TARGETING HBV CCCDNA WITH VIRAL VECTOR CARRYING HBV-SPECIFIC CRISPR/CAS9

by

Bayan Alrashed

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Fall Semester 2016
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Fairfax, VA

DEDICATION

This is dedicated to my lovely husband Ahmed and my wonderful children: Rana, Abdulaziz and Hamad. Their patience, support and encouragement helped me to exceed all obstacles and to pursue success.

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to the many supporters, friends and professors who have helped to make this happen. My principal investigator, Dr. Yuntao Wu, whose encouragement, guidance, assistance and patience enabled me to learn all the research skills and not only grow as an experimentalist and Biologist but also as independent thinker. Also, I would like to thank my committee members, Dr. Jia Guo and Dr. Ramin M Hakami, who provided me with encouragement and support along the way. Finally, I offer my regards and blessings to all my lab members and professors at George Mason University, who supported me on my journey through the completion of my master's degree. Thank you all, this thesis would not have been possible without you.

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LIST OF ABBREVIATIONS AND SYMBOLS

Hepatitis B Virus ..... HBV
Hepatitis B Surface Antigen ..... HBsAg
Hepatitis B Core Antigen ..... HBcAg
Hepatitis B Polymerase ..... Pol
Hepatitis B Virus Protein X ..... HBxAg
Deoxyribose Nucleic Acid ..... DNA
Single stranded Deoxyribose Nucleic Acid ..... ssDNA
Double stranded Deoxyribose Nucleic Acid ..... dsDNA
Relaxed Circular DNA ..... rcDNA
Covalently Closed Circular DNA ..... cccDNA
Ribonucleic Acid ..... RNA
Messenger RNA ..... mRNA
Pre-genomic RNA ..... pgRNA
Na^+ Taurocholate Cotransporting Polypeptide ..... NTCP
Dulbecco's Modified Eagle Medium ..... DMEM
Poly-human serum albumin ..... pHSA
Interlekin-6 ..... IL-6
Endoplasmic Reticulum ..... ER
Alanine transaminase ..... ALT
Hepatocellular carcinoma ..... HCC
Adeno-Associated virus ..... AAV
Inverted terminal repeats ..... ITRs
Assembly-Activating Protein ..... AAP
Recombinant Adeno-Associated virus ..... rAAV
Adenovirus ..... Ad
Herpes simplex virus ..... HSV
Rep Binding Element ..... RBE
Clustered regularly interspaced short palindromic repeats ..... CRISPR
CRISPR-associated protein Cas9
Guide RNA ..... gRNA
Protospacer Adjacent Motif sequence ..... PAM
Double Strand Break ..... DSB
Non-Homologous End Joining ..... NHEJ
Homology Directed Repair ..... HDR
Human Embryonic Kidney ..... HEK
Green Fluorescent Protein ..... GFP

Cytomegalovirus ..... CMV
Multible Cloning Site ..... MCS
Open Reading Frame ..... ORF
Human Immunodeficiency Virus ..... HIV
Long terminal repeat ..... LTR
6-Carboxy-Fluorescein 6-FAM
Microliter ..... µL
Micromolar ..... µM
Milliliter ..... mL
Polymearse Chain Reaction ..... PCR
Quantiative Polymearse Chain Reaction ..... qPCR
Real Time Quantiative Polymearse Chain Reaction ..... RT-qPCR
Molar ..... M
Multiplicity of infection ..... MOI
Propidium Iodide ..... PI
Human Liver Cancer Cell HepG2
Enzyme-Linked Immunosorbent Assay ..... ELISA
Phosphate-buffered saline ..... PBS
Glyceraldehyde 3-phosphate dehydrogenase ..... GAPDH
Base pair ..... bp
Kilo base ..... kb

ABSTRACT

TARGETING HBV CCCDNA WITH VIRAL VECTOR CARRYING HBV-SPECIFIC CRISPR/CAS9

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George Mason University, 2016

Thesis Director: Dr. Yuntao Wu

Chronic HBV infection is a worldwide public health concern. It is characterized by the persistence of the hepatitis B surface antigen (HBsAg) in serum for more than six months. The persistence of HBsAg is mediated by viral covalently closed circular DNA (cccDNA) in the nuclei of infected cells. Although several genome-editing techniques have demonstrated the possibility to inhibit HBV viral replication, attempts to completely clear nuclear cccDNA remain unsuccessful. Recent studies have suggested the possibility to use the Clustered Regularly Interspaced Short Palindromic Repeats system (CRISPR/Cas9) to suppress HBV infection; however, effective delivery of this tool for in vivo targeting is a major issue. To examine the possibility to utilize the CRISPR/Cas9 system for HBV clearance, we plan to generate a mouse model of chronic HBV infection by using a recombinant Adeno-associated virus that expresses HBV (rAAV-HBV). It has been previously suggested that a rAAV-HBV virus can establish persistent HBV

infection in mice. We would like to use this model to test whether viral vectors that carry HBV-specific CRISPR/Cas9 can target HBV cccDNA in vivo. To this end, the recombinant AAV-HBV virus was produced by an AAV helper free expression system in HEK293T cells. As a control, I also produced a rAAV-GFP virus from co-transfection of HEK293T cells. My results show that the recombinant AAV-GFP virus can effectively infect human liver HepG2 cells and express high levels of green fluorescent protein. I also examined the rAAV-HBV virus for expressing HBV genes in HepG2 cells by western blot and quantitative real-time PCR (qPCR). While western blots showed defined bands of HBsAg in HepG2 cells, qPCR of infected HepG2 culture supernatants and cell lysates yielded a low amount of HBV genomes. We therefore demonstrated the possibility to use rAAV-HBV vector to delivery HBV genome into liver cells. However, the efficiency is relatively low, likely resulting from the large size of the HBV genome that may affect the AAV packaging efficiency. Multiple approaches to improve the efficiency of the rAAV-HBV vector are currently being tested.

INTRODUCTION

Hepatitis B Virus

Hepatitis B virus belongs to Hepadnaviridae family. It known as the smallest enveloped DNA virus that infects hepatocytes. The virus classified into nine genotypes A to I based on sequence analysis. The viral particles divided into noninfectious particles that are small, 22 nm in diameter, spherical structures and filaments (Liang, 2009). The infectious HBV virion (Dane particle) was visualized by D. S. Dane (Dane, Cameron, & Briggs, 1970). The size of the Dane particle is about 42 nm in diameter. It has lipid envelope, which contains Hepatitis B surface antigen (HBsAg) that surrounds the hepatitis B core antigen (HBcAg), the polymerase and the viral DNA genome (Liang, 2009). HBV genome is about 3.2 kilo base pairs in length, double-stranded relaxed circular DNA (rcDNA) with a complete minus strand, where the 5' has the reverse transcriptase/polymerase, and incomplete plus strand along with the major mRNAs. There are direct repeats (DR1 and DR2) in the 5' ends of both strands, which play a role in viral replication (Datta, Chatterjee, Veer, & Chakravarty, 2012).

The HBV genome consists of four overlapping open reading frames. The first open reading frame includes pre-S1, pre-S2 and S regions that encode HBsAg. The second open reading frame includes pre-core and core regions that encode HBcAg and HBeAg (hepatitis B e antigen), which play a critical role in infection progress via viral nucleocapsid synthesis. The third open reading frame encodes the polymerase (Pol) that

includes DNA polymerase (DNA Pol) and reverse transcriptase (RT). The Pol protein stimulates HBV genome and minus-strand synthesis, and simplifies viral replication. The fourth open reading frame encodes Hepatitis B x protein, which might play a role in HBV infection (Liang, 2009) (Figure 1).

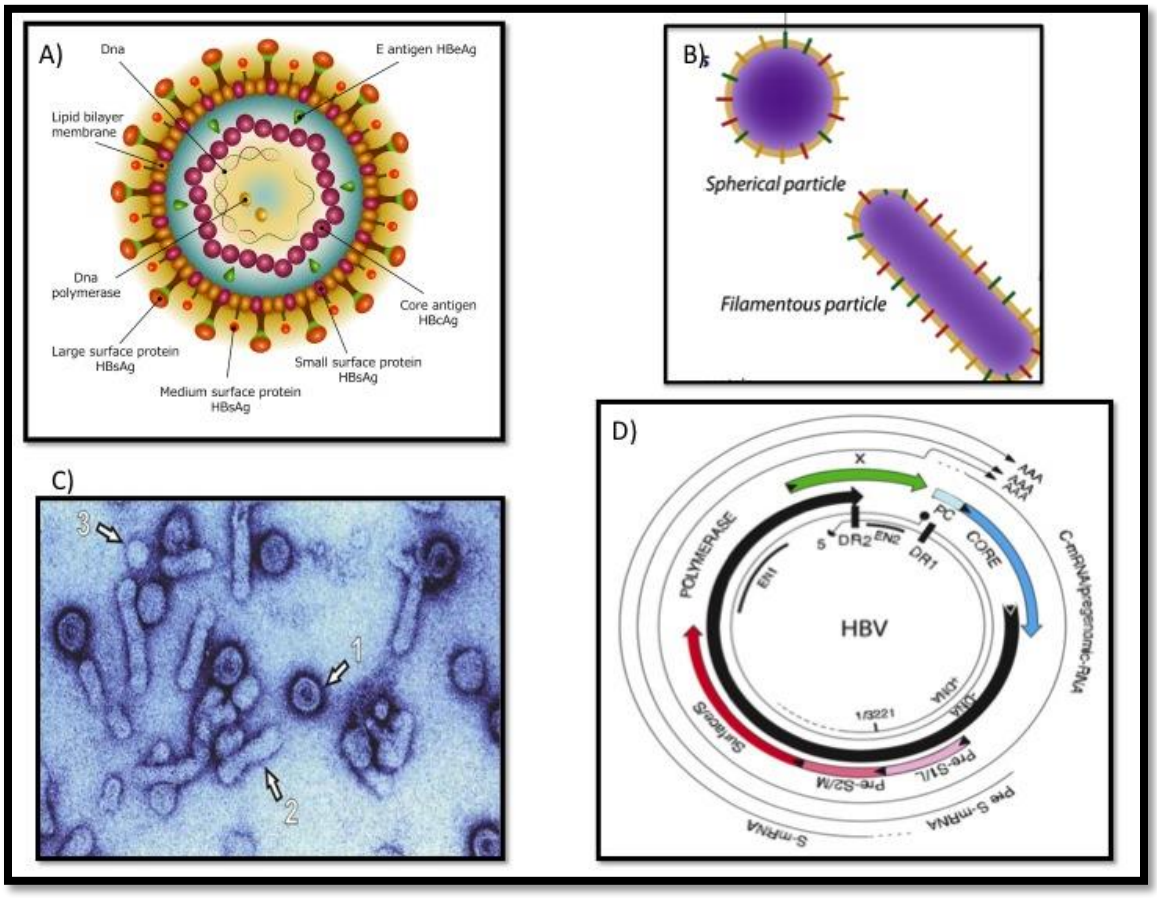

Figure 1: Schematic of Hepatitis B Virus particles and genome.
(A) Dane particle consists of double-stranded DNA genome surrounded by hepatitis B core antigen and covered with lipid bilayer membrane, which contains Hepatitis B surface protein (S, M, L). (B) Non-infectious HBV particles that are spherical in structures or filaments ("Stock Photos, Royalty-Free Images and Vectors - Shutterstock," n.d.). (C) Electron Microscope of Hepatitis B Virus Particles: 1- infectious virions (Dane particle), 2 and 3- non-infectious empty particles ("Universitäts Klinikum Heidelberg: 1. Morphology & Genome Organization," n.d.). (D) Double-stranded relaxed circular HBV genome (rcDNA) with a complete minus strand and incomplete plus strand along with the major mRNAs and the four overlapping open reading frames (Seeger & Mason, 2015a).

The viral life cycle starts from entering hepatocytes, the primary cells for HBV infection, via binding to a specific liver receptor called NTCP. Several studies (in vitro) have been done to prove that elimination of this receptor from hepatocytes leads to unsuccessful infections. Further, there are other receptors that are known as HBV-binding receptors such as poly-human serum albumin (pHSA), carboxypeptidase D, fibronectin and interlekin-6 (IL-6). After host and virus membrane fusion and in the cytosol, the virus nucleo capsid, which has the rcDNA with the viral polymerase, transport to the cell nucleus. In the host nucleus, the rcDNA repaired by the host cellular repair mechanisms to form covalently closed circular DNA (cccDNA) via completion of the plus strand and ligation of both strands. The cccDNA works as a template for viral genome transcription including pre-genomic and sub-genomic RNAs. The sub-genomic RNAs translate into envelope proteins, X protein and pre-core protein (HBeAg). The pre-genomic RNA (pgRNA) translates into core protein and Pol protein, which binds to the 5' of its mRNA in an event which triggers the viral RNA and polymerase into core particles that are then assembled by the core protein (Datta et al., 2012). The packaging of pgRNA depends on host factors such as hsp90 complex. The polypeptides of hsp90 complex help the polymerase to bind to the epsilon on pgRNA. The epsilon also plays a role in the initiation of viral DNA synthesis (Seeger & Mason, 2000). In the viral nucleocapsid, the rcDNA synthesizes via the pgRNA reverse transcript mechanism. The newly made DNA is released into the cell nucleus and converts into cccDNA for viral replication at the early time of infection when the envelope protein concentrations are low. On the other hand after the concentrations of the envelope proteins increase, the viral nucleocapsid

binds to viral envelope proteins that accumulate in the ER membrane for HBV virion assembly (Figure2). Formation of cccDNA, which has the ability to persist within heptocyte nucleus, is the symbol of HBV chronic infection (Datta et al., 2012). As a result of this persistence, more than 250 million people are infected with the Hepatitis B virus worldwide.

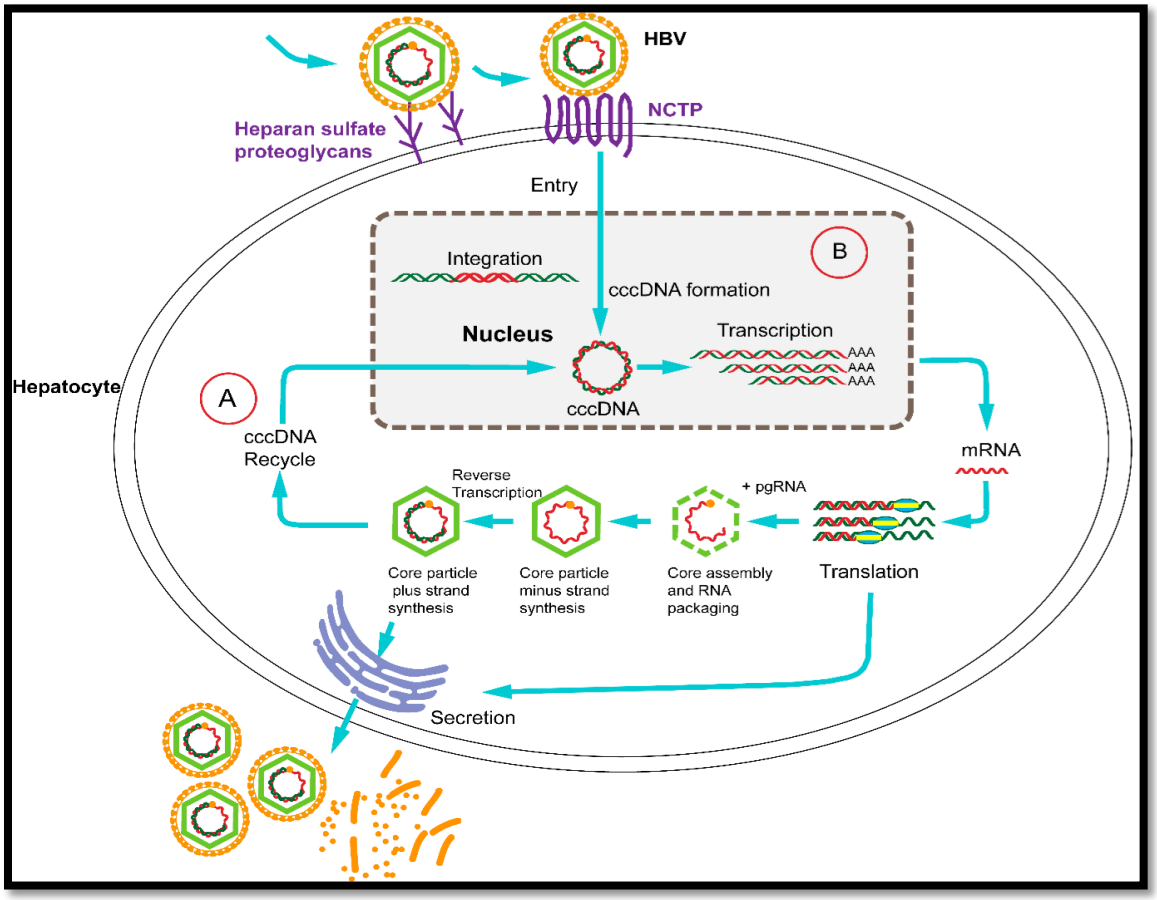

Figure 2: HBV Life Cycle.
During initiation of infection, the rcDNA converts into cccDNA that serves as template for the transcription of viral RNA. The viral mRNAs translate into envelope proteins that locate in the ER, X protein, precore protein and pgRNA. The pgRNA cotes by the viral capsid and transcripts into rcDNA via reverse transcript mechanism. Then the core protein binds to viral envelope proteins in the ER membrane to form HBV virion and leaves the cell via Golgi components. On the other hand, the rcDNA might enter host nucleus for cccDNA cycle (Lin, Zhang, & Li, 2015).

The viral infection could be divided into acute and chronic infection, which might cause cirrhosis or hepatocellular carcinoma (HCC) (Ye et al., 2015). The differences between the two types of infection mostly depend on the appearance of HBV proteins. The first difference is the appearance and persistence of Hepatitis B surface antigen (HBsAg), a protein can be found on the surface of the virus, in the blood or the serum. In acute infection, this protein can be found in serum during the first weeks of infection and then disappears while in chronic infection; HBsAg persists in serum for more than six months. The reason of this persistence is the ability of Hepatitis B virus cccDNA to integrate into host genome and transcript mRNA that specific for HBsAg (Seeger & Mason, 2015b). Also, the high titer of HBeAg, a protein responsible for HBV replication, and HBV genome in serum of infected individual is a characteristic of chronic infection. On the other hand, the production of antibodies against HBV proteins such as anti-HBeAg and anti-HBcAg is a feature of acute infection. Moreover, the high levels of ALT (Alanine transaminase, which is a biomarker of healthy liver) is evidence of liver injury (*Guidelines for the Prevention, Care and Treatment of Persons with Chronic Hepatitis B Infection*, 2015; Liang, 2009). Many studies have been focusing on the elimination of cccDNA in order to inhibit viral replication and gene expression (Dong et al., 2015) (Kennedy, Kornepati, & Cullen, 2015) (Seeger & Sohn, 2014). There are different therapeutic agents against chronic HBV infection such as alpha interferon that suppresses viral replication and decreases viral mRNA via a stimulation of antiviral immune response. Moreover, nucleoside analog therapy is used to stop viral DNA synthesis and

reduce the number of infected cells (Seeger & Mason, 2000). Both therapeutic agents are effective against Hepatitis B infection; however, they can neither eliminate Hepatitis B virus completely (cccDNA) nor be used as life-long treatment. The only known way to eliminate cccDNA is killing all infected hepatocytes, which will lead to liver failure and death. In order to find the best therapeutic agent, the most recent approach is to effectively study the molecular biology of the Hepatitis B virus in order to establish infection by Adeno-Associated Virus Vector carrying the HBV genome.

Adeno-Associated Virus

Adeno-Associated virus (AAV) is a small, non-envelope DNA virus about 22-25nm in diameter. It is a member of Parvoviridae family and Dependoviruse genus. AAV divided into 12 human serotypes and 100 non-human serotypes. Each serotype has specific receptor and co-receptors for attachment and entry, which explains the tropism of this virus (Daya & Berns, 2008). The viral genome is 4.7kb in size, consists of two inverted terminal repeats (ITRs), each about 145-bp, and encodes two genes. The ITRs form T shaped, hairpin structures, and encode the cis-acting elements, which play critical roles in virus replication, double-strand DNA synthesis and genome packaging. The first viral gene is Rep gene that encodes four regulatory proteins (Rep78, Rep68, Rep52, and Rep 40). Those proteins play a critical role in virus replication, gene expression and virion assembly. The second gene is Cap gene that encodes three capsid proteins (VP1, VP2, and VP3) and the assembly-activating protein (AAP) that plays a role in capsid formation. Additionally, there are three viral promoters: p5, p19 and p40 (Cervelli, Backovic, & Galli, 2011) (Arbuthnot, 2015) (Figure 3). The virus does not encode

polymerase for replication and gene expression; however, it depends on the host polymerase.

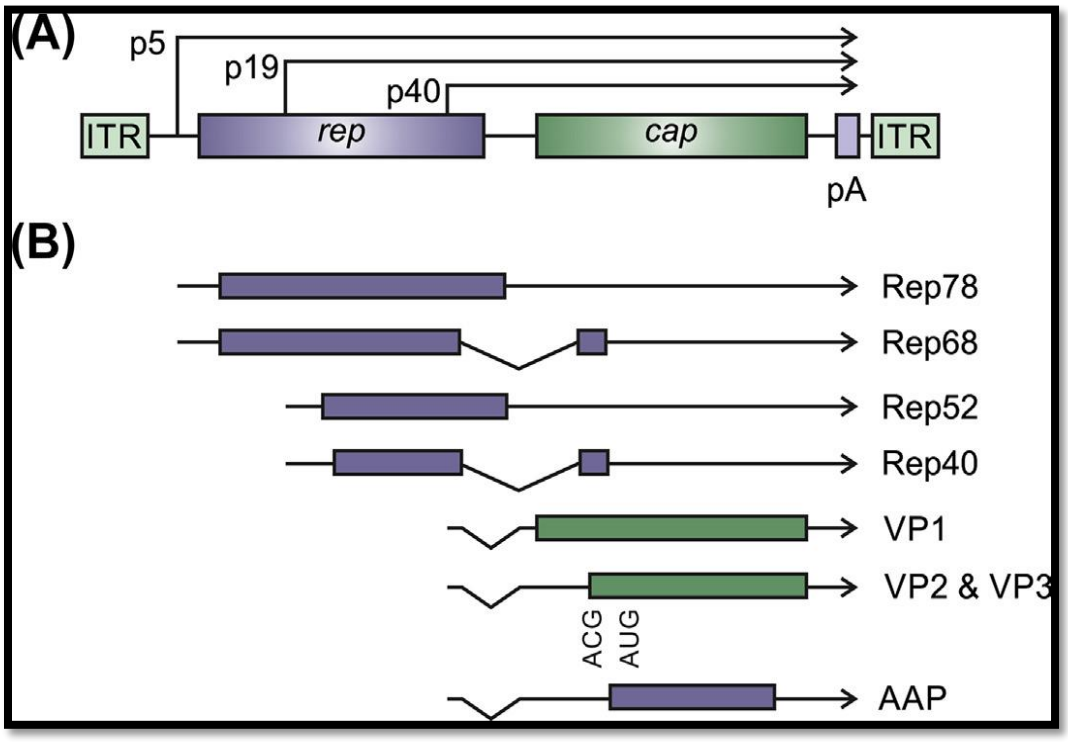

Figure 3: Adeno-Associated Virus Genome.
(A) The viral genome contains two inverted terminal repeats and encodes two genes (Rep and Cap), which transcript under three promoters (p5, p19 and p40). (B) The Rep gene encodes four proteins: Rep78, Rep68, Rep52 and Rep40. The Cap gene encodes four viral proteins: VP1, VP2, VP3 and AAP (Arbuthnot, 2015)

After entering the host cell, part of the virus including ITR and Rep genes will integrate into specific region in host chromosome 19 (latency) and wait for helper virus such as Adenovirus (Ad) or Herpes Simplex virus (HSV), which induces changes on the cellular environment, to start AAV gene expression and replication (this is a rare event). Because of the ability to delete rep and cap genes from the viral genome, the production

of recombinant Adeno-Associated Virus (rAAV), which is successfully being used as a viral vector for gene therapy which includes co-transfection with three plasmids (Figure 4).

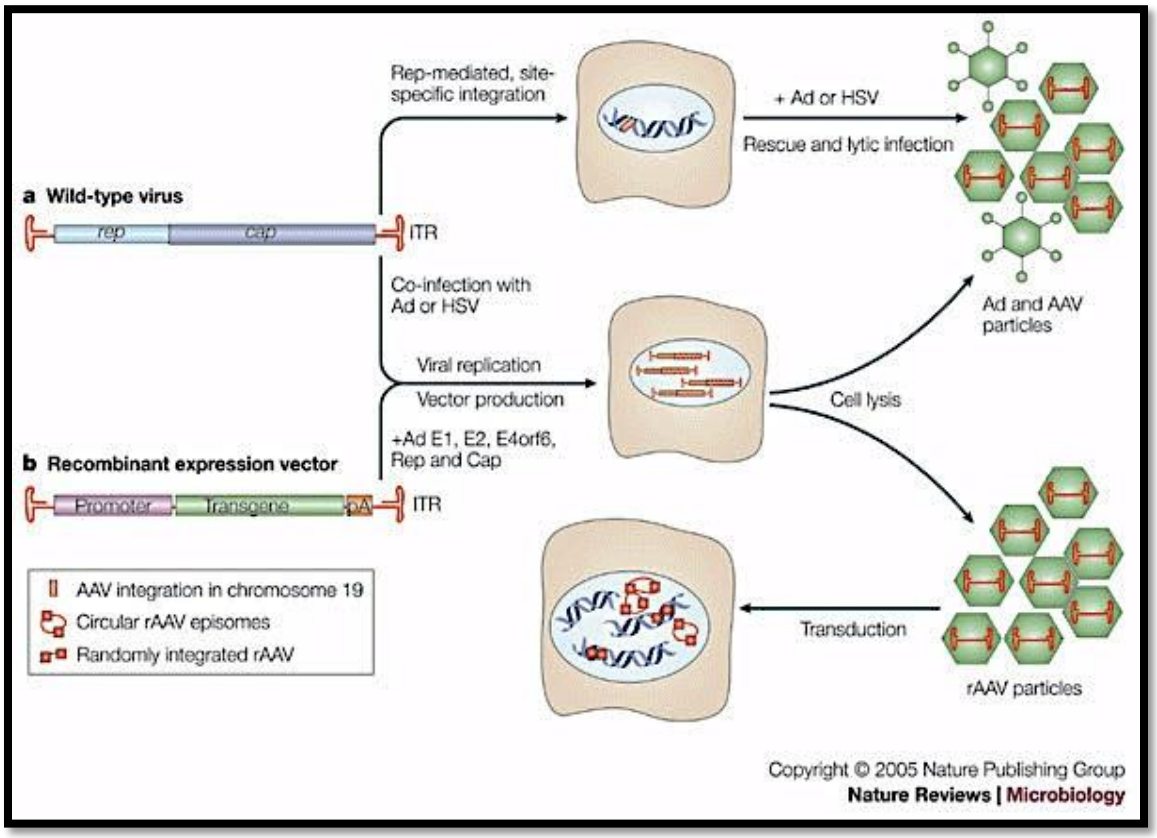

Figure 4: Wild Type and Recombinant Adeno-Associated Virus.

The life cycle of Adeno-Associated virus starts after entering the host cells. The virus takes one of two different pathways to establish infection depending on the presence or absence of helper virus. After entering the cell, the AAV particle has to leave clathrin-coated vesicle (endosome) to start life. The viral linear single-stranded DNA

genome will convert into double-stranded DNA to start viral transcription and gene expression (Daya & Berns, 2008) (Figure 5).

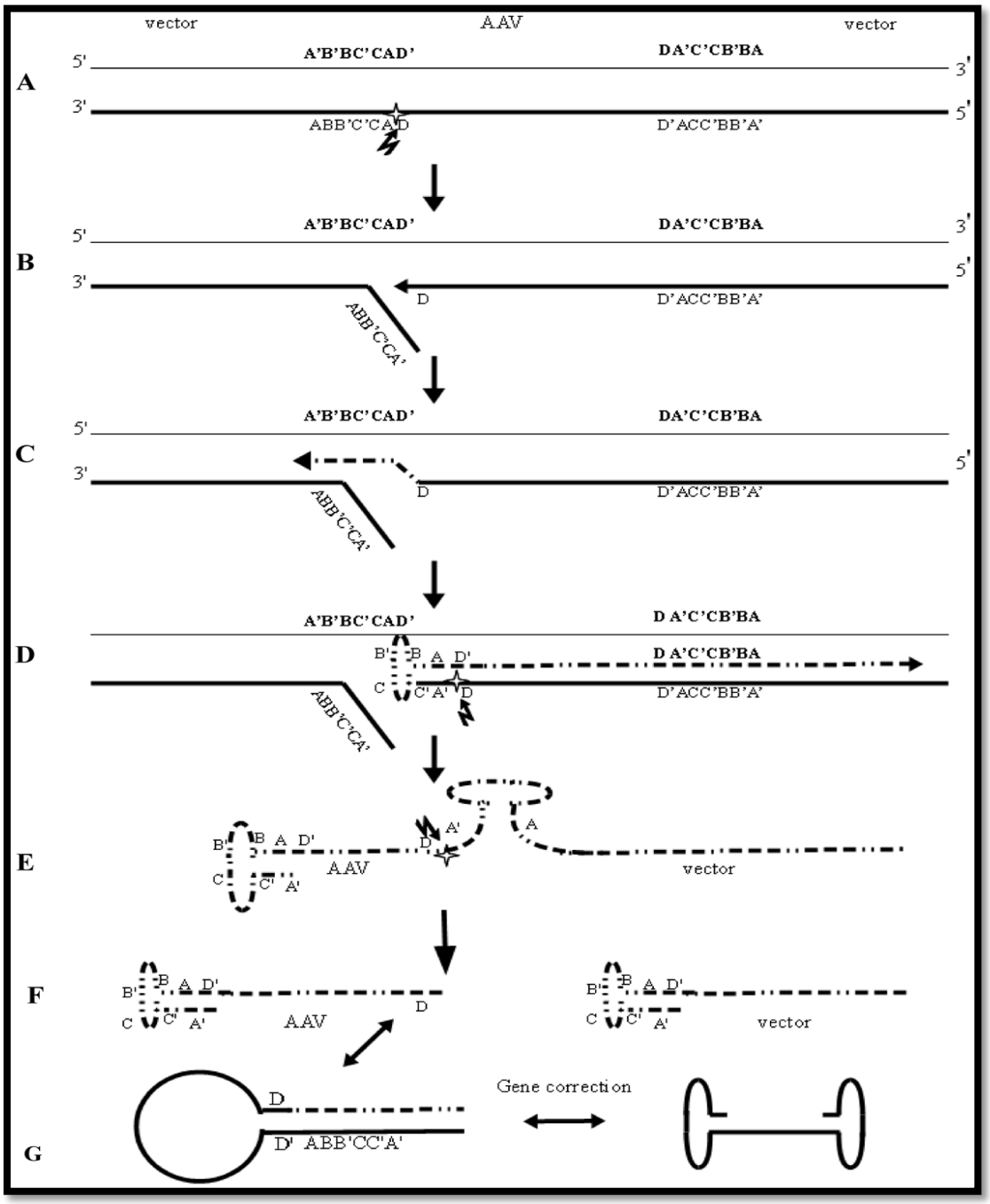

Figure 5: Formation of AAV ssDNA from dsDNA.
Rep68 protein binds to the Rep Binding Element (RBE) on the ITR and nicks one strand. (B) DNA replicates from the free 3'-OH end. (C-D) The replication continues through the template toward the 5' to form hairpin configuration by folding and Rep nick the vector for the second time. (E) Then Rep 68 binds to another RBE and causes third nick after a complete replication. (F) The results are two ssDNA with one ITR for each. (G) The missing ITR can be solved by gene correction (Cervelli et al., 2011).

The virus has the ability to persist in a host cell for a long period of time with stable genome replication. Also, it has no pathogenicity and a very mild immune response. The good safety profile considers AAV as vehicle to transfer genes and as a vector for gene therapy in vivo (Yang et al., 2014). Nowadays, Adeno-Associated virus is being used as vector to carry CRISPR (clustered regularly interspaced short palindromic repeats) that function in genome engineering via DNA editing (Senís et al., 2014).

CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats system (CRISPR) and CRISPR-associated protein (Cas) were discovered in 1987 in E. coli genome. The CRISPR system can be found in archaea and bacterial genomes. This system, which destroys invading DNA or plasmid DNA, known as the adaptive immune system in prokaryotes ("CRISPR_101_eBook_Final.pdf," n.d.). Nowadays, CRISPR/Cas9 system becomes a significant tool in therapeutic applications and genome editing because of its features that include effectiveness, simplicity, and evolution. Many people believe that CRISPR system is the newest defense mechanism against invading viruses in human. To target and cut a specific site on the genome, a short sequence of RNA called Guide RNA (gRNA), (which consists of 20 nucleotides), targets Cas9 to bind to short nucleotide sequence Protospacer Adjacent Motif sequence (PAM), which consists of 3-5 nucleotides

and located immediately downstream (3') of the site targeted by gRNA. Having multiple single gRNAs, which target different sites in genome, increases the efficiency of CRISPR/Cas9 system(Jo, Suresh, Kim, & Ramakrishna, 2015).

As such, the system depends on breaking the double-stranded genome with Cas9 protein. First, Cas9 has to bind to gRNA, which consists of crRNA and tracrRNA. crRNA is the genomic target for cas9 while tracrRNA is the linker between crRNA and Cas9. The sgRNA directs Cas9 toward the targeted DNA. Then Cas9 has to recognize specific 3-5 base pair sequence (PAM). The targeted sequence is going to be cleavage by Cas9. Finally, the double-strand break (DSB) is going to be repaired by either a Non-Homologous End Joining (NHEJ) pathway which is the most common pathway or the Homology Directed Repair (HDR) pathway (Figure 6).

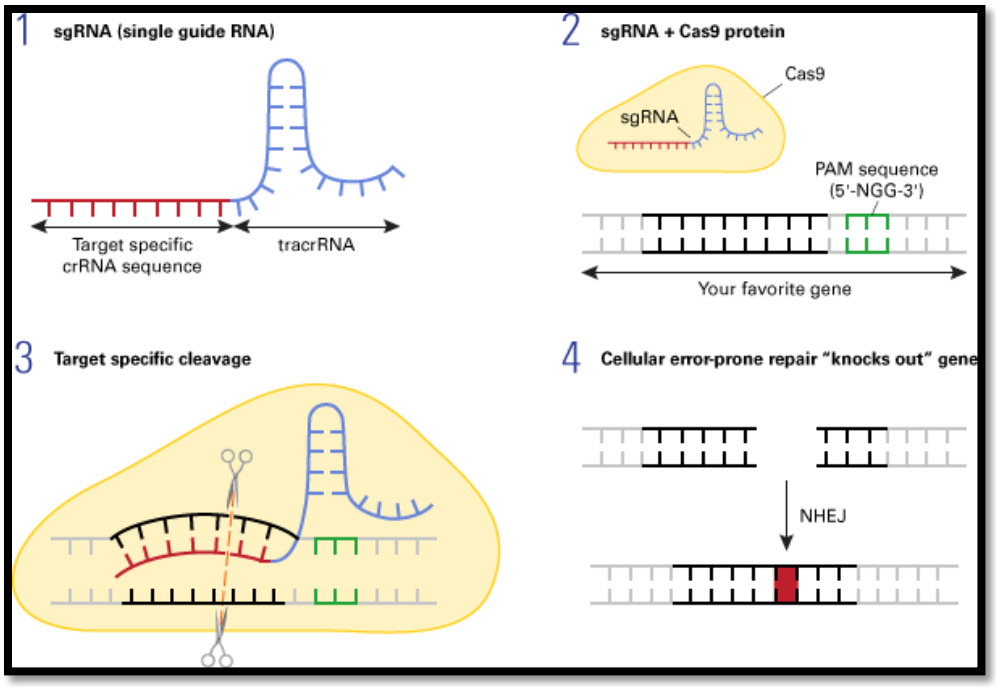

Figure 6: CRISPR/Cas9 System.
1- sgRNA consists of crRNA sequence and tracrRNA sequence. 2- Cas9 linked to crRNA via tracrRNA and then recognize specific sequence calld PAM. The targeted sequence cleavage by Cas9. 4- The DSB repairs by Non-Homologous End Joining (NHEJ) pathway ("CRISPR/Cas9 Overview," n.d.).

There are several methods to deliver CRISPR/Cas9 system (plasmid DNA encoding Cas9 and gRNA) that includes physical and viral methods. Some of the physical methods are electroportation (using physical methods to introduce CRISPR/Cas9 plasmid into target cells), hydrodynamic delivery or so-called microinjection (inject solution that contain large volume of Cas9 and gRNA plasmid into target cells), and lipid-mediated transfection. The viral vectors of CRISPR/Cas9 system include adenoviral vector, Adeno-associated viral vector, and lenti-viral vector (Gori et al., 2015).

Lenti-Viral Vector

Lentiviral vector comes from retroviruses with the ability to infect both dividing and non-dividing cells. Lentivirus consists of capsid, nuclear capsid, tow copies of RNA, membrane associated matrix, envelope proteins, enzymes, and accessory proteins. The life cycle of retrovirus starts from binding to specific receptor on the host cell by using the surface glycoprotein ligand. Then membrane fusion occurs between the viral envelope and the host cell membrane. After entry, the virus loses its capsid and starts reverse transcription to form pre-integration complex (double-stranded DNA and viral proteins). Then the virus enters the host nucleus using PIC and integrates into the host genome. This step followed by transcription and translation of viral genome to assemble new viral particles, which release from the cells and mature outside (Gori et al., 2015).

Lentiviral vector has been used in CRISPR system for a long period of time because of its stability and effectiveness in a wide variety of mammalian cell lines.

To produce lentiviral vector, T293 cells have to be co-transfected with 3 plasmids. The first plasmid encodes the transgene, the second plasmid encodes the viral proteins (packaging plasmid), and the third plasmid encodes the viral envelope protein (vesicular stomatitis virus glycoprotein-G [VSV-G]), which is required for viral tropism. There is a third generation of lenti-viral vectors that require four plasmids for viral production (3 helpers and 1 vector). In this system, the second plasmid divided into two plasmids: one encodes Gag and Pol and another encodes Rev, and this system proves to increase the recombination events ("Lentiviral Vectors - lentiviralvectors.pdf," n.d.).

Thus far the elimination of HBV cccDNA has not been achieved yet. Our studies were initiated o generate a mouse model of chronic HBV infection by using a recombinant Adeno-associated virus that expresses HBV (rAAV-HBV). It has been previously suggested that a rAAV-HBV virus can establish persistent HBV infection in mice. We plan to use this model to test whether viral vectors that carry HBV-specific CRISPR/Cas9 can target HBV cccDNA in vivo. To this end, the recombinant AAV-HBV virus was produced by an AAV helper free expression system in HEK293T cells. As a control, I also produced a rAAV-GFP virus from co-transfection of HEK293T cells. Real-time quantitative PCR and Western Blot methods were utilized to detect HBV infection and quantify HBV genome copy number in infected HepG2 cells. My results show that the recombinant AAV-GFP virus can effectively infect human liver HepG2 cells and express high levels green fluorescent protein. While western blots showed

defined bands of HBsAg in infected HepG2 cells with rAAV-HBV, qPCR yielded a low amount of HBV genome copies. We therefore demonstrated the possibility to use rAAV-HBV vector to delivery HBV genome into liver cells. However, the efficiency is relatively low, likely resulting from the large size of the HBV genome that may affect the AAV packaging efficiency and the viral infectivity.

CHAPTER TWO: MATERIALS AND METHODS

Cell lines and Plasmids

Human Embryonic Kidney 293T cells and HepG2 cells (human liver cancer cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Non-Essential Amino Acid (NEAA) and 1% penicillin-streptomycin (100U/ml) at 37°C with 5% CO_2 incubator. AAV-DJ Helper Free Expression System was provided by Cell Biolabs. The system included four plasmids (pAAV-MCS Expression vector, pAAV-DJ containing Rep and Cap genes, pHelper, and pAAV-GFP as a control).

Plasmid HBV 1.3-mer WT replicon (genotype D, subtype ayw) was a gift from Wang-Shick Ryu (Addgene plasmid # 65459)(Wang, Kim, & Ryu, 2009). This plasmid was used to generate the HBV fragment, which was cloned into the pAAV-MCS Expression vector that contained the inverted terminal repeat of AAV type 2 at both ends. The plasmid AAV/HBV was designed by our lab and constructed commercially by Genewiz (pAAV-MCS Expression vector was digested using Xbal enzyme and pHBV1.3-mer WT replicon was digested with SacI and HindIII) (Figure7-8).

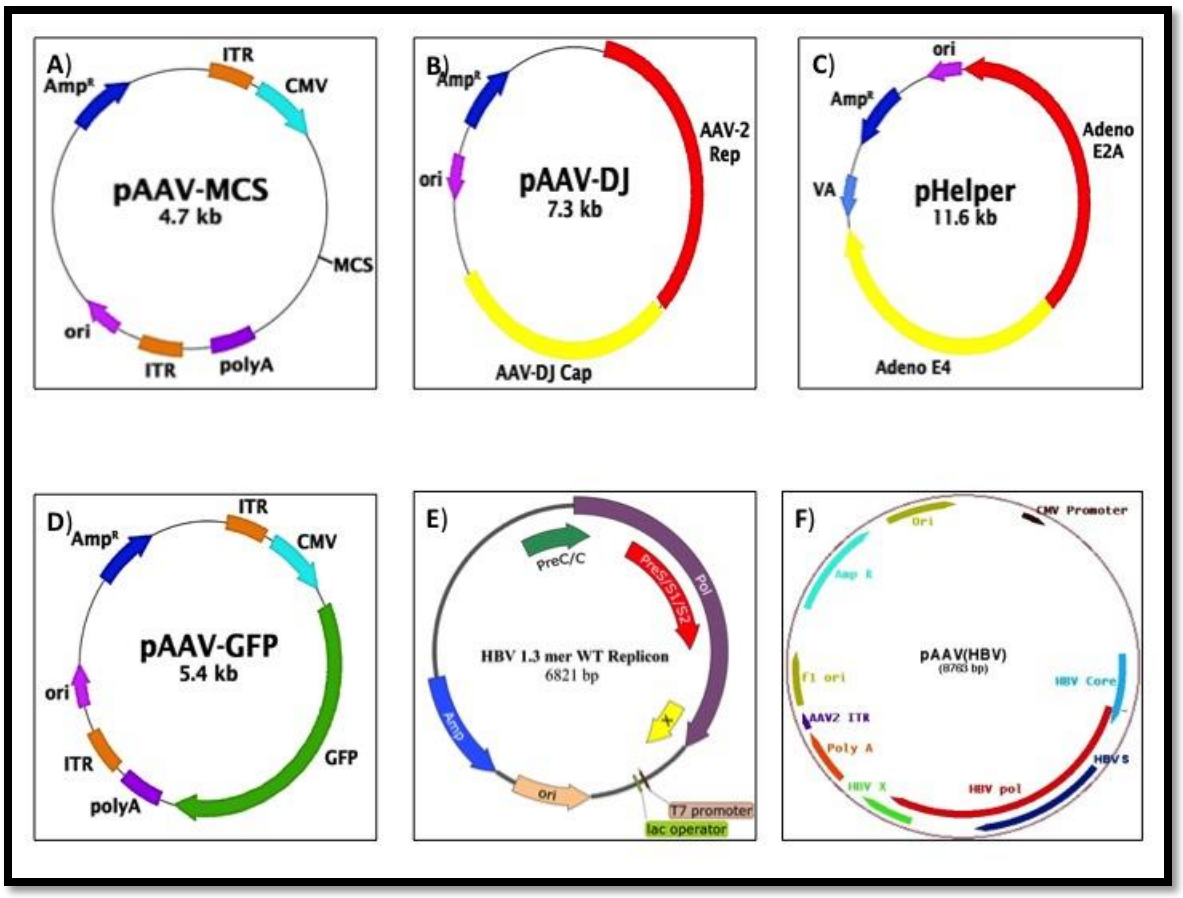

Figure 7: AAV/DJ Helper Free Expression System Plasmids and HBV 1.3-mer WT Replicon Plasmid.
A) AAV- MCS Expression Plasmid: ITRs (Inverted Terminal Repeats), CMV (Cytomegalovirus promoter for gene expression), MCS (Multiple cloning sites for inserting foreign genes), PolyA (Polyadenylation signal sequence), Ori (bacterial plasmid origin of replication), AmpR(Ampicillin resistant gene). The plasmid can be digested with PstI generating three DNA fragments. B) AAV Assembly DJ plasmid: expresses the Rep gene from AAV serotype 2, and Cap gene from a complex library of capsids from eight different wild type AAVs (AAV-2, AAV-4, AAV-5, AAV-8, AAV-9, avian AAV, bovine AAV and caprine AAV). This vector can be digested with SacI, generating three DNA fragments. C) Helper plasmid: expresses E2A and E4 gene from Adenovirus. E2A gene functions in viral DNA replication, viral mRNA processing and export, and AAV promoter regulation. E4 gene causes preferential export of AAV and adenoviral late mRNAs from nucleus. VA gene (Adeno virus associated RNAs) inhibits interferon response. Ori (origin of replication), Amp (Ampicillin resistance gene) The plasmid can be digested with HindIII, generating 2 fragments of DNA. D) AAV- GFP expression plasmid: expresses GFP and can be used as a control to monitor assembly efficiency or transduction effects. The plasmid can be digested with PstI generating three DNA fragments. E) HBV 1.3 –mer WT Replicon plasmid: a mammalian expression vector containing 1.3 units of the HBV genome. PreC/C (Pre core/core protein ORF), PreS/S1/S2 (S protein ORF), X (X gene ORF), Pol (Polymerase/reverse transcriptase protein ORF), Amp (Ampicillin resistance gene). This plasmid can be digested with BamHI, generating 4 fragments of DNA. F) AAV-HBV 1.3-mer WT Replicon plasmid: AAV vector that expresses the entire HBV genome. It used for delivery of HBV to liver cells. Ori/f1 (origins of replication for AAV and HBV respectively), AAV2 ITR (AAV serotype 2 inverted terminal repeat), Poly A (polyadenylation signal sequence), Amp (Ampicillin resistance gene), HBV Core (HBV pre core/core ORF), HBV S (HBV PreS/S1/S2 ORF), HBV pol (HBV polymerase/RT ORF), HBV X (HBV X protein ORF). This plasmid can be digested with NcoI generating three DNA fragments.

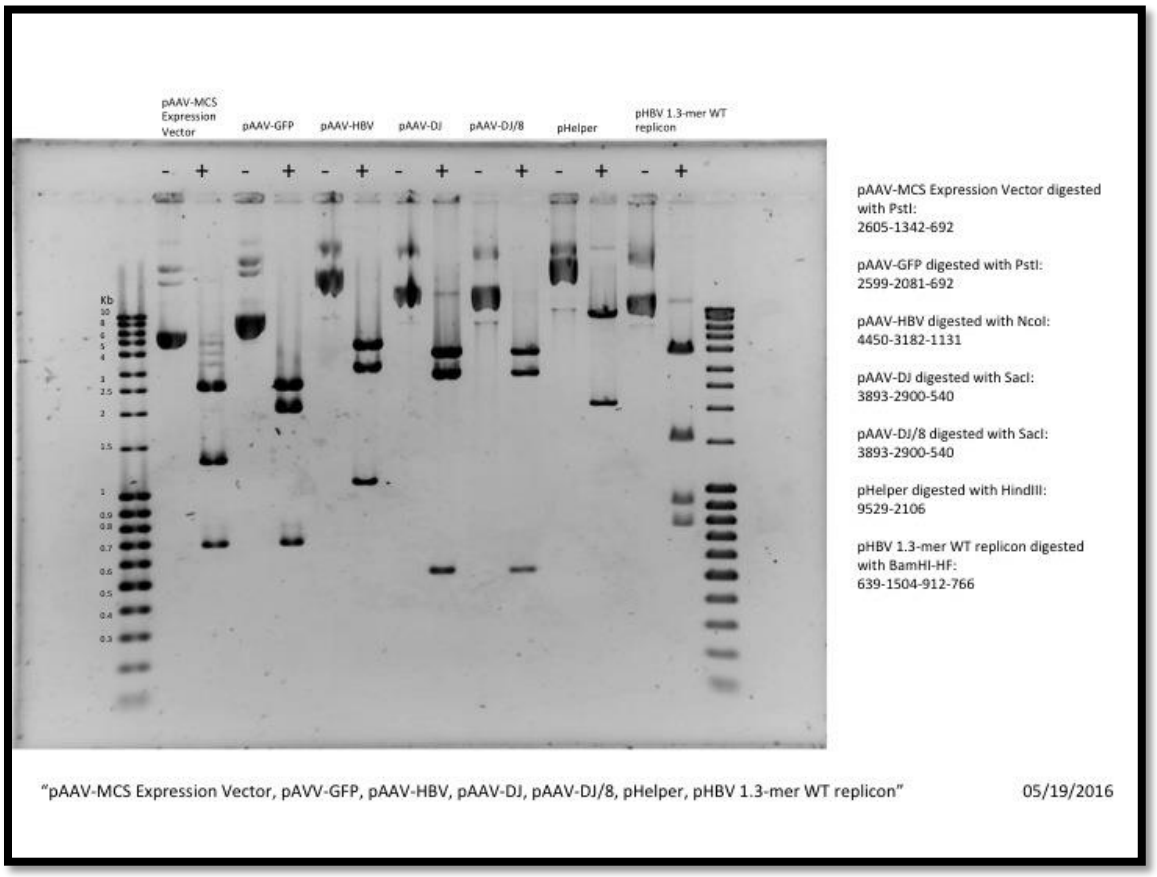

Figure 8: Gel Electrophoresis of plasmids DNA.
Plasmid AAV-MCS Expression vector, pAAV-GFP, pAAV-HBV, pAAV-DJ, pHelper and pHBV1.3-mer WT replicon.

Lenti-CRISPR v2 plasmid that expresses S. pyogenes CRISPR- Cas9 was a gift from FengZhang (Addgene plasmid # 52961(Sanjana, Shalem, & Zhang, 2014). This plasmid was used to express Cas9 and sgRNA specific for HBV. X602-AAV-TBG::NLS-SaCas9-NLS-HA-OLLAS-bGHpA;U6::Bsal-sgRNA that expresses Staphylococcus aureus CRISPR-Cas9 was gift from FengZhang (Addgene #61593)(Ran et al., 2015). This plasmid was used to generate Cas9 and sgRNA specific for HBV. Plasmid CMV-S

that expresses HBV S1 protein and plasmid CMV-M that expresses HBV S1and S2 proteins were gifts from aldevron (Figure 8).

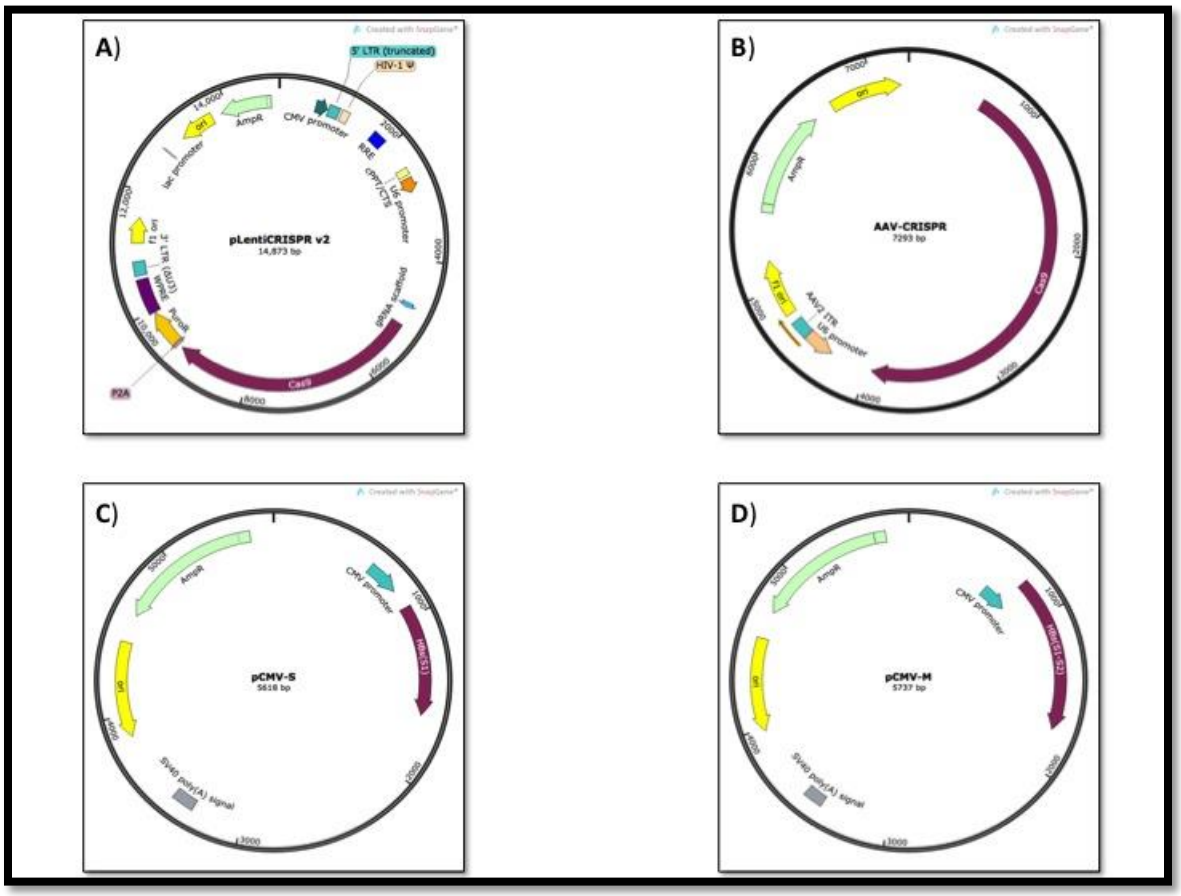

Figure 9: CRISPR-Cas9 Plasmids and HBV Envelope Protein plasmids.
A) Lenti-CRISPR v2 plasmid: expresses Cas9 gene and gRNA. HIV LTRs (long terminal repeat), CMV promoter for gene expression, U6 promoter for sgRNA expression, PuroR (puromycin resistance gene), Ori (bacterial plasmid origin of replication), AmpR (Ampicillin resistant gene). B) AAV-SaCa9 plasmid: expresses Cas9 gene and gRNA. AAV-2 ITR (inverted terminal repeat from serotype 2), U6 Promoter for sgRNA expression, Ori (bacterial plasmid origin of replication), AmpR (Ampicillin resistant gene). C) CMV-S plasmid: expresses HBV Small envelop protein. CMV Promoter for HBs (S1) expression, Ori (bacterial plasmid origin of replication), AmpR (Ampicillin resistant gene). D) CMV-M plasmid: expresses HBV Small and Medium envelop proteins. CMV Promoter for HBs (S1 and S2) expression, Ori (bacterial plasmid origin of replication), AmpR (Ampicillin resistant gene).

Viruses

Recombinant Adeno-associated virus particles were produced in HEK 293 cells that have been passaged 3 times prior to co-transfection and seeded at 2.5 - 3 x 10^6 cells per 10cm dish using AAV/DJ helper-free expression system. When the cells were 75% confluent, they co-transfected with three plasmids: AAV-MCS Expression plasmid (HBV, Cas9 or GFP), AAV/DJ plasmid and Helper plasmid at ratio of 1:1:1 using Lipofectamine 2000 transfection reagent from Invitrogen, Catalog number 11668027. After 48-72 hours, the virus was harvested using 0.5 M EDTA to a final of 10mM and following the manufacture protocol. The rAAV particles were purified and concentrated using affinity chromatograph heparin column (HiTrap Heparin Hp Catalog number 17-0406-01) from GE Healthcare Life Sciences. Several NaCl-Tris Elution Buffers with variety of concentrations of NaCl were used following Journal of Visualized Experiments protocol (Production and Tittering of Recombinant Adeno-associated Viral Vectors) (Figure 10). The virus concentrated using Amicon ultra-4 centrifuge unit with 100,000 MWCO from EMD Millipore catalog number UFC810024 and centrifuged at 2000x g and room temperature to final concentration of 250ul, then supplemented with 250ul of sterile PBS. Finally, the virus filtered through 13mm diameter 0.2uM syringe filter and stored at -80^{o}C (McClure, Cole, Wulff, Klugmann, & Murray, 2011).

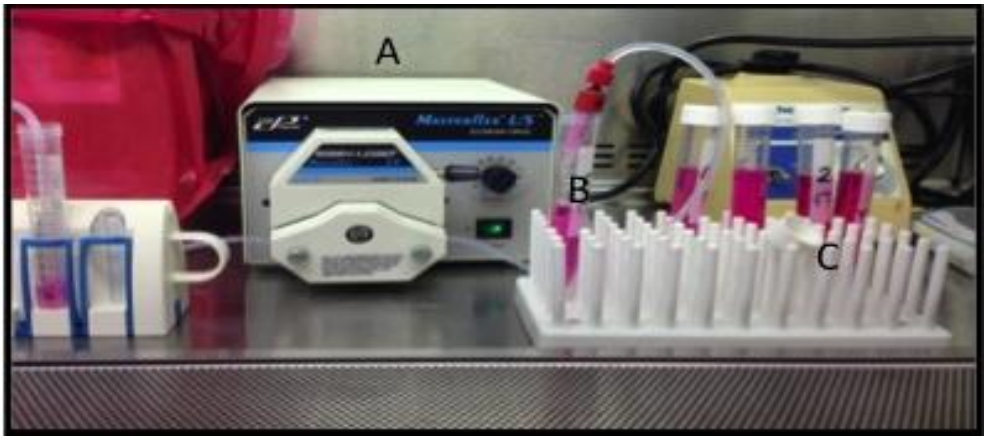

Figure 10: Purification System of Recombinant Adeno-Associated Virus.
A) Masterflex peristaltic pump. B) HiTrap Heparin Column. C) Flow through tubes.

AAV Titer

HEK293 cells were seeded in 12well plates at a 0.17 x 10^6/ well to become 50% confluent the next day. Purified and concentrated AAV-GFP was diluted 10-fold dilution. In time of infection, the cells medium removed and replaced with fresh medium. The experiment has been done in duplicate, each well infected with the appropriate amount of virus. Four days post infection, the cells harvested and prepared for Flow-Cytometry Analysis.

Viral Infection

Cells were seeded in 6 or 12 wells plate at a 0.5 X 10^6 or 0.25 X10^6 density to become 40-50% confluent the next day. The cells were infected with virus at specific MOI and maintained in complete DMEM. The infected cells were incubated at 37^{o}C in 5% CO_2 for the required time in each experiment.

Transfection

HEK293 cells were seeded in 6 wells plate at 700.000 cells per well and incubated at 37^{o}C in 5% CO_2. The next day, when the cells were 65-80% confluent, they transfected with plasmids (pAAV-HBV or pAAV-GFP, pAAV-DJ and pHelper) using Lipofectamine 2000 reagent and following the manufacture's instruction. 10ul/well of Lipofectamine was diluted in 200ul Serum-Free DMEM and then mixed with 4ug/well of plasmids DNA that diluted in 200ul Serum-Free DMEM. After 15minutes incubation at room temperature, the mixture was added to the wells drop wise. After 6-8 hours post transfection, the medium removed from the cells and replaced with complete DMEM. The cells incubated at 37^{o}C in 5% CO_2 for 48-72 hours.

Western Blot

The cells were lysed from 6 wells plate using 200ul/well of blue lyses buffer. Then the lysates were sonicated and heated at 75^{o}C for 10 minutes. The lysates were separated on NuPAGE Novex 4-12% Bis-Tris Protein Gel catalog number NP0322BOX. The gel then transferred into membrane using iBlot dry transfer system from life technologies. Membranes were blocked with 5% milk for 30 minutes and then incubated with HBsAg antibody diluted 1:200 in 2.5% milk overnight at 4^{o}C. The next day, following washes with TBST, the membrane incubated with secondary antibody (Dylight labeled antibody diluted 1:7500 in 2.5% milk) for one hour at room temperature then washed with TBST and PBS. The membrane was visualized using Odyssey imaging system. Then the membrane was stripped for 15 minutes with stripping solution from Thermo Fisher. The membrane washed 2 times with PBS and 2 times with TBST then blocked with 5% milk for 30 minutes. The membrane incubated with Goat polyclonal to

GAPDH from abcam catalog number ab9483 (diluted 1:1000 in 2.5% milk) for one hour at room temperature then washed with TBST and incubated with secondary antibody (Dylight labeled antibody diluted 1:7500 in 2.5% milk) for one hour at room temperature then washed with TBST and PBS. The membrane was visualized by Odyssey imaging system that detects secondary antibodies labeled with fluorescent dyes.

Nucleic Acid Extraction

HBV DNA was extracted from infected or transfected cell supernatant using QIAamp Minieulte Virus spin kit from QIAGEN catalog number 51104 and following manufacture's protocol. In addition, HBV DNA was extracted from cell culture using Wizard SV Genomic DNA Purification System from Promega catalog number A2360 and following manufacture's instructions.

PCR Primers and Probe Design

The HBV primers and probe were specific for S region. The primers and probe were designed according to TaqMan primers and probe instructions. The forward primer (1785-1807) 22 bases is 5'-ATGGGGCAGAATCTTTCCACCA-3', the revers primer (1966-1985) 19 bases is 5'-CTCCACCCAAAAGGCCTC-3' and the probe (1823-1853) 30 bases, which labeled with 56-FAM attached to the 5'-end, ZEN, and 3IABkFQ attached to the 3'-end is 5'-/56-FAM/CTTTCCCGA/ZEN/CCACCAGTTGGATCCAGCCTT/3IABkFQ/-3'. The PCR product was 200 base pair in length.

Real Time qPCR

HBV DNA was quantified using TaqMan Universal PCR Master Mix from Applied Bio system (catalog number 4304437) and following manufacturer's protocol. The PCR was done using iCycler iQ Real-Time PCR Detection System from BIO-RAD (catalog number 170-8740). The reaction was performed in 50ul contains 1X TaqMan Universal Master Mix, 500nM forward primer, 500nM revers primer, 300nM probe, 100ng of extracted DNA, and ended with Nuclease-Free water. The samples were collected from transfected or transduced cells in different time point and real time quantitative PCR (qPCR) was preformed to quantify HBV genome copy number (viral replication). Plasmid HBV1.3-mer WT replicon was diluted 10-folds serial dilution (from 10^0 to 10^{-8}) and used as standard curve template for qPCR. The standard curve linear was obtained between 1.3E+10 and 1.3E+3 HBV genome copy number on plasmid HBV1.3-mer WT Replicon (r^2=0.99). Un-infected cells and water samples were utilized as negative controls. The Amplification started with incubation at 50ºC for 2 minutes, then polymerase activation at 95ºC for 10 minutes, followed by 40 cycles: denature at 95ºC for 15 seconds and anneal at 60ºC for 1 minutes.

Genome Copy Number Calculation

Equation 1: copy number calculation for qPCR

Plasmid HBV 1.3-mer WT Replicon:

Weight (g/mol) = (the size of pHBV 1.3-mer WT replicon) (330Da X 2 nucleotide/bp)

= (6821bp) (330 Da X 2 nucleotide/bp) = 4501860 g/mol

Copy number = Weight (g/mol) / Avogadro's number 6.02214199 x 10^{23}

= 4501860 g/mol / Avogadro's number 6.02214199 x 10^{23} = 7.47 x 10^{-18}

The number of molecule = concentration of pHBV1.3-mer WT replicon (g/ul) / copy number

= (1 x 10^{-7} g/ul) / 7.47 x 10^{-18} grams/molecules) = 1.3 x 10^{10} molecules/ul

Plasmid AAV-HBV:

Weight (g/mol) = (the size of pAAV-HBV) (330Da X 2 nucleotide/bp)

= (8763bp) (330 Da X 2 nucleotide/bp) = 5783580 g/mol

Copy number = Weight (g/mol) / Avogadro's number 6.02214199 x 10^{23}

= 5783580 g/mol / Avogadro's number 6.02214199 x 10^{23} = 9.60 x 10^{-18}

The number of molecule = concentration of pAAV-HBV (g/ul) / copy number

= (1 x 10^{-7} g/ul) / 9.6 x 10^{-18} grams/molecules) = 1 x 10^{10} molecules/ul

CHAPTER THREE: RESULTS

Establishing HBV Infection in HepG2 Cell Line.

In 2015, Dan Yang and others established HBV infection in mouse model using recombinant Adeno-Associated virus (AAV) vector carrying 1.3 copies of HBV genome(Yang et al., 2014). The study demonstrated long-term HBV infection (more than 30 weeks) associated with liver fibrosis, which characterized by the presence of HBsAg and HBeAg, or HBcAg in the mouse serum and/or liver. There are other ways to establish mouse model such as HBV transgenic mice by micro-injecting the whole viral genome or partial copy of HBV genome into fertilized eggs of inbred mice (Akbar & Onji, 1998). This mouse model has been used to study the effects of different drugs on HBV infection; however, it is not an effective model because the viral clearance is difficult because the HBV genome persists in every cell. Also, some studies utilized adenoviral vectors carrying HBV genome into mouse liver. Even though HBV infection was established, there was no persistent infection because of the immune response against the vectors capsid (Ye et al., 2015). Many other studies established HBV infection via hydrodynamic injection (HDI) of HBV genome into the tail vein of mouse. Having HBV model will help to understand HBV biology and develop new treatment strategies. In our lab, we wanted to know if AAV vector, serotype DJ, carrying HBV genome has the ability to avoid host immune response and establish effective HBV

infection (Yang et al., 2014). We used HepG2 cell line as a model to generate HBV infection using AAV vector.

First, we examined the AAV-DJ Helper Free Expression System by producing rAAV-GFP particles in HEK293 cells. The cells were co-transfected with three plasmids (pAAV-GFP, pAAV-DJ and pHelper) at a ratio 1:1:1 using Lipofectamine 2000. Twenty-four and forty-eight hours post transfection, the particles visualized under Florescent Microscope (Figure 11).

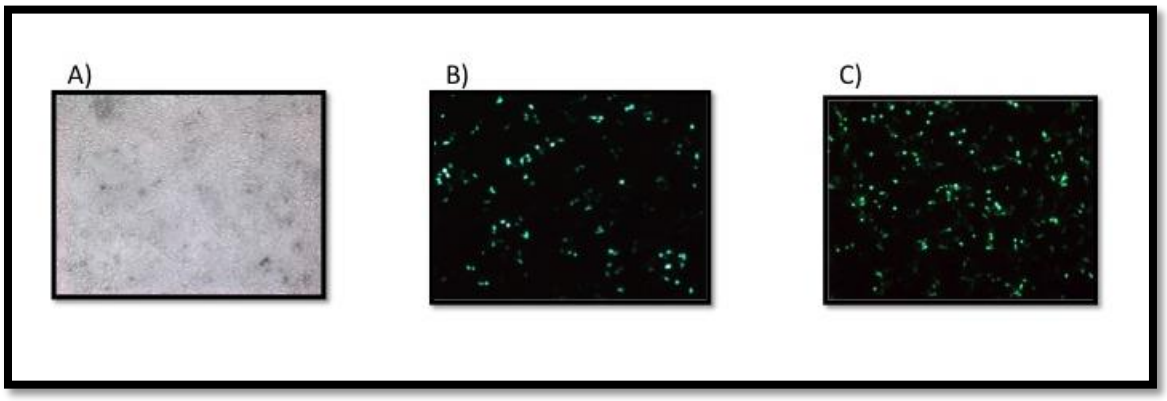

Figure 11: rAAV-GFP Particles.
HEK293 cells were co-transfected with pAAV-GFP, pAAV-DJ and pHelper at ratio 1:1:1 using Lipofectamine 2000. (A) Un-transfected HEK293 cells (negative control). (B) 24 hours post transfection. (C) 48 hours post transfection.

Next, we measured the AAV vector titer using reporter gene (GFP) and Flow Cytometry analysis. HEK293 cells were infected with AAV-GFP (10 – folds dilution) in 12 well plates. Four days post infection; the cells were neutralized, harvested and stained with propidium iodide (PI). The results demonstrated that the rAAV vector titer could exceed 1-2 X $10^{10\text{-}11}$ IU/ml (Figure12).

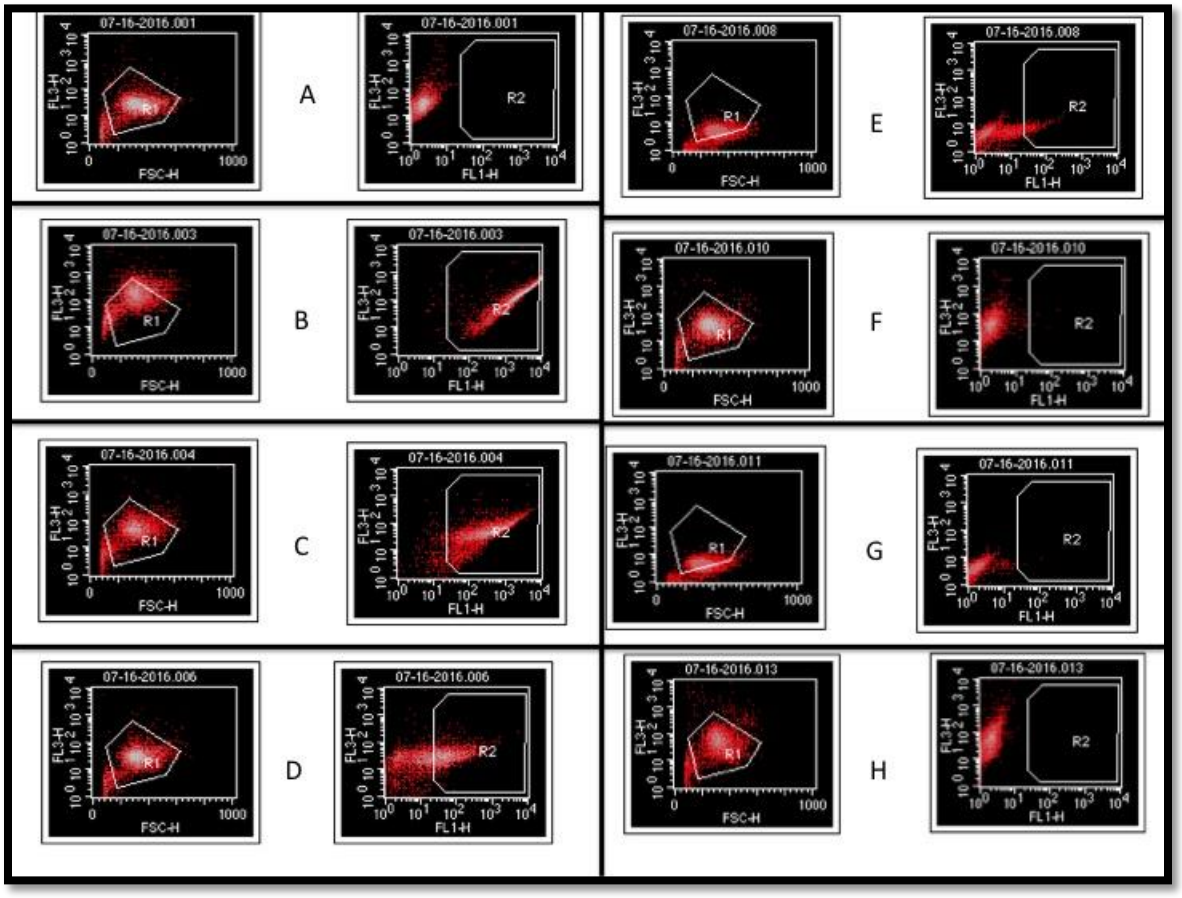

Figure 12: rAAV Titer
HEK 293 cells were infected with rAAV-GFP virus (10-folds dilution). (A) Negative control: HEK 293 cells . (B) 10^{-1} AAV-GFP: the infection ratio is 92.61%. (C) 10^{-2} AAV-GFP: the infection ratio is 88.92%. (D) 10^{-3} AAV-GFP: the infection ratio is 47.63%. (E) 10^{-4} AAV-GFP: the infection ratio is 6.53%. (F) 10^{-5} AAV-GFP: the infection ratio is 0.52%. (G) 10^{-6} AAV-GFP: the infection ratio is 0.39%. (H) 10^{-7} AAV-GFP: the infection ratio is 0.04%.

Then we wanted to quantify HBV genome copy number in plasmid AAV-HBV. Plasmid AAV-HBV 100ng/10ul was subjected to10-folds dilution and real time qPCR was done on each sample. The results demonstrated the quantification of HBV Genome in plasmid AAV-HBV, which started from 1E+10 (Table 1) (Figure13).

Table 1: HBV Genome Copy Number in Plasmid AAV-HBV.

Samples	Dilution	Genome Copy Number	Threshold Cycle Ct
10	10^{0}	1E+10	12.2
9	10^{-1}	1E+9	17.6
8	10^{-2}	1E+8	21
7	10^{-3}	1E+7	23.7
6	10^{-4}	1E+6	27.5
5	10^{-5}	1E+5	30.6
4	10^{-6}	1E+4	34.2
3	10^{-7}	1E+3	36.6
2	10^{-8}	1E+2	39.8

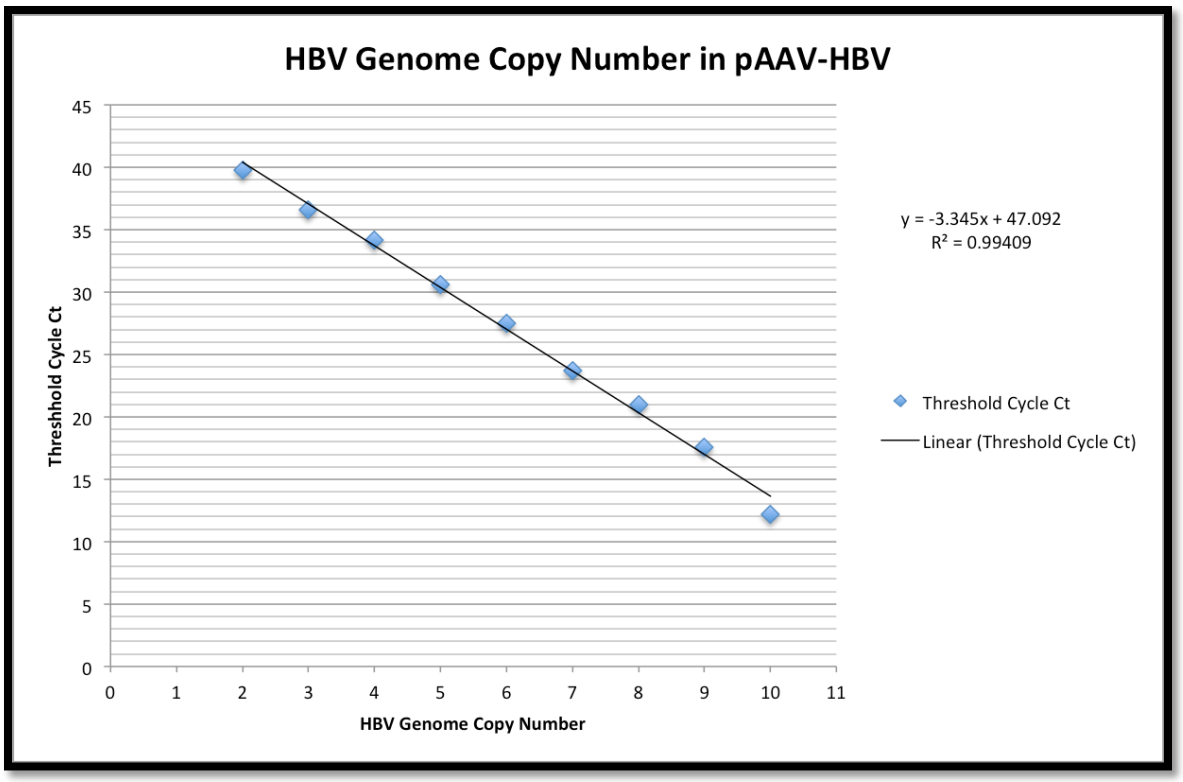

Figure 13: HBV Genome Copy Number in Plasmid AAV-HBV.
Plasmid AAV-HBV was diluted 10-folds dilution. The HBV genome copy number was quantified in 5ul of undiluted sample (100ng/10ul) and 5ul of each diluted sample from 10^{-1}-10^{-8} using TaqMan Universal Master Mix and following manufacture instructions. The genome copy number started from 1E+10

Next, we transfected HEK293 cells in 6 well plate with pAAV-HBV contains the ITR of AAV serotype 2 at both ends and a fragment that comprised 1.3 copies of HBV, pAAV-DJ (rep-cap genes), and pHelper to produce rAAV-HBV Vector. Forty-eight hours post transfection; the HBV DNA was extracted from cells using Wizard SV Genomic DNA Purification System and from supernatant utilizing QIAamp Minieulte Virus spin kit to conform that rAAV-HBV vector carried 1.3 copies of HBV genome (genotype D, serotype ayw). Real Time qPCR was done on the extracted DNA to measure viral replication and quantify HBV genome copy number. First, plasmid

HBV1.3-mer WT Replicon 100ng/10ul was diluted 10-folds dilution and used as standard curve template. The HBV Genome started from 1.3E+10 in 100ng/10ul plasmid HBV1.3-mer WT Replicon (Table 2) (Figure14).

Table 2:HBV Genome Copy Number in Plasmid HBV1.3-mer WT Replicon.

Samples	Dilution	Threshold Cycle Ct	Genome copy number
10	10^{0}	9.3	1.3E+10
9	10^{-1}	13.1	1.3E+9
8	10^{-2}	16.5	1.3E+8
7	10^{-3}	20.1	1.3E+7
6	10^{-4}	24	1.3E+6
5	10^{-5}	28.6	1.3E+5
4	10^{-6}	33	1.3E+4
3	10^{-7}	37.9	1.3E+3

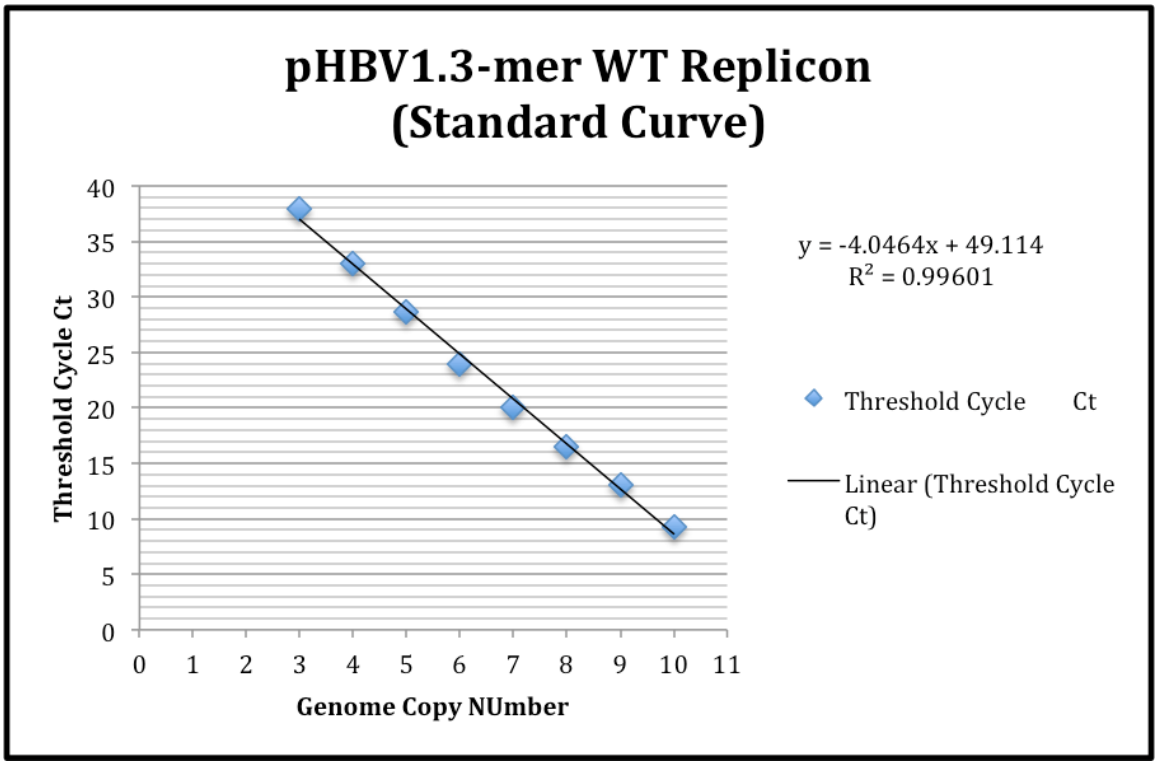

Figure 14:Plasmid HBV1.3-mer WT Replicon as Standard Curve for Real-Time PCR Products.
Plasmid HBV1.3 –mer WT Replicon was diluted 10-folds dilution. The HBV genome copy number was quantified in 5ul of undiluted sample (100ng/10ul) and 5ul of each diluted sample from 10^{-1}-10^{-7} using TaqMan Universal Master Mix and following manufacture instructions. The genome copy number started from 1.3E+10.

Real-Time qPCR results showed that the quantification of HBV genome in rAAV-HBV vector is 1.3E+7 (Table 3) (Figure 15).

Table 3: HBV Genome Copy Number in rAAV-HBV Vector.

		Dilution	Threshold Cycle Ct	HBV Genome Copy Number
Standard	10	10^{0}	9.5	1.3E+10
	9	10^{-1}	14.2	1.3E+9
	8	10^{-2}	18.8	1.3E+8
	7	10^{-3}	22.8	1.3E+7

Curve	**6**	10^{-4}	**26.1**	**1.3E+6**
	5	10^{-5}	**30**	**1.3E+5**
	4	10^{-6}	**34.9**	**1.3E+4**
	3	10^{-7}	**38.8**	**1.3E+3**
Samples	**AAV-HBV transfected cells**		**22.6**	
	AAV-HBV transfected cells		**23.9**	
	AAV-HBV supernatant		**22.9**	
	AAV-HBV supernatant		**22.9**	
	HEK293 supernatant DNA		**N/A**	
	HepG2 Cell DNA		**39.6**	
	Water		**N/A**	

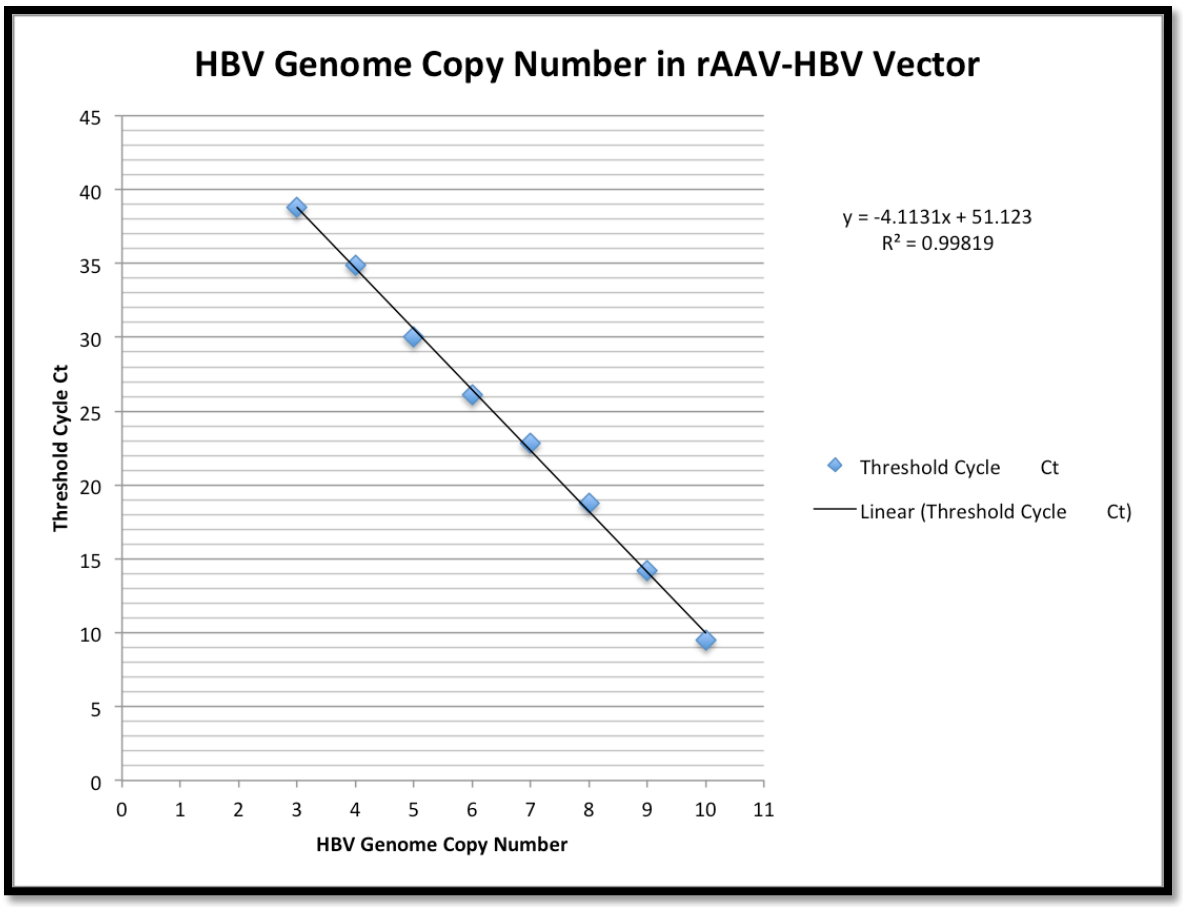

Figure 15:HBV Genome Copy Number in rAAV-HBV Vector.
HEK293 cells in six well plate were co-transfected with pAAV-HBV, pAAV-DJ and pHelper at ratio 1:1:1 using Lipofectamine 2000. 48 hours post transfection; viral DNA was extracted from supernatant and cell lysate. Real-Time qPCR was done on each sample. Plasmid HBV1.3-mer WT Replicon was used as standard curve. The quantification of HBV Genome copy number in rAAV-HBV Vector was 1.3E+7.

Next, HEK 293 cells were seeded in 6 well plate and co-transfected with pAAV-HBV, pAAV-DJ and pHelper at ratio 1:1:1 using Lipofectamine 2000. Forty-eight hours post-transfection, cells washed with 1% sterile PBS and lysed with Blue lyses Buffer. The cell lysate was sonicated and heated at 75^{o}C. Western Blot was done on the samples using HBsAg antibody to detect HBV Surface protein. HEK 293 cells and HepG2 cells were used as negative controls. GAPDH antibody was used as control in this experiment.

48 hours post-transfection, the result revealed the accumulation of HBV surface antigen in HEK293 cells (Figure 16).

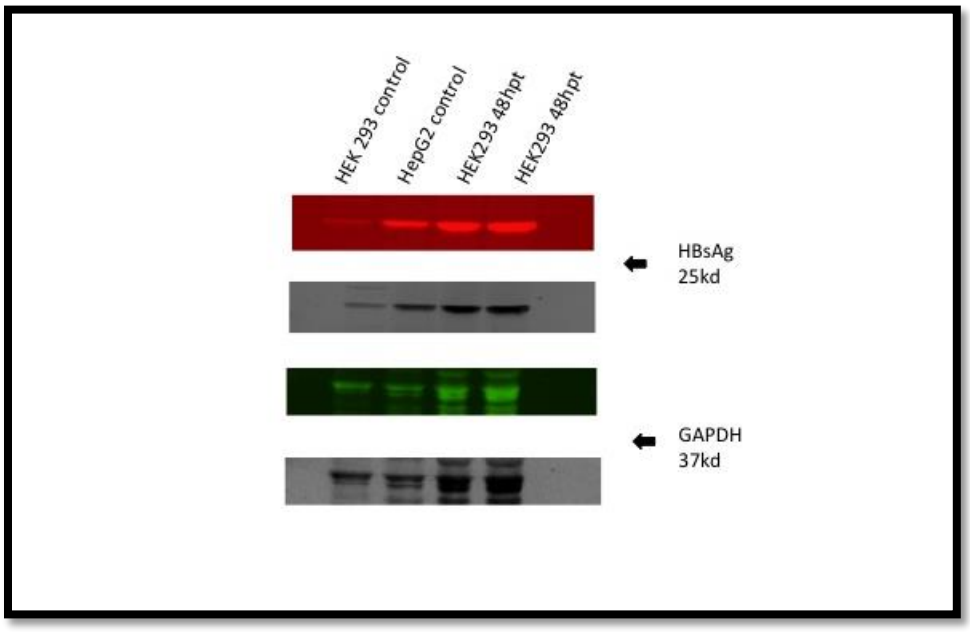

Figure 16: HBsAg in transfected HEK293 Cells.
HEK293 Cells were transfected with pAAV-HBV, pAAV-DJ and pHelper. 48 hours post-transfection; HBV Surface Antigen was presented in both samples. GAPDH enzyme was used as positive control. Un-transfected HepG2 cells and HEK293 cells were used as negative control.

Then, we wanted to conform that AAV-DJ Helper Free Expression System can work in HepG2 cell line. We seeded HepG2 cells at 0.25 X 10^6 densities in a 12 well plate. The cells were infected with rAAV-GFP: un-purified virus stock, purified virus stock and concentrated virus stock to compare the activity of the rAAV-GFP vector. Fluorescent Microscope images were taken after twenty-four and seventy-two hours post infection. Seventy-two hours post infection; the cells were harvested and run through

Flow Cytometry. The GFP was expressed in the cells at a different ratio depending on the virus stock. The results indicate that rAAV-GFP Vector (different stocks) is capable of infecting HepG2 cells (Figure 17).

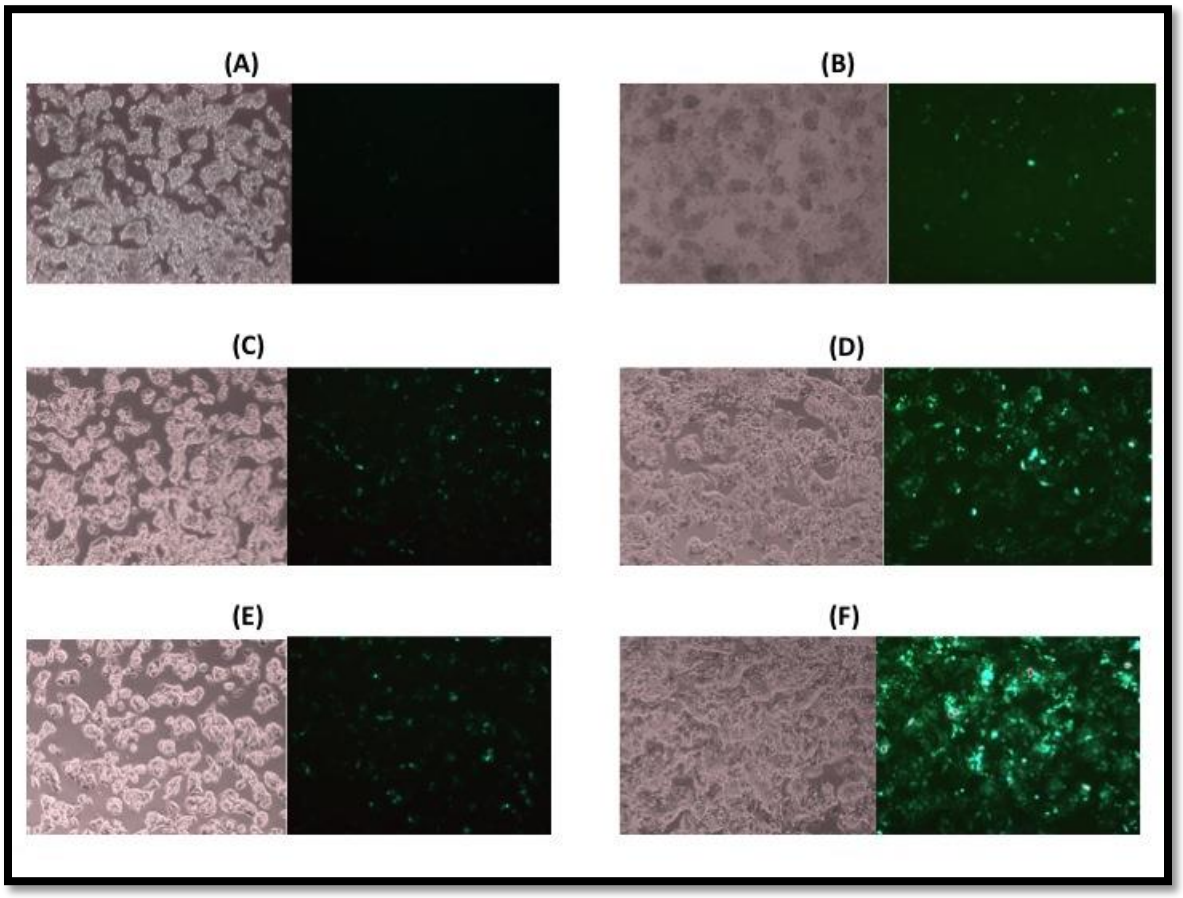

Figure 17: HepG2 Infected with Recombinant AAV-GFP.
HepG2 cells were seeded in 12 well plate at 0.25 X10^{6}/well to become 40% confluent the next day. The GFP expressed in the cells at different ratio after 24 and 72 hours post infection. A) Cells infected with un-purified rAAV-GFP after 24h. B) Cells infected with un-purified rAAV-GFP after 72h. C) Cells infected with purified rAAV-GFP after 24h. D) Cells infected with purified rAAV-GFP after 72h. E) Cells infected with concentrated rAAV-GFP after 24h. F) Cells infected with concentrated rAAV-GFP after 72h.

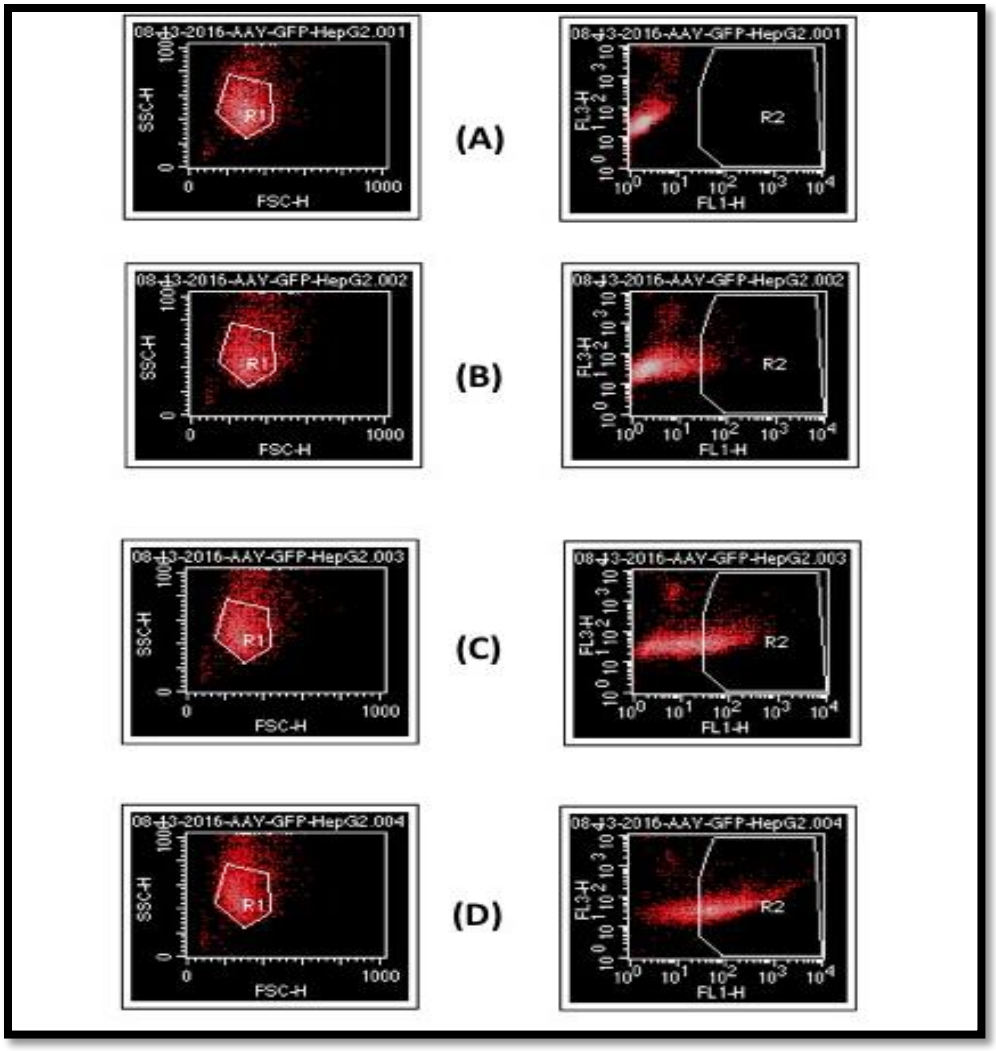

Figure 18: Flow Cytometry Analysis of HepG2 Cells Infected with rAAV-GFP.
HepG2 cells were infected with different types of rAAV-GFP. 72 hours post infection, the samples were harvested, sustained with PI and run through Flow Cytometry. A) Un-infected HepG2 cells were used as negative control. B) HepG2 cells infected with un-purified rAAV-GFP: the infection ratio is 6% C) HepG2 cells infected with purified rAAV-GFP: the infection ratio is 34.45% D) HepG2 infected with concentrated rAAV-GFP: the infection ratio is 67.95%.

Next, HepG2 cells were seeded at 0.25 x 10^6 / well in 12 well plate and were infected with rAAV/HBV virus to study the possibility of AAV vector to mediate delivery of HBV genome into HepG2 cells. Twenty-four hours post infection; cells were washed three times with DMEM-Serum Free and maintained in complete DMEM for

several days. Viral DNA was extracted from supernatant and cell lysates. The HBV Genome copy numbers were measured in extracted DNA by real-time quantitative PCR (qPCR). Two and Four days post infection, the results showed low HBV genome copy number that quantified around 1.3E+4 and 1.3E+5 in 100ng/10ul-extracted DNA from supernatant and cell lysate. (Table 4) (Figure19).

Table 4: The Quantification of HBV Genome Copy Number in HepG2 cells infected with rAAV-HBV:

		Dilution	Threshold cycle Ct	HBV Genome copy number
Standard Curve	10	10^{0}	10.2	1.3E+10
	9	10^{-1}	15.2	1.3E+9
	8	10^{-2}	19.6	1.3E+8
	7	10^{-3}	23.9	1.3E+7
	6	10^{-4}	27.6	1.3E+6
	5	10^{-5}	31.7	1.3E+5
	4	10^{-6}	36.5	1.3E+4
	3	10^{-7}	40	1.3E+3
Samples	DNA from Supernatant		34.5	
	DNA from Supernatant		33	
	DNA from Cells		33	
	DNA from Cells		31.8	
	HepG2 DNA		38.3	
	Water		N/A	

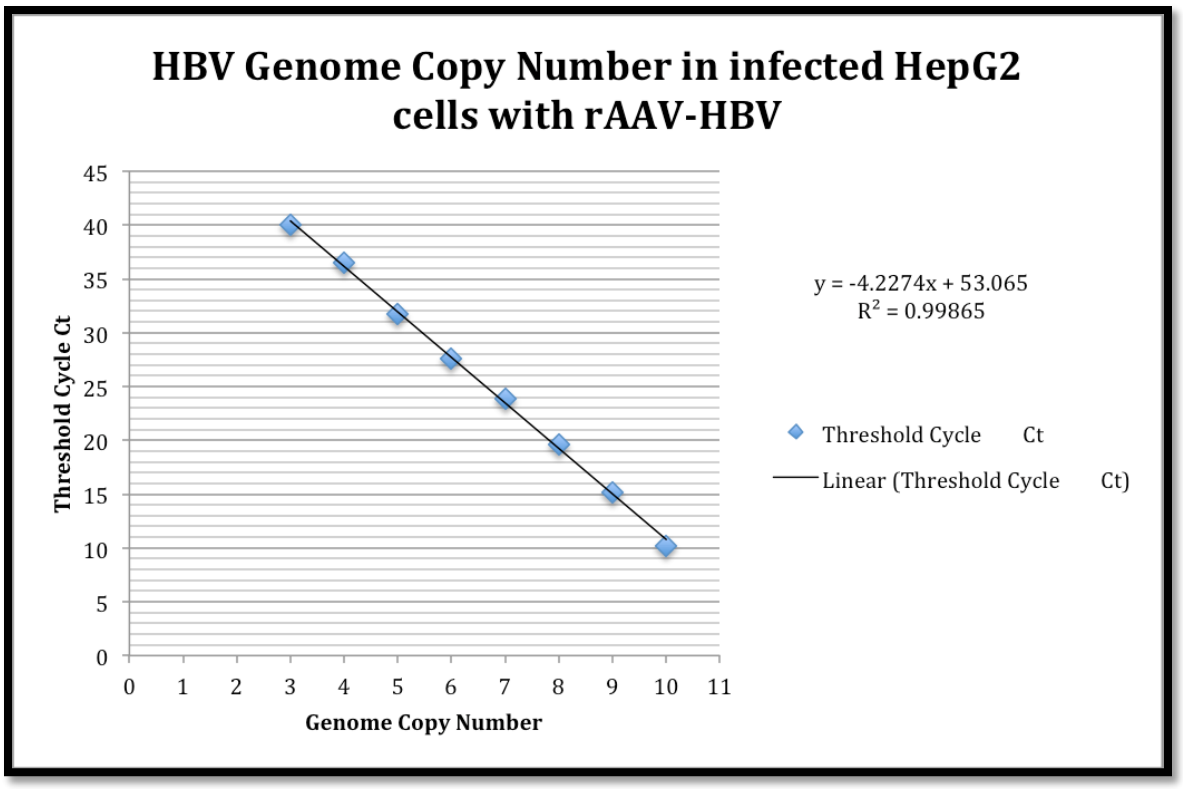

Figure 19: HBV Genome Copy Number in infected HepG2 cells with rAAV-HBV.
HepG2 cells in 12 well plate were infected with rAAV-HBV. Two and four days post infection; viral DNA was extracted from supernatant and cell lysate. Real-Time qPCR was done on each sample. Plasmid HBV1.3-mer WT Replicon was used as standard curve. The quantification of HBV Genome copy number were around 1.3E+5.

Furthermore, four days post infection, western blot analysis was done on infected HepG2 cells with rAAV-HBV. The results revealed low expression of HBsAg; however, there was expression of the HBV surface antigen in the negative control (Figure 20).

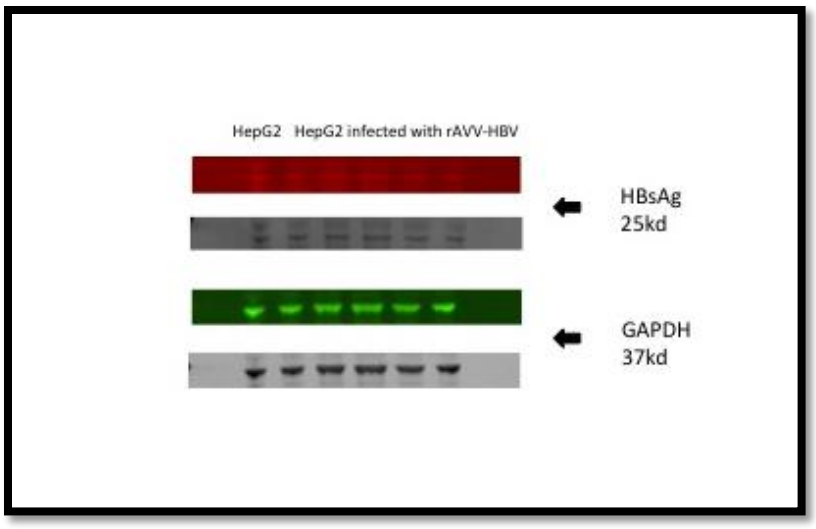

Figure 20: HepG2 Cells Infected With rAAV-HBV.
HepG2 cells were infected with rAAV-HBV. Un-infected HepG2 cells were used as negative control. HBsAg was expressed 4 days post infection in all samples including the negative control.

Our results demonstrate the possibility of establishing HBV infection using recombinant Adeno-Associated virus. However, the low infectivity of rAAV-HBV related to the limited packaging capacity of the AAV vector and the larger AAV-HBV genome.

Construction of Plasmid lenti-CRISPR/spCas9 and HBV-specific gRNA.

Several studies suggested the potential of genome editing technologies such as zinc finger nucleases (ZFNs) and transcription activator-like effector nuclease (TALENs) in cleaving HBV genome(Cradick, Keck, Bradshaw, Jamieson, & McCaffrey, 2010) (Bloom, Mussolino, & Arbuthnot, 2015). Both systems are complicated because of the requirement of engineering specific protein pairs for each target site (Peng, Lu, & Yang, 2015). However, there is another gene-editing tool called CRISPR/Cas9 system that has the ability to cleave any viral DNA by designing gRNA matches the target DNA, which

lead to eliminate viral infection. The CRISPR/Cas9 system can be induced into cells as DNA or carried in viral vector. Many studies used lenti-viral vector carrying CRISPR/Cas9 system specific for HBV to cleavage viral DNA by carefully selecting target sites and specific gRNAs to avoid off-target cleavage, which appear as result of homologs sites between viral DNA and host genome, also as consequence of long-term expression of CRISPR system.

In our study, we designed sgRNAs to target HBV S region (GCTCCTACCTTGTTGGCGTC), Core region (TCTAGAAGATCGTACTGA) and Pol region (CTGTTGTTAGACGACGAGGC). Each sgRNA was designed and cloned into plasmid lenti-CRISPR v2 that has Streptococcus pyogenes Cas9 commercially by GeneCopoeia.

Construction of Plasmid AAV-CRISPR/saCas9 and HBV-specific gRNA.

The recombinant AAV vector is the most promising tool in gene therapy. The ability to infect both dividing and non-dividing cells, the non-pathogenicity, low immune response, and the high level of tropism make it an effective vector. As we know that the packaging capacity of AAV is lower than 5kb and the size of Streptococcus pyogene Cas9 is 4.1, which indicate the difficulty to package sgRNA. However, many laboratories nowadays are investigating the uses of AAV as vector for CRISPR system and working to solve this problem. A recent study by indicated the possibility of designing effective AAV vector carrying Cas9 derived from Streptococcus aurous for gene editing (Ran et al., 2015).

In our lab, we designed sgRNAs to target HBV S region (CCCCAAAAGGCCTCCGTGCGG) and Pol region (TCGCCTCGCAGACGAAGGTCT). Each sgRNA was designed and cloned into plasmid AAV that encodes Streptococcus aurous Cas9 commercially by GeneCopoeia. We are going to use the AAV Helper Free Expression System that includes three plasmids (pAAV-Expression vector (Cas9 gene and sgRNA), pAAV-DJ (Rep and Cap genes) and pHelper) to produce this vector.

CHAPTER FOUR: DISCUSION

Hepatitis B virus infects more than 250 million people worldwide. HBV chronic infection, which is characterized by the persistence of Hepatitis B surface antigen (HBsAg) in serum for more than six months, is mediated by the persistence of viral covalently closed circular DNA (cccDNA). Several genome-editing techniques have demonstrated the possibility to inhibit viral replication, but they did not completely clear cccDNA molecules in nuclei of infected cells. Recent studies also suggested the possibility to use the Clustered Regularly Interspaced Short Palindromic Repeats system (CRISPR/Cas9) to suppress HBV infection; however, effective delivery of this tool for in vivo targeting is a major issue. We plan to design and use a lentiviral vector-based system to deliver HBV specific CRISPR/Cas9 for in vivo targeting of HBV cccDNA for viral eradication. For this purpose, we are going to use a recombinant Adeno-associated virus harboring HBV (rAAV-HBV) mouse model that can generate persistent HBV infection. This model will help to test our Hepatitis B treatment strategies using CRISPR/Cas9. Here, we demonstrated via in-vitro studies the possibility of establishing an HBV infection in a hepatic cell line utilizing rAAV-HBV. First as a control in our project, we produced a recombinant Adeno-associated virus that expresses Green fluorescent protein (rAAV-GFP) with high titter using AAV helper free expression system. Then we designed AAV-HBV plasmid by cloning HBV1.3-mer WT replicon genome into AAV-

MCS Expression plasmid. The rAAV-HBV vector was produced in HEK293 cells using AAV helper free expression system. This system is highly effective in hepatic cell line. The GFP was expressed in most of the infected cells with rAAV-GFP and the Flow Cytometry analysis showed high infectivity rate. HepG2 cells were infected with rAAV-HBV to establish stable HBV infection. The HBV genome copy number was measured by real-time qPCR and the HBsAg was detected by western blot method. The results demonstrate low HBV infectivity. The reason behind that is the limited packaging capacity of the AAV vector. Several previous studies suggested that the maximum capacity of AAV is 5 kb (Wu, Yang, & Colosi, 2009). In our design, the size of the AAV-HBV genome is 6 kb, this large size is a reason of viral degradation by the cell's proteasome. After attachment of the AAV virion to specific receptor on the cell surface, the virus has to follow clathrin-dependent endocytosis pathway, then to escape from the late endosome and traffic to the nucleus. However, if the virus could not escape the late endosome, it will be degraded by the lysosome. On the other hand, the virus might be degraded by the proteasome after escaping the late endosome as a result of encoding a large genome (Figure 21). Studies done by Grieger and Samulski suggested the uses of a proteasome inhibitor during the time of infection which will help to avoid large genome degradation (Grieger & Samulski, 2005). This technique might help to package a large genome into AAV virion and maintain the infectivity of the virus.

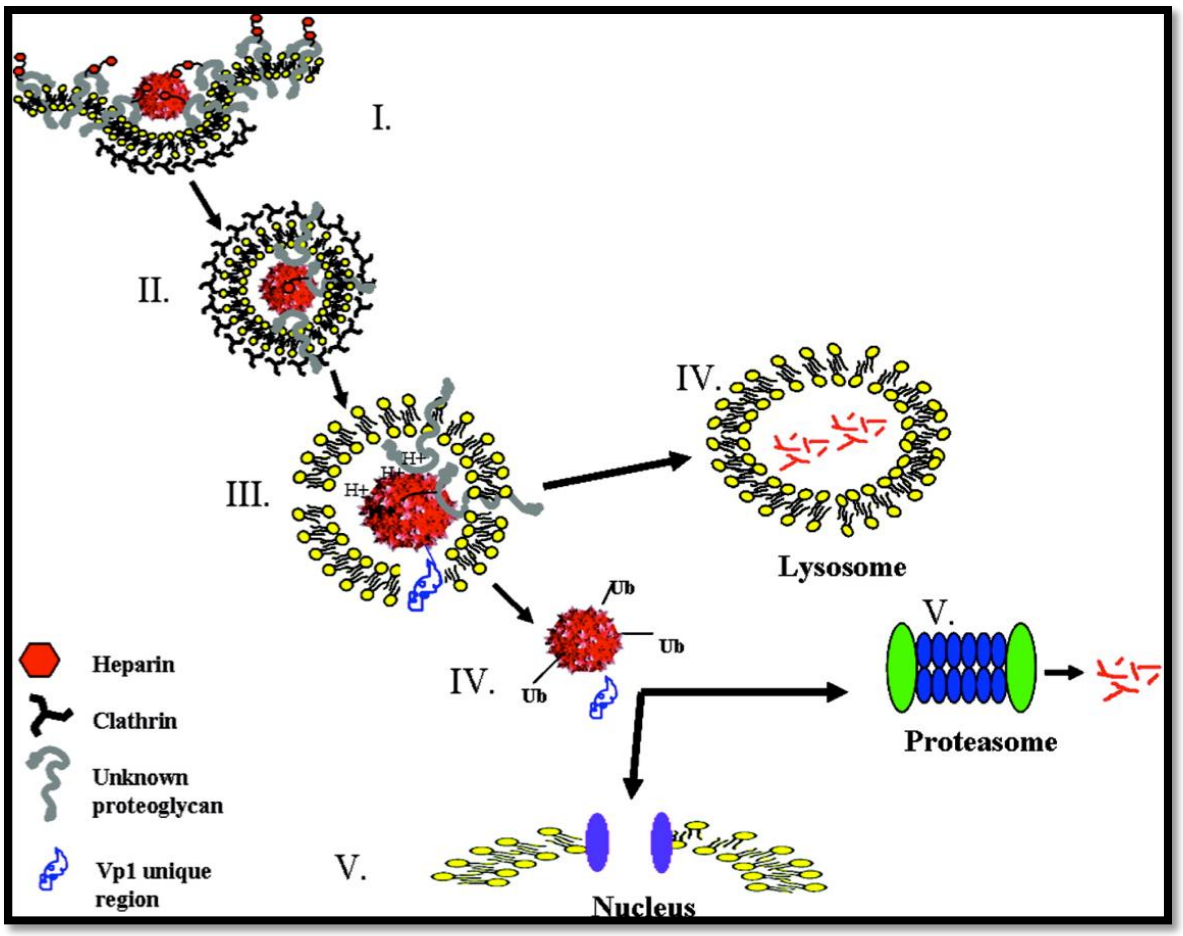

Figure 21: AAV Trafficking to the nucleus.
(I) AAV virion binds to specific primary receptor and secondary on the cell surface. (II) AAV follows clathrin—dependent endocytosis pathway (early endosome). (III) The early endosome matures into late endosome and the AAV has to escape in this point. (IV) The AAV may either fail to escape and become degraded by the lysosome, or escape into the cytoplasm toward the cell nucleus. (V) The AAV virion might be recognized and degraded by the cytoplasm proteasome before reaching the nucleus especially if encoding large genome.

CHAPTER FIVE: FUTURE DIRECTION

Re-design the AAV-HBV plasmid.

The packaging capacity of the AAV vector restrictions the size of the transgene. Several previous studies suggested that recombinant Adeno-associated virus is capable to package up to 5kb. However, encapsidating large genome might affect the infectivity of the virus. The AAV vector with large genome will be degraded by the cell proteasome. As the size of the AAV-HBV genome in our design is 6kb, we need to redesign AAV-HBV plasmid that encodes AAV-HBV genome lowers than 5kb to increase the infectivity of the vector.

Production of lentiviral vectors carrying spCas9 and gRNA specific for HBV and pseudotyped with HBV envelope proteins.

In this study, we are planning to design lenti-viral vectors that pseudotyped with HBV envelope proteins to eliminate HBV cccDNA. The sgRNAs were chosen to target HBV S region, Core region and Pol region. Each sgRNA was designed and cloned into plasmid lenti-CRISPR v2 that has Streptococcus pyogenes Cas9. Then, HEK 293 cells in six10 cm dishes will be co-transfected with three plasmids to produce these vectors.

Production of rAAV vector carrying saCas9 and gRNA specific for HBV.

In our lab, we are planning to design two AAV vectors that can carry Streptococcus aurous Cas9 and sgRNA specific for HBV (S region and Pol region). We are going to use the AAV Helper Free Expression System to produce these vectors.

Examine the effectiveness of viral vectors against HBV- cccDNA in a hepatic cell line.

After establishing stable HBV infection on hepatic cell line, the cells will be treated with either lentiviral vector/CRISPER system or AAV/CRISPR system. The HBV genome copy number and HBsAg expression are going to be detected and measured using Western blot, enzyme-linked immunosorbent assay (ELISA), and real-time quantitative PCR (qPCR).

Establish HBV Mouse Model.

After testing the vector in vitro, C57BL/6 mice are going to be injected with the rAAV/HBV vector (concentration $10^{12 \text{ or } 13}$) diluted in phosphate-buffered saline via tail vein using hydrodynamic injection. The mouse serum is going to be harvested by retro-orbital bleeding. The serum alanine aminotransferase activity (ALT), which is associated with HBV transgene expression, is going to be determined. The HBsAg levels in mouse serum are going to be detected and measured by enzyme-linked immunosorbent assay (ELISA). Also, the HBV DNA is going to be extracted from the serum and the liver then analyzed by real-time quantitative PCR (qPCR) using HBV-specific primers and probe. Moreover, we are going to examine to mouse liver tissues by immunohistochemistry to detect the expression of HBcAg.

Examine the effectiveness of CRISPR-Cas9 viral vectors against HBV-cccDNA in vivo.

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