNase A added to 100 µg/mL and the solution stored at 4 °C.

○ **Buffer 2**

A litre of buffer 2 was prepared by dissolving 8.0 g NaOH pellets, and 10 g SDS in a litre of dH₂O. The solution was autoclaved at 121°C and 1kg/cm² for 20 minutes followed by storage at rtm .

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○ **Buffer 3**

Three hundred and eighty grams of guanidine HCl and 49 g potassium acetate were dissolved in distilled water, the pH was adjusted to 4.5. The volume was adjusted to 1 L using dH₂O. The buffer was stored at 4 °C.

○ **Wash buffer**

Wash buffer was prepared by dissolving 1.2 g Tris Base in 80% ethanol, the pH was adjusted to 7.56 and the volume of the solution was made up to 1 L with dH₂O. The solution was stored at rtm.

(d) TAE gel electrophoresis running buffer

The 50× Tris-acetate-EDTA (TAE) was prepared by dissolving 242 g Tris Base, addition of 57.1 mL glacial acetic acid and 100 mL of 0.5M EDTA. The pH was adjusted to 8 and volume was adjusted to 1L with dH₂O.

5.1.3 General tissue culture media and solutions

(a) FBS

To prepare the working stock of FBS, heat inactivated FBS was filter sterilised, aliquoted into 50 mL and stored at -20 °C.

(b) 1000× Penicillin/Streptomycin

To prepare 1000× Penicillin/Streptomycin (Pen/Strep, 100 000 U/mL; 100 000 µg/ml) 0.9 g Penicillin and 1.5 g Streptomycin were dissolved in 15 mL of 1 × PBS. It was filter sterilised and stored at -20 °C.

(b) DMEM

High or low DMEM medium stock was prepared through dissolving five sachets of DMEM powder, (Life technologies, CA, USA) and 18.5 g of NaHCO₃ was in 4750 ml

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of SABAX water. The pH of the medium was adjusted to 6.7-6.8 using HCl. This was followed by filter sterilisation using VacuCap 90 PF (PALL Life Sciences, NY, USA) filter device, into 500 ml sterile schott bottles. The prepared medium was store at 4 °C.

Complete DMEM was prepared by adding 50 mL of FBS (Foetal Bovine serum) in 449 mL DMEM. This was followed by addition of 0.5 mL of Pen/Strep (Penicillin 100 U/mL; Streptomycin 100 µg/mL) and stored at 4 °C.

(c) 0.5 × TrypleE™Express

To prepare TrypleE™ [(1X) (Life Technologies, CA, USA)], the TrypleE™ was diluted 2-fold using Saline-EDTA solution. This was followed by filter sterilisation and the solution was stored at 4 °C.

(d) Freezing solution

The cells freezing solution was freshly prepared through addition of 5.6 mL complete media (DMEM), 3.4 mL FBS and 1 mL of DMSO. Thereafter, the solution was filter sterilized.

(e) 1 × Phosphate buffer saline (PBS)

One times phosphate buffer saline (PBS) was prepared by dissolving 1 tablet of PBS (Sigma-Aldrich, MO, USA) in 200 mL of distilled water. The solution was autoclaved at 121 °C and 1kg/cm² for 20 minutes.

(f) Polyethyleneimine (PEI) max

To prepare PEI_{max}, 1 g of Pei 'max' was dissolved in 900 mL distilled water. The pH was adjusted to 7.0 with 1 M NaOH. The volume was adjusted up to 1 L through addition of dH₂O. This was followed by filter sterilization using 0.22 µm filter membrane. The PEI_{max} was stored at 4 °C for a period of 9 months.

5.1.4 AAV harvesting and purification solutions

(a) Viral Lysate Buffer

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Lysate buffer was prepared by dissolving 0.19 g MgCl_2 (2 mM) in 50 mL of Tris HCl (pH 8.5). The volume was adjusted to a litre through using dH_2O . The solution was then autoclaved at 121 °C 1kg/cm² for 20 minutes and stored at 4 °C until required.

(b) Viral precipitation solution

To prepare 5 × PEG, 500 g Polyethylene glycol molecular biology grade (Sigma Aldrich, MO, USA) and 115 g NaCl were dissolved in 1 L of dH_2O . Followed by autoclave at 121 °C, 1kg/cm² for 30 minutes. The viral precipitation solution was stored at 37 °C.

(c) PBS-MK

A litre of PBS-MK was prepared by adding 0.1 g MgCl_2 (1mM) and 0.19 g KCl (2.5 mM) to 1 L of 1× PBS. This was followed by filter sterilisation and the solution was stored at room temperature.

(d) PBS-MK-NaCl

One molar concentration of PBS-MK-NaCl was prepared by dissolving 58.44 g of sodium chloride (1M) in 500 mL of PBS-MK. Followed by filter sterilisation and storage at room temperature.

(e) Phenol red

The phenol red was prepared by dissolving 0.5 g phenol in 100 mL dH_2O and stored at room temperature.

(f) Iodixanol

Four iodixanol solutions were prepared at different percentages: 60%, 40%, 25% and 15%. The 60% iodixanol solution was prepared by mixing 60 mL iodixanol (Optiprep, Progen, Biotechnik, GmbH) and 2.5 $\mu\text{L/mL}$ phenol red (Sigma Aldrich). whereas the 40% iodixanol was prepared through mixing 30 mL iodixanol and 15 mL PBS-MK. The 25% iodixanol was prepared by adding 20 mL iodixanol, 28 mL

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PBS-MK and 120 μ L phenol red. Then 15% iodixanol solution was prepared through adding 12 mL iodixanol and 36 mL PBS-MK-NaCl.

5.1.5 Immunohistochemistry solutions

(a) Triton X (0.1%)

Triton X (0.1 %) (Sigma-Aldrich , MO,USA) was prepared by adding 10 μ L of triton X to 10 mL of 1 $\times$ PBS.

(b)BSA (1%)

BSA (1%) (Roche Applied Science, Mannheim, Germany) was prepared by adding 0.1g Bovine serum albumin (BSA) to 10 mL 1 $\times$ PBS.

(c) DAPI

4',6-diamidino-2-phenylindole (DAPI) was prepared by dissolving 1 mg of DAPI into 1ml of dH₂O. This was filter sterilised and used to prepare 1 $\times$ DAPI by diluting 1:1000 in 1 $\times$ PBS.

5.1.6 Southern and northern blot analysis solutions

(a) Lysis buffer

Lysis buffer was prepared by dissolving 58.44 g of NaCl (1 M), 0.1211g Trizma Base (100mM) and 10 g Sodium dodecyl sulfate (1%SDS) 800 ml of dH₂O before adding 40 mL 1 M EDTA. The volume was adjusted to 1L with distilled water. This was autoclaved at 121 $^{\circ}$ C, 1kg/cm² for 20 minutes and stored at 4 $^{\circ}$ C,

(b) 20 $\times$ SSC buffer

One hundred and seventy-five grams of NaCl and 88 g of sodium citrate dihydrate were dissolved in 800 mL of distilled water. Citric acid was used to adjust the pH to 7. The volume was adjusted to 1 L with distilled water. 5 $\times$ and 1 $\times$ SSC buffer were prepared by diluting the 20 $\times$ SSC buffer.

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(c) Denaturation buffer

Denaturation buffer was prepared by dissolving 88 g of NaCl (1.5 M) and 20 g NaOH (0.5 M) in 1 L dH₂O and stored at room temperature.

(d) Neutralisation buffer

Neutralisation buffer was prepared by dissolving 88 g of NaCl and 60.51 g of Tris Base (0.5M) (Sigma-Aldrich, MO, USA) in distilled water. The pH of the solution was adjusted to 7 using HCl. The final volume was adjusted to 1L with dH₂O stored at room temperature.

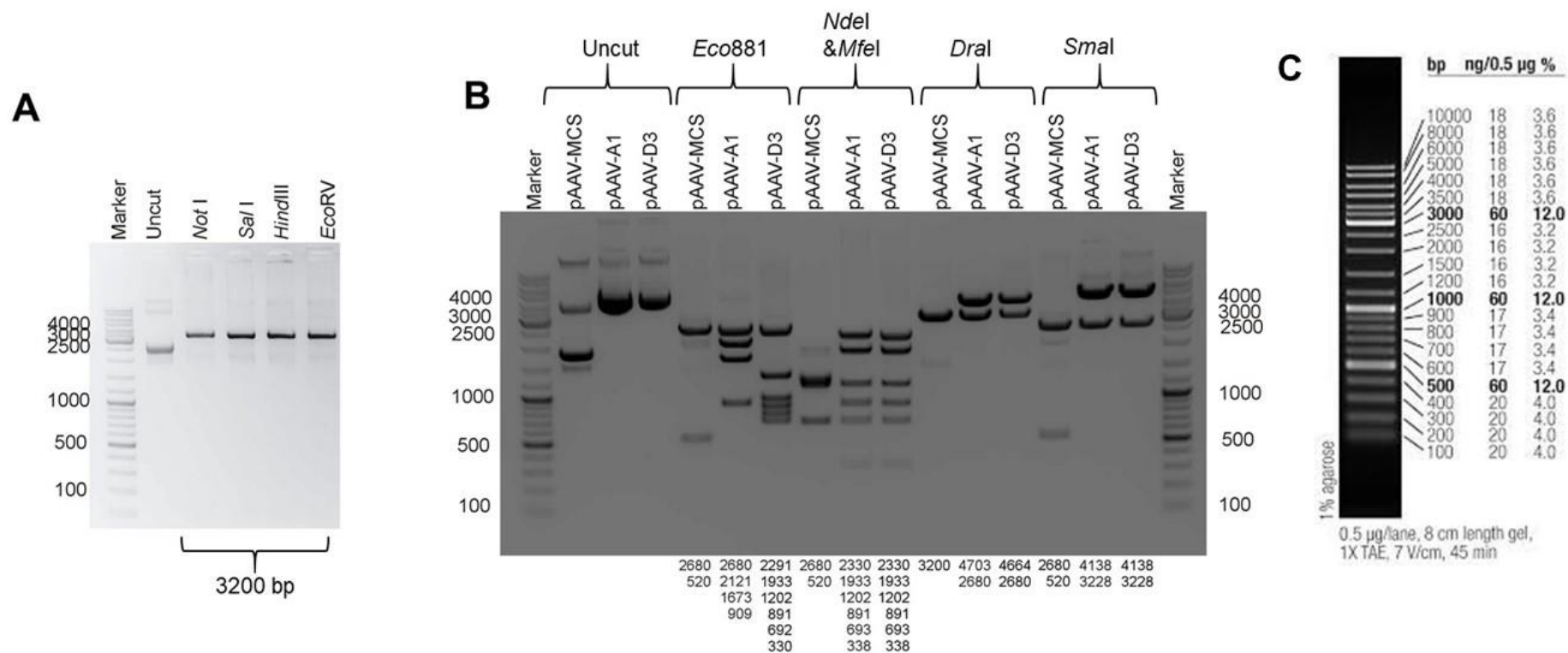
(e) Diethyl pyrocarbonate (DEPC) water

0.1% Diethylpyrocarbonate water was prepared by adding two of millilitre Diethylpyrocarbonate (Sigma, MO, USA) in 2L distilled water and stirred at Room temperature for 1 h. This was then autoclaved for 30 minutes at 121 °C and 1kg/cm²

(f) 10 × MOPS buffer

MOPS buffer was prepared by dissolving 41.86 of MOPS free acid ultra pure; 4.1 g of sodium acetate, 3.76 g of ethylenediaminetetraacetic acid in distilled water. The pH was then adjusted to 7.0 using NaOH. The volume of the solution was adjusted to 1L with dH₂O. This was followed by autoclaving the solution at 121 °C and 1kg/cm² for 20 minutes. It was stored in dark covered with foil at room temperature.

5.2. Digestion of pAAV-MCS and pAAV carrying HBV plasmids



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Figure 5.1: Enzymatic digestion of pAAV-MCS, pAAV-A1 and pAAV-D3.

A: Lane 1 (Molecular weight marker O' Gene ruler DNA ladder mix), **B:** The pAAV-A1 and pAAV-D3 plasmids carrying greater-than-genome length sequence of subgenotype A1 or D3 were digested with *Eco88I*/*DraI*/*NotI* or *MfeI* and *SmaI*. lane 1 (O' Gene ruler DNA ladder mix), lane 2 and lane 17 (O' Gene ruler DNA ladder mix). C, O' Gene ruler 1Kb DNA ladder, reference ladder)

5.3 Transfection of pAAV plasmids carrying greater-than HBV genome length sequence

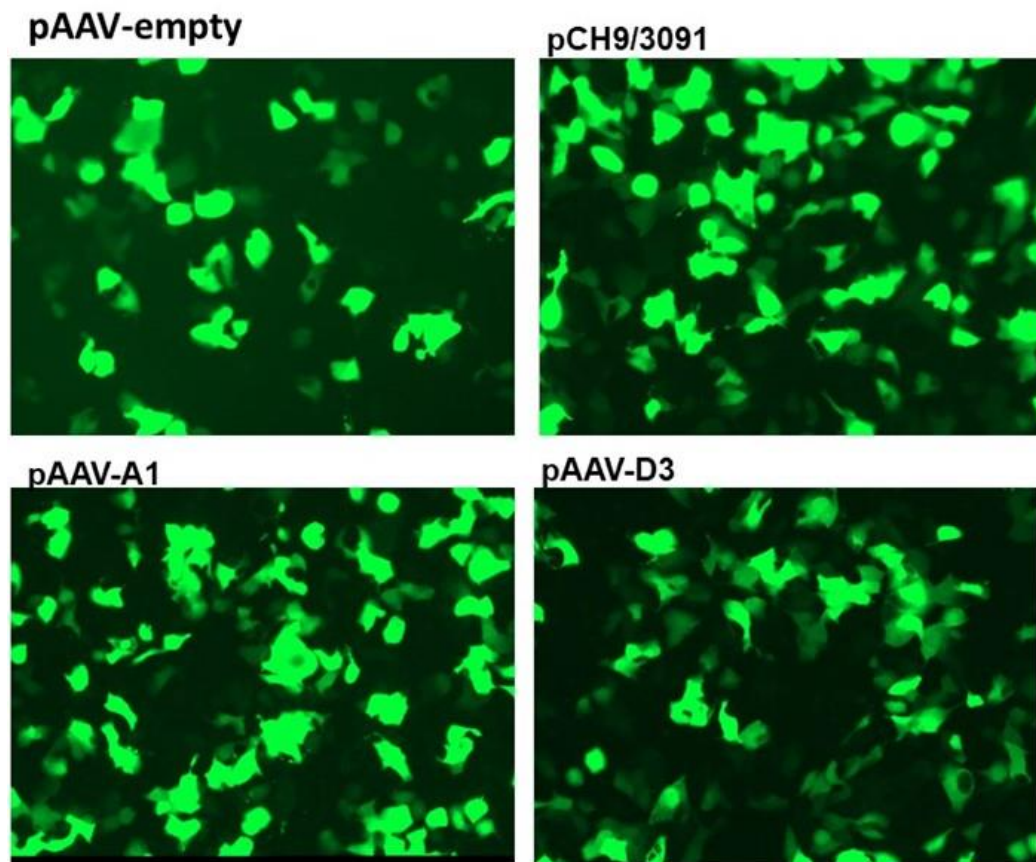


Figure 5.2: Detection of expressed GFP in cultured liver derived cell line by immunofluorescence microscopy.

Fluorescence microscopy was used to assess transfection efficiency of pAAV plasmids. Huh 7 cells were co-transfected with 100 ng of pAAV-A1, pAAV-D3, pAAV-empty or pCH-9/3091 and 50 ng of pAAV-CAG-GFP. Cells were imaged 24 hours after transfection. Representative images of pAAV-A1, pAAV-D3 and pAAV-empty transfections are shown.

5.4 Large-scale production of AAV vectors

Table 5.1: Second batch of recombinant AAV vectors quantified by qPCR

Second batch			
rAAV vectors	Average VPs/mL	Volume prep (mL)	Total VPs
AAV2-A1	7.2E+12	1.0	7.42E+12
AAV2-D3	2.8E+13	1.2	3.36E+13
AAV2-mTTR-BCDE	1.2E+12	1.3	1.56E+12
AAV2-GFP	6.80 E+12	1.0	6.80E+12
AAV2-miR31,5,8,9	2.160E+12	1.2	2.592E+12
AAV2-empty	1.1E+12	1.0	1.1E+12
AAV8-A1	6.78 E+12	1.5	1.017E+13
AAV8-D3	1.63E+13	1.2	1.95E+13
AAV8-miR31,5,8,9	4.48E+10	1.5	6.72E+11
AAV8-mTTR-BCDE	6.8E+12	1.0	6.8E+12
AAV8-Empty	3.62 E+12	1.2	4.34E+12

Table 5.2: Third batch of recombinant AAV vectors quantified by qPCR

Third batch			
rAAV vectors	Average VPs/mL	Volume prep (mL)	Total VPs
AAV8-A1	1.78E+12	1.0	1.7E+12
AAV8-D3	1.93E+13	1.2	2.316E+13
AAV8-mTTR-BCDE	2.2E+12	1.3	2.86E+12
AAV8-miR31,5,8,9	2.16E+12	1.2	2.592E+12
AAV8-empty	1.32E+12	1.0	1.32.E+12

5.5 Ethics approval



STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2019/02/6/C

APPLICANT: Ms S Limani

SCHOOL: Molecular Medicine and Haematology

DEPARTMENT:

LOCATION:

PROJECT TITLE: Using Adeno-associated viruses (AAVs) to model HBV replication in cell culture and in vivo (Mice)

Number and Species

120X 20-25g Female NMRI mice

Approval was given for the use of animals for the project described above at an AESC meeting held on 2019/02/26. This approval remains valid until 2021/03/13.

Unreported changes to the application may invalidate the clearance given by the AESC.

An annual progress report must be provided.

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

Signed: _____

(Chairperson AESC)

Date: _____

18/3/2019

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

Signed: _____

(Registered Veterinarian)

Date: _____

15 March 2019

cc: Supervisor: Dr B Maepa and Prof A Ely
Director: CAS

Works 2000/la/n0015/AESCcert.wps

5.6 Injection of AAV vectors bearing HBV results in low HBV gene expression

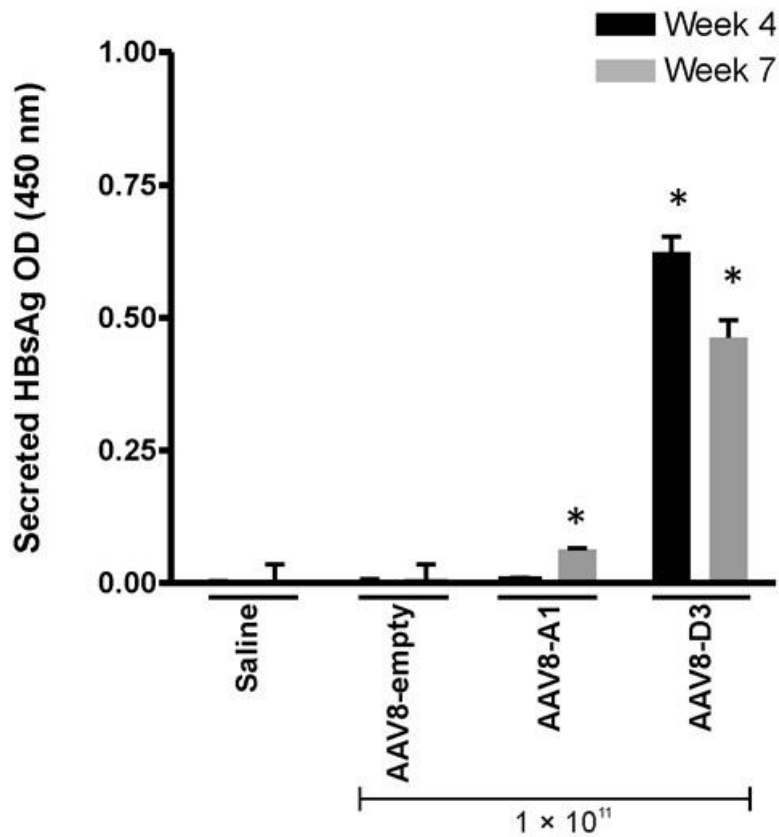


Figure 5.3: HBsAg expression in mice transduced with AAV carrying HBV genome.

Mice injected with 1×10^{11} viral particle equivalents (VPEs) of HBV genome bearing AAV. Mice were bled and 100 μ l serum used for HBsAg ELISA. *: $P \leq 0.05$.

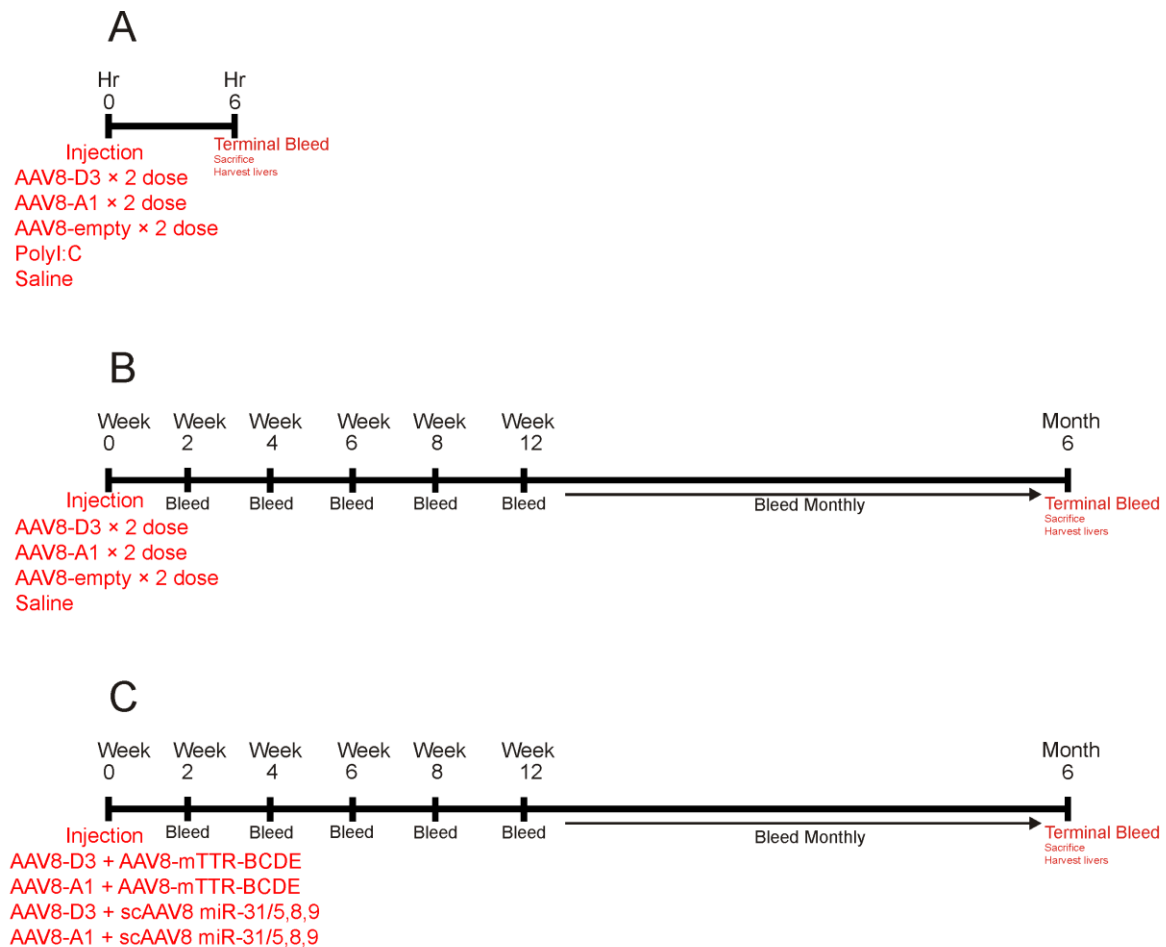


Figure 5.4: Sequence and timing as per experimental design.

A. Determination of inflammatory response. Mice were injected with AAV vectors bearing HBV genome sequence (AAV8-A1, AAV8-D3, AAV8-empty) at either 5×10^{11} VPEs/mouse or 9×10^{11} VPEs/mouse, Poly I:C (positive control) or Saline (negative control). Mice were bled and sacrificed 6 hours post-infection. **B.** Long term HBV gene expression and replication in mice. Mice were injected with AAV vectors bearing HBV genome sequence (AAV8-A1, AAV8-D3, AAV8-empty) at either 5×10^{11} VPEs/mouse or 9×10^{11} VPEs/mouse or Saline. Mice were bled as shown in the diagram. Six months post-injection, mice were sacrificed, and liver was harvested. **C.** Silencing of AAV mediated HBV gene expression and replication inhibition. Mice were co-injected with AAV vectors bearing HBV genome sequence (AAV8-A1, AAV8-D3, AAV8-empty) either at 5×10^{11} VPEs/mouse or 9×10^{11} VPEs/mouse and AAV8-mTTR-BCDE (negative control) or scAAV8 miR-31/5,8,9. Mice were bled as shown in the diagram. Six months post-injection, mice were sacrificed, and livers were harvested.

5.7 Schematic diagram showing HBV targets

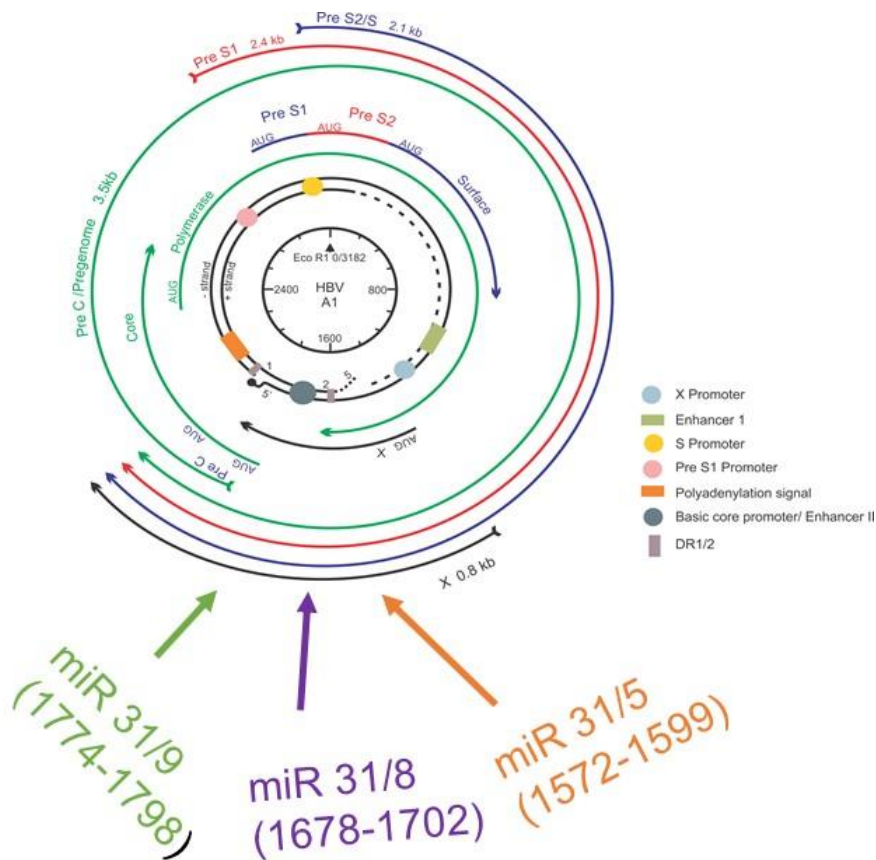


Figure 5.5: Schematic illustration of HBV targets.

The Relaxed circular DNA found in the virion with + and – strands. The nucleotide coordinates are indicated in a clockwise direction from *EcoRI* site. The HBx transcripts shown as outer arrow. The region of pri-miR targeted are indicated by axial arrow Adapted from (Maepa et al., 2017)].

5.8 HBV genotype D HBcAg amino acid sequence alignment

clustalw.aln

CLUSTAL 2.1 multiple sequence alignment

```

pCH-9/3091      MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAIL
KM519455        MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAIL
LC519824        MDIDPYKEFGASLELLSFLPADFFPSVRDLLDTASALYRDALESPEHCTPHHTALRQAIL
*****:*****:*****:*****:*****:*****

pCH-9/3091      CWGELMTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGRETVIEWYLV
KM519455        CWGELMTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGRETVIEWYLV
LC519824        CWGDLMTLATWVGVLQDPASRELVVNYVNTNMGLKFRQLLWFHVSCLTFGREVVEYLV
***:*****:*****:***.*****:*****.*:***

pCH-9/3091      SFGVWIRTPPAYRPPNAPILSTLPETTVLRRRGRSPRRRTSPRRRRSQSPRRRSQSRE
KM519455        SFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRRTSPRRRRSQSPRRRSQSRE
LC519824        SFGVWIRTPQAYRPPNAPILSTLPETTVVRRRGRTPRRTSPRRRRSQSPRRRSQSRE
***** *****:*****:*****:*****:*****

pCH-9/3091      SQC
KM519455        SQC
LC519824        SQC

```

Figure 5.6: CLUSTAL multiple sequence alignment of selected HBcAg sequences.

pCH-9/3091 contain HBV sequence of Genotype D, and is the most used sequence. KM519455 (Subgenotype D3 used in this study) and LC519824 (Genotype D) HBcAg sequences are from genotype D3 used in this study. The amino acid residues that form arginine rich region within the C-terminus are highlighted in green. The point of mutation within the implicated region is highlighted in yellow and in bold letter.

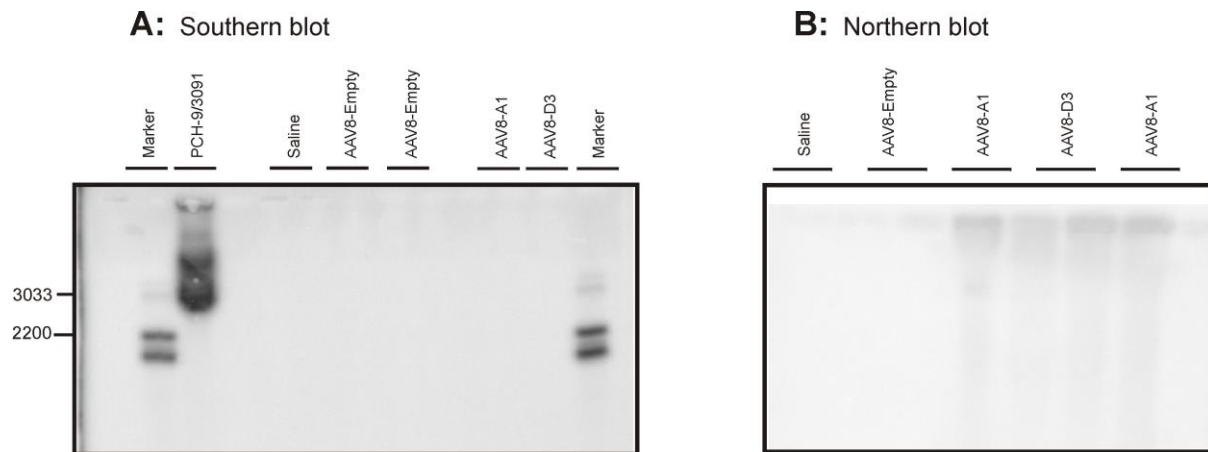


Figure 5. 7 : Southern and Northern blot Analysis of HBV replication and transcription in vivo

Southern (A) and Northern (B) blot analysis was performed using genomic DNA (A) or Total RNA (B) from livers of mice injected with saline or AAV8-empty, or AAV8-A1 or AAV8-D3 was extracted 8 weeks post injection. The pCH-9/3091 plasmid carrying HBV sequence was also included on agarose gel as the control to show probe hybridisation during a Southern blot analysis.

REFERENCES

- AL-SADEQ, D. W., TALEB, S. A., ZAIED, R. E., FAHAD, S. M., SMATTI, M. K., RIZEQ, B. R., AL THANI, A. A., YASSINE, H. M. & NASRALLAH, G. K. 2019. Hepatitis B Virus Molecular Epidemiology, Host-Virus Interaction, Coinfection, and Laboratory Diagnosis in the MENA Region: An Update. *Pathogens*, 8, 63.
- ALLWEISS, L., GASS, S., GIERSCHE, K., GROTH, A., KAH, J., VOLZ, T., RAPP, G., SCHÖBEL, A., LOHSE, A. W. & POLYWKA, S. 2016. Human liver chimeric mice as a new model of chronic hepatitis E virus infection and preclinical drug evaluation. *Journal of hepatology*, 64, 1033-1040.
- ARORA, A., SHARMA, P., TYAGI, P., SINGLA, V., ARORA, V., BANSAL, N., TOSHNIWAL, J. & KUMAR, A. 2013. Hepatitis B virus infection can cause hepatocellular carcinoma in less advanced liver cirrhosis: a comparative study of 142 patients from North India. *Journal of clinical and experimental hepatology*, 3, 288-295.
- ASABE, S., WIELAND, S. F., CHATTOPADHYAY, P. K., ROEDERER, M., ENGLE, R. E., PURCELL, R. H. & CHISARI, F. V. 2009. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. *Journal of virology*, 83, 9652-9662.
- ATCHISON, R. W., CASTO, B. C. & HAMMON, W. M. 1965. Adenovirus-associated defective virus particles. *Science*, 149, 754-755.
- AZUMA, H., PAULK, N., RANADE, A., DORRELL, C., AL-DHALIMY, M., ELLIS, E., STROM, S., KAY, M. A., FINEGOLD, M. & GROMPE, M. 2007. Robust expansion of human hepatocytes in *Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-} mice. *Nature biotechnology*, 25, 903-910.

- BANNISTER, E. G., YUEN, L., LITTLEJOHN, M., EDWARDS, R., SOZZI, V., COLLEDGE, D., LI, X., LOCARNINI, S., HARDIKAR, W. & REVILL, P. A. 2018. Molecular characterization of hepatitis B virus (HBV) in African children living in Australia identifies genotypes and variants associated with poor clinical outcome. *Journal of General Virology*, 99, 1103-1114.
- BARKER, L. F., MAYNARD, J. E., PURCELL, R. H., HOOFNAGLE, J. H., BERQUIST, K. R., LONDON, W. T., GERETY, R. J. & KRUSHAK, D. H. 1975. Hepatitis B virus infection in chimpanzees: titration of subtypes. *Journal of Infectious Diseases*, 132, 451-458.
- BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116, 281-97.
- BARTENSCHLAGER, R. & SCHALLER, H. 1992. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. *The EMBO journal*, 11, 3413-3420.
- BARTUSCH, C., DÖRING, T. & PRANGE, R. 2017. Rab33B controls hepatitis B virus assembly by regulating core membrane association and nucleocapsid processing. *Viruses*, 9, 157.
- BCHINI, R., CAPEL, F., DAUGUET, C., DUBANCHET, S. & PETIT, M. 1990. In vitro infection of human hepatoma (HepG2) cells with hepatitis B virus. *Journal of virology*, 64, 3025-3032.
- BERGERON, A., D'ASTOUS, M., TIMM, D. E. & TANGUAY, R. M. 2001. Structural and functional analysis of missense mutations in fumarylacetoacetate hydrolase, the gene deficient in hereditary tyrosinemia type 1. *Journal of Biological Chemistry*, 276, 15225-15231.

- BERNS, K. I. 2020. The Unusual Properties of the AAV Inverted Terminal Repeat. *Human Gene Therapy*, 31, 518-523.
- BERNSTEIN, E., CAUDY, A. A., HAMMOND, S. M. & HANNON, G. J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409, 363-366.
- BHoola, N. H., REUMANN, K., KEW, M. C., WILL, H. & KRAMVIS, A. 2014. Construction of replication competent plasmids of hepatitis B virus subgenotypes A1, A2 and D3 with authentic endogenous promoters. *Journal of virological methods*, 203, 54-64.
- BILITY, M. T., LI, F., CHENG, L. & SU, L. 2013. Liver immune-pathogenesis and therapy of human liver tropic virus infection in humanized mouse models. *Journal of gastroenterology and hepatology*, 28, 120-124.
- BLOOM, K., ELY, A., MUSSOLINO, C., CATHOMEN, T. & ARBUTHNOT, P. 2013. Inactivation of hepatitis B virus replication in cultured cells and in vivo with engineered transcription activator-like effector nucleases. *Molecular Therapy*, 21, 1889-1897.
- BOHNSACK, M. T., CZAPLINSKI, K. & GÖRLICH, D. 2004. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *Rna*, 10, 185-191.
- BOURHILL, T., ARBUTHNOT, P. & ELY, A. 2016. Successful disabling of the 5' UTR of HCV using adeno-associated viral vectors to deliver modular multimeric primary microRNA mimics. *Journal of virological methods*, 235, 26-33.
- BRUSS, V. & GANEM, D. 1991. The role of envelope proteins in hepatitis B virus assembly. *Proceedings of the National Academy of Sciences*, 88, 1059-1063.

- BÜNING, H. 2013. Gene therapy enters the pharma market: the short story of a long journey. *EMBO molecular medicine*, 5, 1-3.
- BURWITZ, B. J., WETTENGEL, J. M., MÜCK-HÄUSL, M. A., RINGELHAN, M., KO, C., FESTAG, M. M., HAMMOND, K. B., NORTHRUP, M., BIMBER, B. N. & JACOB, T. 2017a. Hepatocytic expression of human sodium-taurocholate cotransporting polypeptide enables hepatitis B virus infection of macaques. *Nature communications*, 8, 1-10.
- BURWITZ, B. J., WETTENGEL, J. M., MUCK-HAUSL, M. A., RINGELHAN, M., KO, C., FESTAG, M. M., HAMMOND, K. B., NORTHRUP, M., BIMBER, B. N., JACOB, T., REED, J. S., NORRIS, R., PARK, B., MOLLER-TANK, S., ESSER, K., GREENE, J. M., WU, H. L., ABDULHAQQ, S., WEBB, G., SUTTON, W. F., KLUG, A., SWANSON, T., LEGASSE, A. W., VU, T. Q., ASOKAN, A., HAIGWOOD, N. L., PROTZER, U. & SACHA, J. B. 2017b. Hepatocytic expression of human sodium-taurocholate cotransporting polypeptide enables hepatitis B virus infection of macaques. *Nat Commun*, 8, 2146.
- CAI, C. W., LOMONOSOVA, E., MORAN, E. A., CHENG, X., PATEL, K. B., BAILLY, F., COTELLE, P., MEYERS, M. J. & TAVIS, J. E. 2014. Hepatitis B virus replication is blocked by a 2-hydroxyisoquinoline-1, 3 (2H, 4H)-dione (HID) inhibitor of the viral ribonuclease H activity. *Antiviral research*, 108, 48-55.
- CARMONA, S., ELY, A., CROWTHER, C., MOOLLA, N., SALAZAR, F. H., MARION, P. L., FERRY, N., WEINBERG, M. S. & ARBUTHNOT, P. 2006. Effective inhibition of HBV replication in vivo by anti-HBx short hairpin RNAs. *Molecular Therapy*, 13, 411-421.

- CHAN, H. L., HUI, A., WONG, M., TSE, A. M., HUNG, L. C., WONG, V. W. & SUNG, J. J. 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut*, 53, 1494-1498.
- CHEN, B.-F. 2016. Different pre-S deletion patterns and their association with hepatitis B virus genotypes. *World journal of gastroenterology*, 22, 8041.
- CHEN, C.-C., SUN, C.-P., MA, H.-I., FANG, C.-C., WU, P.-Y., XIAO, X. & TAO, M.-H. 2009. Comparative study of anti-hepatitis B virus RNA interference by double-stranded adeno-associated virus serotypes 7, 8, and 9. *Molecular Therapy*, 17, 352-359.
- CHEN, M. & OU, J.-H. 1995. Cell type-dependent regulation of the activity of the negative regulatory element of the hepatitis B virus core promoter. *Virology*, 214, 198-206.
- CHEN, M., SÄLLBERG, M., HUGHES, J., JONES, J., GUIDOTTI, L. G., CHISARI, F. V., BILLAUD, J.-N. & MILICH, D. R. 2005. Immune tolerance split between hepatitis B virus precore and core proteins. *Journal of virology*, 79, 3016-3027.
- CHENG, C.-P., LEE, P.-F., LIU, W.-C., WU, I.-C., CHIN, C.-Y., CHANG, T.-T. & TSENG, V. S. 2012. Analysis of precore/core covariances associated with viral kinetics and genotypes in hepatitis B e antigen-positive chronic hepatitis B patients. *PloS one*, 7, e32553.
- CHISARI, F., FILIPPI, P., MCLACHLAN, A., MILICH, D., RIGGS, M., LEE, S., PALMITER, R., PINKERT, C. & BRINSTER, R. 1986. Expression of hepatitis B virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. *Journal of virology*, 60, 880-887.

- CHISARI, F., PINKERT, C., MILICH, D., FILIPPI, P., MCLACHLAN, A., PALMITER, R. & BRINSTER, R. 1985. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. *Science*, 230, 1157-1160.
- CHISARI, F. V. 1995. Hepatitis B virus transgenic mice: insights into the virus and the disease. *Hepatology*, 22, 1316-1325.
- CHISARI, F. V., FILIPPI, P., BURAS, J., MCLACHLAN, A., POPPER, H., PINKERT, C. A., PALMITER, R. D. & BRINSTER, R. L. 1987. Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. *Proceedings of the National Academy of Sciences*, 84, 6909-6913.
- CHOI, J.-G., BHARAJ, P., ABRAHAM, S., MA, H., YI, G., YE, C., DANG, Y., MANJUNATH, N., WU, H. & SHANKAR, P. 2015. Multiplexing seven miRNA-based shRNAs to suppress HIV replication. *Molecular Therapy*, 23, 310-320.
- CHOU, H.-H., CHIEN, W.-H., WU, L.-L., CHENG, C.-H., CHUNG, C.-H., HORNG, J.-H., NI, Y.-H., TSENG, H.-T., WU, D. & LU, X. 2015. Age-related immune clearance of hepatitis B virus infection requires the establishment of gut microbiota. *Proceedings of the National Academy of Sciences*, 112, 2175-2180.
- CHU, C.-M., YEH, C.-T., SHEEN, I.-S. & LIAW, Y.-F. 1995. Subcellular localization of hepatitis B core antigen in relation to hepatocyte regeneration in chronic hepatitis B. *Gastroenterology*, 109, 1926-1932.
- CRISSIEN, A. M. & FRENETTE, C. 2014. Current management of hepatocellular carcinoma. *Gastroenterology & hepatology*, 10, 153.

- CROWTHER, C., MOWA, B. & ARBUTHNOT, P. 2016. Hepatic delivery of artificial micro RNAs using helper-dependent adenoviral vectors. *SiRNA Delivery Methods*. Springer.
- DANDRI, M., BURDA, M. R., TÖRÖK, E., POLLOK, J. M., IWANSKA, A., SOMMER, G., ROGIERS, X., ROGLER, C. E., GUPTA, S. & WILL, H. 2001. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology*, 33, 981-988.
- DANDRI, M. & PETERSEN, J. 2017. Animal models of HBV infection. *Best Practice & Research Clinical Gastroenterology*, 31, 273-279.
- DAYA, S. & BERNS, K. I. 2008. Gene therapy using adeno-associated virus vectors. *Clinical microbiology reviews*, 21, 583-593.
- DE OLIVEIRA, A. P. & FRAEFEL, C. 2010. Herpes simplex virus type 1/adeno-associated virus hybrid vectors. *The open virology journal*, 4, 109.
- DECORSIÈRE, A., MUELLER, H., VAN BREUGEL, P. C., ABDUL, F., GEROSSIER, L., BERAN, R. K., LIVINGSTON, C. M., NIU, C., FLETCHER, S. P. & HANTZ, O. 2016. Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. *Nature*, 531, 386.
- DECORSIERE, A., MUELLER, H., VAN BREUGEL, P. C., ABDUL, F., GEROSSIER, L., BERAN, R. K., LIVINGSTON, C. M., NIU, C., FLETCHER, S. P., HANTZ, O. & STRUBIN, M. 2016. Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. *Nature*, 531, 386-9.
- DEVERMAN, B. E., PRAVDO, P. L., SIMPSON, B. P., KUMAR, S. R., CHAN, K. Y., BANERJEE, A., WU, W.-L., YANG, B., HUBER, N. & PASCA, S. P. 2016. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nature biotechnology*, 34, 204-209.

- DIENSTAG, J. L., GOLDIN, R. D., HEATHCOTE, E. J., HANN, H., WOESSNER, M., STEPHENSON, S. L., GARDNER, S., GRAY, D. F. & SCHIFF, E. R. 2003. Histological outcome during long-term lamivudine therapy. *Gastroenterology*, 124, 105-117.
- DION, S., BOURGINE, M., GODON, O., LEVILLAYER, F. & MICHEL, M.-L. 2013. Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. *Journal of virology*, 87, 5554-5563.
- DOITSH, G. & SHAUL, Y. 2004. Enhancer I predominance in hepatitis B virus gene expression. *Molecular and cellular biology*, 24, 1799-1808.
- DOMINGUEZ II, J. M., HU, P., CABALLERO, S., MOLDOVAN, L., VERMA, A., OUDIT, G. Y., LI, Q. & GRANT, M. B. 2016. Adeno-associated virus overexpression of angiotensin-converting enzyme-2 reverses diabetic retinopathy in type 1 diabetes in mice. *The American journal of pathology*, 186, 1688-1700.
- DUAN, D., LI, Q., KAO, A. W., YUE, Y., PESSIN, J. E. & ENGELHARDT, J. F. 1999. Dynamin is required for recombinant adeno-associated virus type 2 infection. *J Virol*, 73, 10371-6.
- DUPINAY, T., GHEIT, T., ROQUES, P., COVA, L., CHEVALLIER-QUEYRON, P., TASAHSU, S. I., LE GRAND, R., SIMON, F., CORDIER, G. & WAKRIM, L. 2013. Discovery of naturally occurring transmissible chronic hepatitis B virus infection among *Macaca fascicularis* from Mauritius Island. *Hepatology*, 58, 1610-1620.
- DURANTE, D., CARROUÉE-DURANTE, S., WERLE-LAPOSTOLLE, B., BRUNELLE, M. N., PICHOU, C., TRÉPO, C. & ZOULIM, F. 2004. A new

- strategy for studying in vitro the drug susceptibility of clinical isolates of human hepatitis B virus. *Hepatology*, 40, 855-864.
- EL-SERAG, H. B. 2012. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology*, 142, 1264-1273. e1.
- EL-SERAG, H. B. 2020. Epidemiology of hepatocellular carcinoma. *The Liver: Biology and Pathobiology*, 758-772.
- ELY, A., NAIDOO, T. & ARBUTHNOT, P. 2009. Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nucleic acids research*, 37, e91-e91.
- ELY, A., NAIDOO, T., MUFAMADI, S., CROWTHER, C. & ARBUTHNOT, P. 2008a. Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo. *Molecular Therapy*, 16, 1105-1112.
- ELY, A., NAIDOO, T., MUFAMADI, S., CROWTHER, C. & ARBUTHNOT, P. 2008b. Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo. *Mol Ther*, 16, 1105-12.
- EREN, R., ILAN, E., NUSSBAUM, O., LUBIN, I., TERKIELTAUB, D., ARAZI, Y., BEN-MOSHE, O., KITCHINZKY, A., BERR, S. & GOPHER, J. 2000. Preclinical evaluation of two human anti-hepatitis B virus (HBV) monoclonal antibodies in the HBV-trimera mouse model and in HBV chronic carrier chimpanzees. *Hepatology*, 32, 588-596.
- FERRARI, C., PENNA, A., BERTOLETTI, A., VALLI, A., ANTONI, A. D., GIUBERTI, T., CAVALLI, A., PETIT, M. & FIACCADORI, F. 1990. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *The Journal of Immunology*, 145, 3442-3449.

- FUNG, S. K. & LOK, A. S. 2004. Drug insight: Nucleoside and nucleotide analog inhibitors for hepatitis B. *Nature Clinical Practice Gastroenterology & Hepatology*, 1, 90-97.
- GALLE, P. R., HAGELSTEIN, J., KOMMERELL, B., VOLKMANN, M., SCHRANZ, P. & ZENTGRAF, H. 1994. In vitro experimental infection of primary human hepatocytes with hepatitis B virus. *Gastroenterology*, 106, 664-673.
- GAO, G., VANDENBERGHE, L. H., ALVIRA, M. R., LU, Y., CALCEDO, R., ZHOU, X. & WILSON, J. M. 2004. Clades of Adeno-associated viruses are widely disseminated in human tissues. *Journal of virology*, 78, 6381-6388.
- GE, J.-H., ZHANG, L.-Z., LI, J.-X., LIU, H., LIU, H.-M., HE, J., YAO, Y.-C., YANG, Y.-J., YU, H.-Y. & HU, Y.-P. 2004. Replication and gene expression of mutant hepatitis B virus in a transgenic mouse containing the complete viral genome with mutant s gene. *World journal of gastroenterology: WJG*, 10, 3141.
- GERLICH, W. H. 2013. Medical virology of hepatitis B: how it began and where we are now. *Virology journal*, 10, 239.
- GILADI, H., KETZINEL-GILAD, M., RIVKIN, L., FELIG, Y., NUSSBAUM, O. & GALUN, E. 2003. Small interfering RNA inhibits hepatitis B virus replication in mice. *Molecular Therapy*, 8, 769-776.
- GRIMM, D., LEE, J. S., WANG, L., DESAI, T., AKACHE, B., STORM, T. A. & KAY, M. A. 2008. In vitro and in vivo gene therapy vector evolution via multispecies interbreeding and retargeting of adeno-associated viruses. *Journal of virology*, 82, 5887-5911.
- GRIMM, D., STREETZ, K. L., JOPLING, C. L., STORM, T. A., PANDEY, K., DAVIS, C. R., MARION, P., SALAZAR, F. & KAY, M. A. 2006. Fatality in mice due to

- oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*, 441, 537-541.
- GRIMM, D., THIMME, R. & BLUM, H. E. 2011. HBV life cycle and novel drug targets. *Hepatology international*, 5, 644-653.
- GRIPON, P., DIOT, C. & GUGUEN-GUILLOUZO, C. 1993. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology*, 192, 534-540.
- GRIPON, P., DIOT, C., THEZE, N., FOUREL, I., LOREAL, O., BRECHOT, C. & GUGUEN-GUILLOUZO, C. 1988. Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. *Journal of virology*, 62, 4136-4143.
- GRIPON, P., RUMIN, S., URBAN, S., LE SEYEC, J., GLAISE, D., CANNIE, I., GUYOMARD, C., LUCAS, J., TREPO, C. & GUGUEN-GUILLOUZO, C. 2002. Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences*, 99, 15655-15660.
- GUHA, C., MOHAN, S., ROY-CHOWDHURY, N. & ROY-CHOWDHURY, J. 2004. Cell culture and animal models of viral hepatitis. Part I: hepatitis B. *Lab animal*, 33, 37-46.
- GUIDOTTI, L. G., MARTINEZ, V., LOH, Y.-T., ROGLER, C. E. & CHISARI, F. V. 1994. Hepatitis B virus nucleocapsid particles do not cross the hepatocyte nuclear membrane in transgenic mice. *Journal of virology*, 68, 5469-5475.
- GUIDOTTI, L. G., ROCHFORD, R., CHUNG, J., SHAPIRO, M., PURCELL, R. & CHISARI, F. V. 1999. Viral clearance without destruction of infected cells during acute HBV infection. *Science*, 284, 825-829.

- GÜNTHER, S., PIWON, N., IWANSKA, A., SCHILLING, R., MEISEL, H. & WILL, H. 1996. Type, prevalence, and significance of core promoter/enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. *Journal of virology*, 70, 8318-8331.
- GUZMAN-COTTRILL, J. A., RAVIN, K. A., BRYANT, K. A., ZERR, D. M., KOCIOLEK, L. & SIEGEL, J. D. 2013. Infection prevention and control in residential facilities for pediatric patients and their families. *Infection Control & Hospital Epidemiology*, 34, 1003-1041.
- HABIG, J. W. & LOEB, D. D. 2003. Template switches during plus-strand DNA synthesis of duck hepatitis B virus are influenced by the base composition of the minus-strand terminal redundancy. *Journal of virology*, 77, 12412-12420.
- HABIG, J. W. & LOEB, D. D. 2006. Sequence identity of the direct repeats, DR1 and DR2, contributes to the discrimination between primer translocation and in situ priming during replication of the duck hepatitis B virus. *Journal of molecular biology*, 364, 32-43.
- HAMASAKI, K., NAKAO, K., MATSUMOTO, K., ICHIKAWA, T., ISHIKAWA, H. & EGUCHI, K. 2003. Short interfering RNA-directed inhibition of hepatitis B virus replication. *FEBS letters*, 543, 51-54.
- HAMMOND, S. M., BERNSTEIN, E., BEACH, D. & HANNON, G. J. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*, 404, 293-6.
- HAN, J., LEE, Y., YEOM, K.-H., KIM, Y.-K., JIN, H. & KIM, V. N. 2004. The Drosha-DGCR8 complex in primary microRNA processing. *Genes & development*, 18, 3016-3027.

- HANAHAN, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of molecular biology*, 166, 557-580.
- HANTZ, O., PARENT, R., DURANTEL, D., GRIPON, P., GUGUEN-GUILLOUZO, C. & ZOULIM, F. 2009. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. *Journal of General Virology*, 90, 127-135.
- HECKEL, J. L., SANDGREN, E. P., DEGEN, J. L., PALMITER, R. D. & BRINSTER, R. L. 1990. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell*, 62, 447-456.
- HEERMANN, K., GOLDMANN, U., SCHWARTZ, W., SEYFFARTH, T., BAUMGARTEN, H. & GERLICH, W. 1984. Large surface proteins of hepatitis B virus containing the pre-s sequence. *Journal of virology*, 52, 396-402.
- HELSEN, N., DEBING, Y., PAESHUYSE, J., DALLMEIER, K., BOON, R., COLL, M., SANCHO-BRU, P., CLAES, C., NEYTS, J. & VERFAILLIE, C. M. 2016. Stem cell-derived hepatocytes: A novel model for hepatitis E virus replication. *Journal of hepatology*, 64, 565-573.
- HORDEAUX, J., WANG, Q., KATZ, N., BUZA, E. L., BELL, P. & WILSON, J. M. 2018. The neurotropic properties of AAV-PHP. B are limited to C57BL/6J mice. *Molecular Therapy*, 26, 664-668.
- HSU, H. Y., CHANG, M. H., HSIEH, K. H., LEE, C. Y., LIN, H. H., HWANG, L. H., CHEN, P. J. & CHEN, D. S. 1992. Cellular immune response to HBcAg in mother-to-infant transmission of hepatitis B virus. *Hepatology*, 15, 770-776.
- HU, J., LIN, Y.-Y., CHEN, P.-J., WATASHI, K. & WAKITA, T. 2019. Cell and animal models for studying hepatitis B virus infection and drug development. *Gastroenterology*, 156, 338-354.

- HUANG, H.-C., CHEN, C.-C., CHANG, W.-C., TAO, M.-H. & HUANG, C. 2012a. Entry of hepatitis B virus into immortalized human primary hepatocytes by clathrin-dependent endocytosis. *Journal of virology*, 86, 9443-9453.
- HUANG, L.-R., WU, H.-L., CHEN, P.-J. & CHEN, D.-S. 2006. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. *Proceedings of the National Academy of Sciences*, 103, 17862-17867.
- HUANG, L. R., GÄBEL, Y. A., GRAF, S., ARZBERGER, S., KURTS, C., HEIKENWALDER, M., KNOLLE, P. A. & PROTZER, U. 2012b. Transfer of HBV genomes using low doses of adenovirus vectors leads to persistent infection in immune competent mice. *Gastroenterology*, 142, 1447-1450. e3.
- HUANG, Y.-H., FANG, C.-C., TSUNNEYAMA, K., CHOU, H.-Y., PAN, W.-Y., SHIH, Y.-M., WU, P.-Y., CHEN, Y., LEUNG, P. S. & GERSHWIN, M. E. 2011. A murine model of hepatitis B-associated hepatocellular carcinoma generated by adeno-associated virus-mediated gene delivery. *International journal of oncology*, 39, 1511-1519.
- HUANG, Y. 2017. Preclinical and clinical advances of GalNAc-decorated nucleic acid therapeutics. *Molecular Therapy-Nucleic Acids*, 6, 116-132.
- HUTVÁGNER, G. & ZAMORE, P. D. 2002. A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, 297, 2056-2060.
- HUY, T. T. T., NGOC, T. T. & ABE, K. 2008. New complex recombinant genotype of hepatitis B virus identified in Vietnam. *Journal of virology*, 82, 5657-5663.
- ILAN, E., BURAKOVA, T., DAGAN, S., NUSSBAUM, O., LUBIN, I., EREN, R., BEN-MOSHE, O., ARAZI, J., BERR, S. & NEVILLE, L. 1999. The hepatitis B virus-trimera mouse: A model for human HBV infection and evaluation of anti-HBV therapeutic agents. *Hepatology*, 29, 553-562.

- INUZUKA, T., TAKAHASHI, K., CHIBA, T. & MARUSAWA, H. 2014. Mouse models of hepatitis B virus infection comprising host-virus immunologic interactions. *Pathogens*, 3, 377-389.
- IWAMOTO, M., WATASHI, K., TSUKUDA, S., ALY, H. H., FUKASAWA, M., FUJIMOTO, A., SUZUKI, R., AIZAKI, H., ITO, T. & KOIWAI, O. 2014. Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP. *Biochemical and biophysical research communications*, 443, 808-813.
- JACOBS, R. *Adeno-associated viral vectors expressing anti-HBV TALENs from an mTTR promoter*.
- JAZAYERI, M. S., DORNAN, E. S., BONER, W., FATTOVICH, G., HADZIYANNIS, S. & CARMAN, W. F. 2004. Intracellular distribution of hepatitis B virus core protein expressed in vitro depends on the sequence of the isolate and the serologic pattern. *The Journal of infectious diseases*, 189, 1634-1645.
- JOO, K. I., FANG Y FAU - LIU, Y., LIU Y FAU - XIAO, L., XIAO L FAU - GU, Z., GU Z FAU - TAI, A., TAI A FAU - LEE, C.-L., LEE CL FAU - TANG, Y., TANG Y FAU - WANG, P. & WANG, P. 2011. Enhanced real-time monitoring of adeno-associated virus trafficking by virus-quantum dot conjugates.
- KANE, M. 1995. Global programme for control of hepatitis B infection. *Vaccine*, 13, S47-S49.
- KAWAI, K., HORIIKE, N., MICHITAKA, K. & ONJI, M. 2003. The effects of hepatitis B virus core promoter mutations on hepatitis B core antigen distribution in hepatocytes as detected by laser-assisted microdissection. *Journal of hepatology*, 38, 635-641.

- KENNERDELL, J. R. & CARTHEW, R. W. 1998. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell*, 95, 1017-1026.
- KIMBI, G. C., KRAMVIS, A. & KEW, M. C. 2004. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *Journal of general virology*, 85, 1211-1220.
- KLEIN, C., BOCK, C. T., WEDEMEYER, H., WÜSTEFELD, T., LOCARNINI, S., DIENES, H. P., KUBICKA, S., MANNS, M. P. & TRAUTWEIN, C. 2003. Inhibition of hepatitis B virus replication in vivo by nucleoside analogues and siRNA. *Gastroenterology*, 125, 9-18.
- KNIPE, D., HOWLEY, P., GRIFFIN, D., LAMB, R., MARTIN, M., ROIZMAN, B. & STRAUS, S. 2013. Fields Virology. Vol I+ II. Lippincott, Williams & Wilkins.
- KOCZOT, F. J., CARTER, B. J., GARON, C. F. & ROSE, J. A. 1973. Self-complementarity of terminal sequences within plus or minus strands of adenovirus-associated virus DNA. *Proceedings of the National Academy of Sciences*, 70, 215-219.
- KOIKE, K., MORIYA, K., IINO, S., YOTSUYANAGI, H., ENDO, Y., MIYAMURA, T. & KUROKAWA, K. 1994. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. *Hepatology*, 19, 810-819.
- KONISHI, M., WU, C. H. & WU, G. Y. 2003. Inhibition of HBV replication by siRNA in a stable HBV-producing cell line. *Hepatology*, 38, 842-850.
- KOTIN, R. M., SINISCALCO, M., SAMULSKI, R. J., ZHU, X. D., HUNTER, L., LAUGHLIN, C. A., MCLAUGHLIN, S., MUZYCZKA, N., ROCCHI, M. & BERNIS, K. I. 1990. Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A*, 87, 2211-5.

- KRAJDEN, M., MCNABB, G. & PETRIC, M. 2005. The laboratory diagnosis of hepatitis B virus. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 16.
- KRAMVIS, A. 2014. Genotypes and genetic variability of hepatitis B virus. *Intervirology*, 57, 141-150.
- KRAMVIS, A., KOSTAKI, E.-G., HATZAKIS, A. & PARASKEVIS, D. 2018. Immunomodulatory function of HBeAg related to short-sighted evolution, transmissibility, and clinical manifestation of hepatitis B virus. *Frontiers in microbiology*, 9, 2521.
- KULIK, L. & EL-SERAG, H. B. 2019. Epidemiology and management of hepatocellular carcinoma. *Gastroenterology*, 156, 477-491. e1.
- KUMAR, A. G., SRIDHARAN, K. & THIRUNALASUNDARI, T. 2007. Prevalence pattern of blood borne hepatitis group of viruses in liver disease patients. *World Journal of Medical Sciences*, 2, 33-38.
- LADNER, S. K., OTTO, M. J., BARKER, C. S., ZAIFERT, K., WANG, G.-H., GUO, J.-T., SEEGER, C. & KING, R. W. 1997. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. *Antimicrobial agents and chemotherapy*, 41, 1715-1720.
- LAGO, B. V., MELLO, F. C., KRAMVIS, A., NIEL, C. & GOMES, S. A. 2014. Hepatitis B virus subgenotype A1: evolutionary relationships between Brazilian, African and Asian isolates. *PloS one*, 9.
- LAI, C.-L., CHIEN, R.-N., LEUNG, N. W., CHANG, T.-T., GUAN, R., TAI, D.-I., NG, K.-Y., WU, P.-C., DENT, J. C. & BARBER, J. 1998. A one-year trial of

- lamivudine for chronic hepatitis B. *New England Journal of Medicine*, 339, 61-68.
- LANFORD, R. E., GUERRA, B., CHAVEZ, D., GIAVEDONI, L., HODARA, V. L., BRASKY, K. M., FOSDICK, A., FREY, C. R., ZHENG, J. & WOLFGANG, G. 2013. GS-9620, an oral agonist of Toll-like receptor-7, induces prolonged suppression of hepatitis B virus in chronically infected chimpanzees. *Gastroenterology*, 144, 1508-1517. e10.
- LE BEC, C. & DOUAR, A. 2006. Gene therapy progress and prospects—vectorology: design and production of expression cassettes in AAV vectors. *Gene therapy*, 13, 805-813.
- LEE, W. M. 1997. Hepatitis B virus infection. *New England journal of medicine*, 337, 1733-1745.
- LI, C. & SAMULSKI, R. J. 2020. Engineering adeno-associated virus vectors for gene therapy. *Nature Reviews Genetics*, 1-18.
- LI, F., NIO, K., YASUI, F., MURPHY, C. M. & SU, L. 2017. Studying HBV Infection and Therapy in Immune-Deficient NOD-Rag1^{-/-} IL2RgammaC-null (NRG) Fumarylacetoacetate Hydrolase (Fah) Knockout Mice Transplanted with Human Hepatocytes. *Hepatitis B Virus*. Springer.
- LI, H., ZHUANG, Q., WANG, Y., ZHANG, T., ZHAO, J., ZHANG, Y., ZHANG, J., LIN, Y., YUAN, Q. & XIA, N. 2014. HBV life cycle is restricted in mouse hepatocytes expressing human NTCP. *Cellular & molecular immunology*, 11, 175-183.
- LIANG, W., MENKE, A. L., DRIESSEN, A., KOEK, G. H., LINDEMAN, J. H., STOOP, R., HAVEKES, L. M., KLEEMANN, R. & VAN DEN HOEK, A. M. 2014.

- Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. *PloS one*, 9, e115922.
- LIANG, X., BI, S., YANG, W., WANG, L., CUI, G., CUI, F., ZHANG, Y., LIU, J., GONG, X. & CHEN, Y. 2009. Epidemiological serosurvey of hepatitis B in China—declining HBV prevalence due to hepatitis B vaccination. *vaccine*, 27, 6550-6557.
- LIAW, Y.-F. & CHU, C.-M. 2009. Hepatitis B virus infection. *The lancet*, 373, 582-592.
- LIM, L. P., LAU, N. C., GARRETT-ENGELE, P., GRIMSON, A., SCHELTER, J. M., CASTLE, J., BARTEL, D. P., LINSLEY, P. S. & JOHNSON, J. M. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, 433, 769-73.
- LIM, S. S., VOS, T., FLAXMAN, A. D., DANAEI, G., SHIBUYA, K., ADAIR-ROHANI, H., ALMAZROA, M. A., AMANN, M., ANDERSON, H. R. & ANDREWS, K. G. 2012. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The lancet*, 380, 2224-2260.
- LIN, C. L. & KAO, J. H. 2011. The clinical implications of hepatitis B virus genotype: Recent advances. *Journal of gastroenterology and hepatology*, 26, 123-130.
- LIU, F., SONG, Y. & LIU, D. 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene therapy*, 6, 1258-1266.
- LIU, Y., JOO, K. I. & WANG, P. 2013. Endocytic processing of adeno-associated virus type 8 vectors for transduction of target cells. *Gene Therapy*, 20, 308-317.

- LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods*, 25, 402-408.
- LOHMANN, J. U., ENDL, I. & BOSCH, T. C. 1999. Silencing of developmental genes in Hydra. *Developmental biology*, 214, 211-214.
- LU, S. N., CHEN, T. M., LEE, C. M., WANG, J. H., TUNG, H. D. & WU, J. C. 2003. Molecular epidemiological and clinical aspects of hepatitis D virus in a unique triple hepatitis viruses (B, C, D) endemic community in Taiwan. *Journal of medical virology*, 70, 74-80.
- MABIT, H., DUBANCHET, S., CAPEL, F., DAUGUET, C. & PETIT, M.-A. 1994. In vitro infection of human hepatoma cells (HepG2) with hepatitis B virus (HBV): spontaneous selection of a stable HBV surface antigen-producing HepG2 cell line containing integrated HBV DNA sequences. *Journal of general virology*, 75, 2681-2689.
- MAEPA, M. B., BLOOM, K., ELY, A. & ARBUTHNOT, P. 2021. Hepatitis B virus: promising drug targets and therapeutic implications. *Expert Opinion on Therapeutic Targets*, 1-16.
- MAEPA, M. B., ELY, A., GRAYSON, W. & ARBUTHNOT, P. 2017. Sustained inhibition of HBV replication in vivo after systemic injection of AAVs encoding artificial antiviral primary microRNAs. *Molecular Therapy-Nucleic Acids*, 7, 190-199.
- MAGNIUS, L. O. & NORDER, H. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology*, 38, 24-34.

- MAK, D. & KRAMVIS, A. 2020. Molecular characterization of hepatitis B virus isolated from Black South African cancer patients, with and without hepatocellular carcinoma. *Archives of Virology*, 165, 1815-1825.
- MAO, T., KUSEFOGLU, D., HOOKS, B. M., HUBER, D., PETREANU, L. & SVOBODA, K. 2011. Long-range neuronal circuits underlying the interaction between sensory and motor cortex. *Neuron*, 72, 111-123.
- MARGOLIS, H. S. 1998. Hepatitis B virus infection. *Bulletin of the World Health Organization*, 76, 152.
- MARIMANI, M. D., ELY, A., BUFF, M. C., BERNHARDT, S., ENGELS, J. W. & ARBUTHNOT, P. 2013. Inhibition of hepatitis B virus replication in cultured cells and in vivo using 2'-O-guanidinopropyl modified siRNAs. *Bioorganic & medicinal chemistry*, 21, 6145-6155.
- MARION, P., SALAZAR, F., LIITTSCHWAGER, K., BORDIER, B., SEEGER, C., WINTERS, M., COOPER, A. & CULLEN, J. 2003. A transgenic mouse lineage useful for testing antivirals targeting hepatitis B virus. *Frontiers in Viral Hepatitis*. Elsevier.
- MARTINEZ, M. A., GUTIERREZ, A., ARMAND-UGON, M., BLANCO, J., PARERA, M., GOMEZ, J., CLOTET, B. & ESTE, J. A. 2002. Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. *AIDS*, 16, 2385-90.
- MARTINO, A. T., SUZUKI, M., MARKUSIC, D. M., ZOLOTUKHIN, I., RYALS, R. C., MOGHIMI, B., ERTL, H. C., MURUVE, D. A., LEE, B. & HERZOG, R. W. 2011. The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the

- liver. *Blood, The Journal of the American Society of Hematology*, 117, 6459-6468.
- MATSUZAKI, Y., KONNO, A., MOCHIZUKI, R., SHINOHARA, Y., NITTA, K., OKADA, Y. & HIRAI, H. 2018. Intravenous administration of the adeno-associated virus-PHP. B capsid fails to upregulate transduction efficiency in the marmoset brain. *Neuroscience letters*, 665, 182-188.
- MAYNARD, J. E., BERQUIST, K. R., KRUSHAK, D. H. & PURCELL, R. H. 1972. Experimental infection of chimpanzees with the virus of hepatitis B. *Nature*, 237, 514-515.
- MCAULIFFE, V. J., PURCELL, R. H. & GERIN, J. L. 1980. Type B hepatitis: a review of current prospects for a safe and effective vaccine. *Reviews of infectious diseases*, 2, 470-492.
- MCCAFFREY, A. P., NAKAI, H., PANDEY, K., HUANG, Z., SALAZAR, F. H., XU, H., WIELAND, S. F., MARION, P. L. & KAY, M. A. 2003. Inhibition of hepatitis B virus in mice by RNA interference. *Nature biotechnology*, 21, 639-644.
- MCCARTY, D., MONAHAN, P. E. & SAMULSKI, R. J. 2001a. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene therapy*, 8, 1248-1254.
- MCCARTY, D. M., FU, H., MONAHAN, P. E., TOULSON, C. E., NAIK, P. & SAMULSKI, R. J. 2003. Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. *Gene Ther*, 10, 2112-8.
- MCCARTY, D. M., MONAHAN, P. E. & SAMULSKI, R. J. 2001b. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther*, 8, 1248-54.

- MCKENZIE, T. J., LILLEGARD, J. B. & NYBERG, S. L. Artificial and bioartificial liver support. *Seminars in liver disease*, 2008. © Thieme Medical Publishers, 210-217.
- MENG, X., WISEMAN, B., ELVINGER, F., GUENETTE, D., TOTH, T., ENGLE, R., EMERSON, S. & PURCELL, R. 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. *Journal of clinical microbiology*, 40, 117-122.
- MERCER, D. F., SCHILLER, D. E., ELLIOTT, J. F., DOUGLAS, D. N., HAO, C., RINFRET, A., ADDISON, W. R., FISCHER, K. P., CHURCHILL, T. A. & LAKEY, J. R. 2001. Hepatitis C virus replication in mice with chimeric human livers. *Nature medicine*, 7, 927-933.
- MEULEMAN, P., LIBBRECHT, L., DE VOS, R., DE HEMPTINNE, B., GEVAERT, K., VANDEKERCKHOVE, J., ROSKAMS, T. & LEROUX-ROELS, G. 2005. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology*, 41, 847-856.
- MICHLER, T., GROßE, S., MOCKENHAUPT, S., RÖDER, N., STÜCKLER, F., KNAPP, B., KO, C., HEIKENWALDER, M., PROTZER, U. & GRIMM, D. 2016. Blocking sense-strand activity improves potency, safety and specificity of anti-hepatitis B virus short hairpin RNA. *EMBO molecular medicine*, 8, 1082-1098.
- MIYAKAWA, K., MATSUNAGA, S., YAMAOKA, Y., DAIRAKU, M., FUKANO, K., KIMURA, H., CHIMURO, T., NISHITSUJI, H., WATASHI, K. & SHIMOTOHNO, K. 2018. Development of a cell-based assay to identify

- hepatitis B virus entry inhibitors targeting the sodium taurocholate cotransporting polypeptide. *Oncotarget*, 9, 23681.
- MOL, M. P., GONÇALVES, J. P., SILVA, E. A., SCARPONI, C. F., GRECO, D. B., CAIRNCROSS, S. & HELLER, L. 2016. Seroprevalence of hepatitis B and C among domestic and healthcare waste handlers in Belo Horizonte, Brazil. *Waste Management & Research*, 34, 875-883.
- MOOLLA, N., KEW, M. & ARBUTHNOT, P. 2002. Regulatory elements of hepatitis B virus transcription. *Journal of viral hepatitis*, 9, 323-331.
- MOWA, M. B., CROWTHER, C., ELY, A. & ARBUTHNOT, P. 2014. Inhibition of hepatitis B virus replication by helper dependent adenoviral vectors expressing artificial anti-HBV pri-miRs from a liver-specific promoter. *BioMed research international*, 2014.
- MÜLLER, M., BIRD, T. G. & NAULT, J.-C. 2020. The landscape of gene mutations in cirrhosis and hepatocellular carcinoma. *Journal of Hepatology*.
- MURPHY, C. M., XU, Y., LI, F., NIO, K., RESZKA-BLANCO, N., LI, X., WU, Y., YU, Y., XIONG, Y. & SU, L. 2016. Hepatitis B Virus X Protein Promotes Degradation of SMC5/6 to Enhance HBV Replication. *Cell Rep*, 16, 2846-2854.
- NASSAL, M. 2015. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut*, 64, 1972-1984.
- NASSAL, M., JUNKER-NIEPMANN, M. & SCHALLER, H. 1990. Translational inactivation of RNA function: discrimination against a subset of genomic transcripts during HBV nucleocapsid assembly. *Cell*, 63, 1357-1363.
- NELSON, C. E., HAKIM, C. H., OUSTEROUT, D. G., THAKORE, P. I., MOREB, E. A., RIVERA, R. M. C., MADHAVAN, S., PAN, X., RAN, F. A. & YAN, W. X.

2016. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*, 351, 403-407.
- NGO, H., TSCHUDI, C., GULL, K. & ULLU, E. 1998. Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences*, 95, 14687-14692.
- NI, Y. & LEMPP, F. 2014. a., Mehrle S, Nkongolo S, Kaufman C, Fälth M, et al. Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. *Gastroenterology*, 146, 1070-1083.
- NI, Y. & URBAN, S. 2017a. Hepatitis B virus infection of HepaRG cells, HepaRG-hNTCP cells, and primary human hepatocytes. *Hepatitis B Virus*. Springer.
- NI, Y. & URBAN, S. 2017b. Stem cell-derived hepatocytes: A promising novel tool to study hepatitis B virus infection. *Journal of hepatology*, 66, 473-475.
- NIE, Y.-Z., ZHENG, Y.-W., MIYAKAWA, K., MURATA, S., ZHANG, R.-R., SEKINE, K., UENO, Y., TAKEBE, T., WAKITA, T. & RYO, A. 2018. Recapitulation of hepatitis B virus–host interactions in liver organoids from human induced pluripotent stem cells. *EBioMedicine*, 35, 114-123.
- NIU, C., LIVINGSTON, C. M., LI, L., BERAN, R. K., DAFFIS, S., RAMAKRISHNAN, D., BURDETTE, D., PEISER, L., SALAS, E. & RAMOS, H. 2017. The Smc5/6 complex restricts HBV when localized to ND10 without inducing an innate immune response and is counteracted by the HBV X protein shortly after infection. *PLoS One*, 12, e0169648.
- NONY, P., CHADEUF, G., TESSIER, J., MOULLIER, P. & SALVETTI, A. 2003. Evidence for packaging of rep-cap sequences into adeno-associated virus

- (AAV) type 2 capsids in the absence of inverted terminal repeats: a model for generation of rep-positive AAV particles. *Journal of virology*, 77, 776-781.
- NORDER, H., COUROUCÉ, A.-M., COURSAGET, P., ECHEVARRIA, J. M., LEE, S.-D., MUSHAHWAR, I. K., ROBERTSON, B. H., LOCARNINI, S. & MAGNIUS, L. O. 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology*, 47, 289-309.
- NORDER, H., COUROUCÉ, A.-M. & MAGNIUS, L. O. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology*, 198, 489-503.
- NOVINA, C. D., MURRAY, M. F., DYKXHOORN, D. M., BERESFORD, P. J., RIESS, J., LEE, S.-K., COLLMAN, R. G., LIEBERMAN, J., SHANKAR, P. & SHARP, P. A. 2002. siRNA-directed inhibition of HIV-1 infection. *Nature medicine*, 8, 681-686.
- OCHIYA, T., TSURIMOTO, T., UEDA, K., OKUBO, K., SHIOZAWA, M. & MATSUBARA, K. 1989. An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes. *Proceedings of the National Academy of Sciences*, 86, 1875-1879.
- OKAMOTO, H., TSUDA, F., SAKUGAWA, H., SASTROSOEWIGNJO, R. I., IMAI, M., MIYAKAWA, Y. & MAYUMI, M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *Journal of general Virology*, 69, 2575-2583.
- ORTEGA-PRIETO, A., SKELTON, J., WAI, S., LARGE, E., LUSSIGNOL, M., VIZCAY-BARRENA, G., HUGHES, D., FLECK, R., THURSZ, M. &

- CATANESE, M. 2018. 3D microfluidic liver cultures as a physiological preclinical tool for hepatitis B virus infection. *Nature communications*, 9, 1-15.
- OSIOWY, C., GILES, E., TANAKA, Y., MIZOKAMI, M. & MINUK, G. Y. 2006. Molecular evolution of hepatitis B virus over 25 years. *Journal of virology*, 80, 10307-10314.
- ÖSTERLUND, P., VECKMAN, V., SIRÉN, J., KLUCHER, K. M., HISCOTT, J., MATIKAINEN, S. & JULKUNEN, I. 2005. Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. *Journal of virology*, 79, 9608-9617.
- PAGANELLI, M., DALLMEIER, K., NYABI, O., SCHEERS, I., KABAMBA, B., NEYTS, J., GOUBAU, P., NAJIMI, M. & SOKAL, E. M. 2013. Differentiated umbilical cord matrix stem cells as a new in vitro model to study early events during hepatitis B virus infection. *Hepatology*, 57, 59-69.
- PANCHOLI, P., LEE, D. H., LIU, Q., TACKNEY, C., TAYLOR, P., PERKUS, M., ANDRUS, L., BROTMAN, B. & PRINCE, A. M. 2001. DNA prime/canarypox boost—based immunotherapy of chronic hepatitis B virus infection in a chimpanzee. *Hepatology*, 33, 448-454.
- PRANGE, R. & STREECK, R. E. 1995. Novel transmembrane topology of the hepatitis B virus envelope proteins. *The EMBO journal*, 14, 247-256.
- QI, Y., GAO, Z., XU, G., PENG, B., LIU, C., YAN, H., YAO, Q., SUN, G., LIU, Y. & TANG, D. 2016. DNA polymerase κ is a key cellular factor for the formation of covalently closed circular DNA of hepatitis B virus. *PLoS pathogens*, 12.
- QIAO, L., SUI, J. & LUO, G. 2018. Robust human and murine hepatocyte culture models of hepatitis B virus infection and replication. *Journal of virology*, 92, e01255-18.

- QING, K., MAH, C., HANSEN, J., ZHOU, S., DWARKI, V. & SRIVASTAVA, A. 1999. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nature medicine*, 5, 71.
- QUARLERI, J. 2014. Core promoter: a critical region where the hepatitis B virus makes decisions. *World Journal of Gastroenterology: WJG*, 20, 425.
- REN, X., XU, Z., LIU, Y., LI, X., BAI, S., DING, N., ZHONG, Y., WANG, L., MAO, P. & ZOULIM, F. 2010. Hepatitis B virus genotype and basal core promoter/precore mutations are associated with hepatitis B-related acute-on-chronic liver failure without pre-existing liver cirrhosis. *Journal of viral hepatitis*, 17, 887-895.
- RIAZ, M., IDREES, M., KANWAL, H. & KABIR, F. 2011. An overview of triple infection with hepatitis B, C and D viruses. *Virology journal*, 8, 1-5.
- RONZITTI, G., GROSS, D.-A. & MINGOZZI, F. 2020. Human Immune Responses to Adeno-Associated Virus (AAV) Vectors. *Frontiers in Immunology*, 11.
- RUMIN, S., GRIPON, P., SEYEC, J. L., CORRAL-DEBRINSKI, M. & GUGUEN-GUILLOUZO, C. 1996. Long-term productive episomal hepatitis B virus replication in primary cultures of adult human hepatocytes infected in vitro. *Journal of viral hepatitis*, 3, 227-238.
- RYAN, J., ABDUL-BASIT, C. S. S. & HERRERA, K. 2015. Considerations in First-Line Therapies for Treatment-Naive Chronic Hepatitis B. *Medical Research Archives*.
- SA-NGIAMSUNTORN, K., THONGSRI, P., PEWKLIANG, Y., WONGKAJORN SILP, A., KONGSOMBOONCHOKE, P., SUTHIVANICH, P., BORWORN PINYO, S. & HONGENG, S. 2019. An Immortalized Hepatocyte-Like Cell Line (imHC)

- Accommodated Complete Viral Lifecycle, Viral Persistence Form, cccDNA and Eventual Spreading of a Clinically-Isolated HBV. *Viruses*, 11, 952.
- SAEIDI, A., ZANDI, K., CHEOK, Y. Y., SAEIDI, H., WONG, W. F., LEE, C. Y. Q., CHEONG, H. C., YONG, Y. K., LARSSON, M. & SHANKAR, E. M. 2018. T-cell exhaustion in chronic infections: reversing the state of exhaustion and reinvigorating optimal protective immune responses. *Frontiers in immunology*, 9, 2569.
- SAKURAI, F., MITANI, S., YAMAMOTO, T., TAKAYAMA, K., TACHIBANA, M., WATASHI, K., WAKITA, T., IJIMA, S., TANAKA, Y. & MIZUGUCHI, H. 2017. Human induced-pluripotent stem cell-derived hepatocyte-like cells as an in vitro model of human hepatitis B virus infection. *Scientific reports*, 7, 45698.
- SAMULSKI, R. J. & MUZYCZKA, N. 2014. AAV-mediated gene therapy for research and therapeutic purposes. *Annual review of virology*, 1, 427-451.
- SANJUÁN, R. & DOMINGO-CALAP, P. 2016. Mechanisms of viral mutation. *Cellular and molecular life sciences*, 73, 4433-4448.
- SCHAEFER, S. 2007. Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World journal of gastroenterology: WJG*, 13, 14.
- SCHNEPP, B. C., JENSEN, R. L., CHEN, C. L., JOHNSON, P. R. & CLARK, K. R. 2005. Characterization of adeno-associated virus genomes isolated from human tissues. *J Virol*, 79, 14793-803.
- SCHÖNEWEIS, K., MOTTER, N., ROPPERT, P. L., LU, M., WANG, B., ROEHL, I., GLEBE, D., YANG, D., MORREY, J. D. & ROGGENDORF, M. 2018. Activity of nucleic acid polymers in rodent models of HBV infection. *Antiviral research*, 149, 26-33.

- SCOTT, T., MOYO, B., NICHOLSON, S., MAEPA, M. B., WATASHI, K., ELY, A., WEINBERG, M. S. & ARBUTHNOT, P. 2017. ssAAVs containing cassettes encoding SaCas9 and guides targeting hepatitis B virus inactivate replication of the virus in cultured cells. *Scientific reports*, 7, 1-11.
- SEEGER, C. & MASON, W. S. 2000. Hepatitis B virus biology. *Microbiol. Mol. Biol. Rev.*, 64, 51-68.
- SELLS, M. A., CHEN, M. L. & ACS, G. 1987a. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proceedings of the National Academy of Sciences*, 84, 1005-1009.
- SELLS, M. A., CHEN, M. L. & ACS, G. 1987b. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci U S A*, 84, 1005-9.
- SELLS, M. A., ZELENT, A. Z., SHVARTSMAN, M. & ACS, G. 1988. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. *J Virol*, 62, 2836-44.
- SHAYAKHMETOV, D. M., LI, Z.-Y., NI, S. & LIEBER, A. 2004. Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors. *Journal of virology*, 78, 5368-5381.
- SHEN, F., LI, Y., WANG, Y., SOZZI, V., REVILL, P. A., LIU, J., GAO, L., YANG, G., LU, M. & SUTTER, K. 2018. Hepatitis B virus sensitivity to interferon- α in hepatocytes is more associated with cellular interferon response than with viral genotype. *Hepatology*, 67, 1237-1252.
- SHIH, Y.-M., SUN, C.-P., CHOU, H.-H., WU, T.-H., CHEN, C.-C., WU, P.-Y., CHEN, Y.-C. E., BISSIG, K.-D. & TAO, M.-H. 2015. Combinatorial RNA interference

- therapy prevents selection of pre-existing HBV variants in human liver chimeric mice. *Scientific reports*, 5, 15259.
- SIOUD, M. 2005. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *Journal of molecular biology*, 348, 1079-1090.
- SLAGLE, B. L., LEE, T. H., MEDINA, D., FINEGOLD, M. J. & BUTEL, J. S. 1996. Increased sensitivity to the hepatocarcinogen diethylnitrosamine in transgenic mice carrying the hepatitis B virus X gene. *Molecular Carcinogenesis: Published in cooperation with the University of Texas MD Anderson Cancer Center*, 15, 261-269.
- SOLOMON, S. D., ADAMS, D., KRISTEN, A., GROGAN, M., GONZÁLEZ-DUARTE, A., MAURER, M. S., MERLINI, G., DAMY, T., SLAMA, M. S. & BRANNAGAN III, T. H. 2019. Effects of patisiran, an RNA interference therapeutic, on cardiac parameters in patients with hereditary transthyretin-mediated amyloidosis: analysis of the APOLLO Study. *Circulation*, 139, 431-443.
- SONG, E., ZHU, P., LEE, S.-K., CHOWDHURY, D., KUSSMAN, S., DYKXHOORN, D. M., FENG, Y., PALLISER, D., WEINER, D. B. & SHANKAR, P. 2005. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nature biotechnology*, 23, 709-717.
- SPEARMAN, C. & SONDERUP, M. W. 2014. Preventing hepatitis B and hepatocellular carcinoma in South Africa: The case for a birth-dose vaccine. *SAMJ: South African Medical Journal*, 104, 610-612.

- SPRENGEL, R., KUHN, C., WILL, H. & SCHALLER, H. 1985. Comparative sequence analysis of duck and human hepatitis B virus genomes. *Journal of medical virology*, 15, 323-333.
- SPRINGER, A. D. & DOWDY, S. F. 2018. GalNAc-siRNA conjugates: leading the way for delivery of RNAi therapeutics. *nucleic acid therapeutics*, 28, 109-118.
- SPRINZL, M. F., OBERWINKLER, H., SCHALLER, H. & PROTZER, U. 2001. Transfer of hepatitis B virus genome by adenovirus vectors into cultured cells and mice: crossing the species barrier. *Journal of virology*, 75, 5108-5118.
- SRIVASTAVA, A., LUSBY, E. & BERNIS, K. I. 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *Journal of virology*, 45, 555-564.
- STEVEN, A. C., CONWAY, J. F., CHENG, N., WATTS, N. R., BELNAP, D. M., HARRIS, A., STAHL, S. J. & WINGFIELD, P. T. 2005. Structure, assembly, and antigenicity of hepatitis B virus capsid proteins. *Advances in virus research*, 64, 125-164.
- SUHAIL, M., ABDEL-HAFIZ, H., ALI, A., FATIMA, K., DAMANHOURI, G. A., AZHAR, E., CHAUDHARY, A. G. & QADRI, I. 2014. Potential mechanisms of hepatitis B virus induced liver injury. *World Journal of Gastroenterology: WJG*, 20, 12462.
- SUMMERS, J. & MASON, W. S. 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell*, 29, 403-415.
- SUN, D., RÖSLER, C., KIDD-LJUNGGREN, K. & NASSAL, M. 2010. Quantitative assessment of the antiviral potencies of 21 shRNA vectors targeting conserved, including structured, hepatitis B virus sites. *Journal of hepatology*, 52, 817-826.

- SUN, K.-X., LI, J., ZHU, F.-C., LIU, J.-X., LI, R.-C., ZHAI, X.-J., LI, Y.-P., CHANG, Z.-J., NIE, J.-J. & ZHUANG, H. 2012. A predictive value of quantitative HBsAg for serum HBV DNA level among HBeAg-positive pregnant women. *Vaccine*, 30, 5335-5340.
- SUN, S. & LI, J. 2017. Humanized chimeric mouse models of hepatitis B virus infection. *International Journal of Infectious Diseases*, 59, 131-136.
- SUN, Y., QI, Y., PENG, B. & LI, W. 2017. NTCP-reconstituted in vitro HBV infection system. *Hepatitis B Virus*. Springer.
- SUREAU, C., ROMET-LEMONNE, J.-L., MULLINS, J. I. & ESSEX, M. 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell*, 47, 37-47.
- SUREAU, C. & SALISSE, J. 2013. A conformational heparan sulfate binding site essential to infectivity overlaps with the conserved hepatitis B virus a-determinant. *Hepatology*, 57, 985-94.
- TAMADA, Y., YATSUHASHI, H., MASAKI, N., NAKAMUTA, M., MITA, E., KOMATSU, T., WATANABE, Y., MURO, T., SHIMADA, M. & HIJIOKA, T. 2012. Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B. *Gut*, 61, 765-773.
- TAO, Y., WU, D., ZHOU, L., CHEN, E., LIU, C., TANG, X., JIANG, W., HAN, N., LI, H. & TANG, H. 2020. Present and future therapies for chronic hepatitis B. *Hepatitis B Virus Infection*. Springer.
- TATEMATSU, K., TANAKA, Y., KURBANOV, F., SUGAUCHI, F., MANO, S., MAESHIRO, T., NAKAYOSHI, T., WAKUTA, M., MIYAKAWA, Y. & MIZOKAMI, M. 2009. A genetic variant of hepatitis B virus divergent from

- known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *Journal of virology*, 83, 10538-10547.
- THUENER, J. 2017. Hepatitis A and B infections. *Primary Care: Clinics in Office Practice*, 44, 621-629.
- TSAI, J. F., CHANG, W. Y., JENG, J. E., HO, M. S., LIN, Z. Y. & TSAI, J. H. 1994. Hepatitis B and C virus infection as risk factors for liver cirrhosis and cirrhotic hepatocellular carcinoma: A case-control study. *Liver*, 14, 98-102.
- TSUKUDA, S. & WATASHI, K. 2020. Hepatitis B virus biology and life cycle. *Antiviral Research*, 104925.
- TUTTLEMAN, J. S., POURCEL, C. & SUMMERS, J. 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell*, 47, 451-460.
- URBAN, S., BREINER, K. M., FEHLER, F., KLINGMÜLLER, U. & SCHALLER, H. 1998. Avian hepatitis B virus infection is initiated by the interaction of a distinct pre-S subdomain with the cellular receptor gp180. *Journal of virology*, 72, 8089-8097.
- VAN DEN BERG, F. T., ELY, A. & ARBUTHNOT, P. 2020. Generating DNA Expression Cassettes Encoding Multimeric Artificial MicroRNA Precursors. *RNA Interference and CRISPR Technologies*. Springer.
- VERRIER, E. R., COLPITTS, C. C., SCHUSTER, C., ZEISEL, M. B. & BAUMERT, T. F. 2016. Cell culture models for the investigation of hepatitis B and D virus infection. *Viruses*, 8, 261.
- VOGEL, A., VAN DEN BERG, I. E., AL-DHALIMY, M., GROOPMAN, J., OU, C. N., RYABININA, O., IORDANOV, M. S., FINEGOLD, M. & GROMPE, M. 2004.

- Chronic liver disease in murine hereditary tyrosinemia type 1 induces resistance to cell death. *Hepatology*, 39, 433-443.
- VON FREYEND, M., UNTERGASSER, A., ARZBERGER, S., OBERWINKLER, H., DREBBER, U., SCHIRMACHER, P. & PROTZER, U. 2011. Sequential control of hepatitis B virus in a mouse model of acute, self-resolving hepatitis BJ Viral Hepat.
- WANG, C.-S., CHANG, T.-T., YAO, W.-J., WANG, S.-T. & CHOU, P. 2012. Impact of increasing alanine aminotransferase levels within normal range on incident diabetes. *Journal of the Formosan Medical Association*, 111, 201-208.
- WANG, G.-H. & SEEGER, C. 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell*, 71, 663-670.
- WEITZMAN, M. D. & LINDEN, R. M. 2011. Adeno-associated virus biology. *Adeno-Associated Virus: Methods and Protocols*, 1-23.
- WHERRY, E. J. & KURACHI, M. 2015. Molecular and cellular insights into T cell exhaustion. *Nature Reviews Immunology*, 15, 486-499.
- WIELAND, S., THIMME, R., PURCELL, R. H. & CHISARI, F. V. 2004. Genomic analysis of the host response to hepatitis B virus infection. *Proceedings of the National Academy of Sciences*, 101, 6669-6674.
- WIELAND, S. F. 2015a. The chimpanzee model for hepatitis B virus infection. *Cold Spring Harb Perspect Med*, 5.
- WIELAND, S. F. 2015b. The chimpanzee model for hepatitis B virus infection. *Cold Spring Harbor perspectives in medicine*, 5, a021469.
- WILL, H., REISER, W., WEIMER, T., PFAFF, E., BÜSCHER, M., SPRENGEL, R., CATTANEO, R. & SCHALLER, H. 1987. Replication strategy of human hepatitis B virus. *Journal of Virology*, 61, 904-911.

- WINER, B. Y., HUANG, T., LOW, B. E., AVERY, C., PAIS, M.-A., HREBIKOVA, G., SIU, E., CHIRIBOGA, L., WILES, M. V. & PLOSS, A. 2017. Recapitulation of treatment response patterns in a novel humanized mouse model for chronic hepatitis B virus infection. *Virology*, 502, 63-72.
- WITT-KEHATI, D., BITTON ALALUF, M. & SHLOMAI, A. 2016. Advances and challenges in studying hepatitis B virus in vitro. *Viruses*, 8, 21.
- XIA, Y., CARPENTIER, A., CHENG, X., BLOCK, P. D., ZHAO, Y., ZHANG, Z., PROTZER, U. & LIANG, T. J. 2017. Human stem cell-derived hepatocytes as a model for hepatitis B virus infection, spreading and virus-host interactions. *Journal of hepatology*, 66, 494-503.
- XIAO, X., LI, J. & SAMULSKI, R. J. 1998. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *Journal of virology*, 72, 2224-2232.
- XIAO, Y., LIU, C., TANG, W., ZHANG, H. & CHEN, X. 2019. Evans blue inhibits HBV replication through a dual antiviral mechanism by targeting virus binding and capsid assembly. *Frontiers in Microbiology*, 10, 2638.
- XIAOFEI, E., STADLER, B. M., DEBATIS, M., WANG, S., LU, S. & KOWALIK, T. F. 2012. RNA interference-mediated targeting of human cytomegalovirus immediate-early or early gene products inhibits viral replication with differential effects on cellular functions. *Journal of virology*, 86, 5660-5673.
- YAMADA, G. & TSUJI, T. 1993. 4. immunological mechanism of chronic liver injury in viral hepatitis. *Internal Medicine*, 32, 911-913.
- YAN, F., WANG, Y., ZHANG, W., CHANG, M., HE, Z., XU, J., SHANG, C., CHEN, T., LIU, J. & WANG, X. 2017. Human embryonic stem cell-derived

- hepatoblasts are an optimal lineage stage for hepatitis C virus infection. *Hepatology*, 66, 717-735.
- YANG, D., LIU, L., ZHU, D., PENG, H., SU, L., FU, Y.-X. & ZHANG, L. 2014. A mouse model for HBV immunotolerance and immunotherapy. *Cellular & molecular immunology*, 11, 71.
- YANG, F., YIN, Y., WANG, F., ZHANG, L., WANG, Y. & SUN, S. 2010. An altered pattern of liver apolipoprotein AI isoforms is implicated in male chronic hepatitis B progression. *Journal of proteome research*, 9, 134-143.
- YANG, P. L., ALTHAGE, A., CHUNG, J. & CHISARI, F. V. 2002. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proceedings of the National Academy of Sciences*, 99, 13825-13830.
- YE, L., KAN, F., YAN, T., CAO, J., ZHANG, L., WU, Z. & LI, W. 2017. Enhanced antiviral and antifibrotic effects of short hairpin RNAs targeting HBV and TGF- β in HBV-persistent mice. *Scientific reports*, 7, 1-12.
- YE, L., YU, H., LI, C., HIRSCH, M. L., ZHANG, L., SAMULSKI, R. J., LI, W. & LIU, Z. 2015. Adeno-associated virus vector mediated delivery of the HBV genome induces chronic hepatitis B virus infection and liver fibrosis in mice. *PloS one*, 10, e0130052.
- YI, R., QIN, Y., MACARA, I. G. & CULLEN, B. R. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development*, 17, 3011-3016.
- YLÄ-HERTTUALA, S. 2012. Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. *Molecular Therapy*, 20, 1831-1832.

- YU, H., YUAN, Q., GE, S.-X., WANG, H.-Y., ZHANG, Y.-L., CHEN, Q.-R., ZHANG, J., CHEN, P.-J. & XIA, N.-S. 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". *PloS one*, 5, e9297.
- YUEN, M.-F., WONG, D. K.-H., SUM, S. S.-M., YUAN, H.-J., YUEN, J. C.-H., CHAN, A. O.-O., WONG, B. C.-Y. & LAI, C.-L. 2005. Effect of lamivudine therapy on the serum covalently closed-circular (ccc) DNA of chronic hepatitis B infection. *American Journal of Gastroenterology*, 100, 1099-1103.
- YUEN, M. F., SCHIEFKE, I., YOON, J. H., AHN, S. H., HEO, J., KIM, J. H., LIK YUEN CHAN, H., YOON, K. T., KLINKER, H. & MANNS, M. 2019. RNA Interference Therapy With ARC-520 Results in Prolonged Hepatitis B Surface Antigen Response in Patients With Chronic Hepatitis B Infection. *Hepatology*.
- YUH, C., CHANG, Y. & TING, L. 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. *Journal of Virology*, 66, 4073-4084.
- ZHANG, Y.-L., CHENG, T., CAI, Y.-J., YUAN, Q., LIU, C., ZHANG, T., XIA, D.-Z., LI, R.-Y., YANG, L.-W. & WANG, Y.-B. 2010. RNA Interference inhibits hepatitis B virus of different genotypes in vitro and in vivo. *BMC microbiology*, 10, 214.
- ZHAO, Y.-Q., LI, G.-H., E35, J.-F. H., QIN, D.-D. & RIZAK, J. D. 2013. Special Issue for Primates and Animal Models of Human Diseases. *Zoological Research*, 34.
- ZHONG, G., YAN, H., WANG, H., HE, W., JING, Z., QI, Y., FU, L., GAO, Z., HUANG, Y. & XU, G. 2013. Sodium taurocholate cotransporting polypeptide mediates woolly monkey hepatitis B virus infection of Tupaia hepatocytes. *Journal of virology*, JVI. 03533-12.
- ZHOU, M., QIN, B., DENG, X. S., ZENG, X. L., LU, Y., HUANG, Z. G., WU, C. C. & MOU, L. S. 2019. hNTCP-expressing primary pig hepatocytes are a valuable

- tool for investigating hepatitis B virus infection and antiviral drugs. *Molecular medicine reports*, 20, 3820-3828.
- ZHOU, M., ZHAO, F., LI, J., CHENG, Z., TIAN, X., ZHI, X., HUANG, Y. & HU, K. 2014. Long-term maintenance of human fetal hepatocytes and prolonged susceptibility to HBV infection by co-culture with non-parenchymal cells. *Journal of virological methods*, 195, 185-193.
- ZHOU, M., ZHAO, K., YAO, Y., YUAN, Y., PEI, R., WANG, Y., CHEN, J., HU, X., ZHOU, Y. & CHEN, X. 2017. Productive HBV infection of well-differentiated, hNTCP-expressing human hepatoma-derived (Huh7) cells. *Virologica Sinica*, 32, 465-475.
- ZHOU, T., GUO, H., GUO, J.-T., CUCONATI, A., MEHTA, A. & BLOCK, T. M. 2006. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. *Antiviral research*, 72, 116-124.
- ZOULIM, F. 2011. Hepatitis B virus resistance to antiviral drugs: where are we going? *Liver International*, 31, 111-116.
- ZOULIM, F. & LOCARNINI, S. 2009. Hepatitis B virus resistance to nucleos (t) ide analogues. *Gastroenterology*, 137, 1593-1608. e2.