SUCCESSFUL DISABLING OF THE 5' UTR OF HCV USING ADENO-ASSOCIATED VIRAL VECTORS TO DELIVER ARTIFICIAL PRIMARY MICRORNA MIMICS

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A dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2015

DECLARATION

I, Tarryn Jackie Bourhill (student number: 348295) declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

…….. ……

……day of ……., 2015

DEDICATION

To my parents and sister for their eternal support and encouragement

Catherine and Kevin Bourhill

Odette de Bruin.

PUBLICATIONS AND PRESENTATIONS

Conferences.

1. Bourhill, Tarryn, Ely, Abdullah, and Arbuthnot, Patrick. Inhibition of hepatitis C virus replication using adeno-associated viral vector to delivery primary microRNA mimics. The Research Day and Postgraduate Expo for the Faculty of Health Sciences. 2013 Sept 17; Parktown, South Africa.
2. Bourhill, Tarryn, Ely, Abdullah, and Arbuthnot, Patrick. Inhibition of hepatitis C virus replication using adeno-associated viral vector to delivery primary microRNA mimics. The Molecular Biosciences Research Thrust Postgraduate Research Day. 2013 Dec 4; Parktown, South Africa.
3. Bourhill, Tarryn, Ely, Abdullah, and Arbuthnot, Patrick. Inhibition of hepatitis C virus replication using adeno-associated viral vector to delivery primary microRNA mimics. The South African Society for Biochemistry and Molecular Biology Congress. 2014 July 6-9; Cape Town, South Africa.
4. Bourhill, Tarryn, Ely, Abdullah, and Arbuthnot, Patrick. Inhibition of hepatitis C virus replication using adeno-associated viral vector to delivery primary microRNA mimics. The Research Day and Postgraduate Expo for the Faculty of Health Sciences. 2014 Sept 17; Parktown, South Africa.
5. Bourhill, Tarryn, Ely, Abdullah, and Arbuthnot, Patrick. Inhibition of hepatitis C virus replication using adeno-associated viral vector to delivery primary microRNA mimics. The Molecular Biosciences Research Thrust Postgraduate Research Day. 2014 Dec 4; Parktown, South Africa.

ABSTRACT

Chronic hepatitis C virus (HCV) infection is a major health concern and is strongly associated with cirrhosis, hepatocellular carcinoma and liver related mortality. The current standard treatment for HCV is a combination of interferon-based therapies and ribavirin, which only produces sustained viral suppression in 40-50% of patients. Thus, the development of new treatments for HCV infection is critical. The HCV genome is the template for both protein translation and viral replication and, being RNA, is amenable to direct genetic silencing by RNA interference (RNAi). HCV is a highly mutable virus with error prone RNA replication and it has been previously reported that the virus can escape RNAi-mediated treatments through various point mutations. This has highlighted the importance of developing RNAi-based therapy that simultaneously targets multiple regions of the HCV genome. Thus, five artificial primary miRNA (pri-miRNA) were designed to mimic the naturally occurring monomeric pri-miRNA-31. The natural guide sequence on the 5' arm of the pri-miRNA-31 was replaced with sequences complementary to different regions of the 5' UTR of HCV. Potent knockdown of an HCV reporter was seen with four of the five constructs, and these were used to generate polycistronic cassettes, which showed impressive silencing of an HCV target. To further their application as a gene therapy recombinant adeno-associated viral (rAAV) vectors that express the polycistronic pri-miRNA mimics were generated. Two different promoter sequences were used to direct the expression of the polycistronic constructs. Ubiquitously expressed CMV and liver-specific mTTR promoters were used to generate rAAVs. All of the vectors enter liver-derived cells efficiently and significantly knock down the expression of an HCV target and showed dramatic inhibition of HCV replicon replication. The expressed polycistronic pri-miRNA mimics did not induce any off-target effects, such as stimulation of the immune

response and saturation of the RNAi pathway. All the pri-miRNA mimics within the polycistronic cassettes were processed according to their intended design. The anti-HCV rAAVs developed have the potential to be an effective therapy that may contribute to the eradication of HCV.

ACKNOWLEDGEMENTS

1. I wish to express my appreciation to my supervisors Dr. Abdullah Ely and Prof. Patrick Arbuthnot for their tireless guidance and encouragement without which I could not have completed my dissertation.

2. I would also like to thank Prof. Volker Lohmann for providing the replicon cell culture HCV model.

3. I would like to thank Prof. Dirk Grimm for providing the AAV packaging plasmid pBS-RVS-GFP, AAV helper plasmid and AAV-2 Capsid plasmid.

4. I would also like to thank the various funding bodies for their contributions to financing my degree, the University of the Witwatersrand postgraduate merit award, the National Research Foundation and Poliomyelitis Research Foundation, the funding of my project.

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LIST OF ABBREVIATIONS

1. Ago	-	Argonaute
2. AmpR	-	Ampicillin Resistance gene
3. ASGP-R	-	Asialoglycoprotein Receptors
4. DC-SIGN	-	Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
5. DGCR8	-	DiGeorge critical region 8
6. DMEM	-	Dulbecco's Modified Eagle Medium
7. dsRNA	-	Double stranded RNA
8. ER	-	Endoplasmic reticulum
9. FCS	-	Foetal calf serum
10. fluc	-	Firefly luciferase
11. GFP	-	Green fluorescent protein
12. HBV	-	Hepatitis B virus
13. HCV	-	Hepatitis C virus
14. IFN-α	-	Interferon-α
15. IL-6	-	Interleukin-6
16. IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
17. LB	-	Luria Bertani
18. LDL	-	Low density lipoproteins
19. LDLR	-	Low density lipoprotein receptor
20. L-SIGN	-	Liver and lymph node-specific intercellular adhesion molecule-3- grabbing nonintegrin

21.MDA-5	-	Melanoma differentiation-associated protein-5
22. NPC1L1	-	Niemann-Pick C1–like 1 cholesterol absorption receptor
23. miRNA	-	microRNA
24. NS	-	Non structural
25. PCR	-	Polymerase chain reaction
26. PEG	-	Polyethylene glycol
27. Pol II	-	RNA polymerase II
28. pre-miRNA	-	Precursor miRNA
29. pri-miRNA	-	Primary miRNA
30. RC	-	RNA replication complex
31. RIG -I	-	Retinoic acid-inducible gene-I
32. RISC	-	RNA-induced silencing complex
33. Rluc	-	*Renilla* luciferase
34. RNAi	-	RNA interference
35. SCID	-	Severe combined immunodeficiency
36. siRNA	-	Small interfering RNA
37. SR-B1	-	Scavenger receptor class B type 1
38. TRBP	-	TAR RNA binding protein
39. TNF	-	Tumour necrosis factor
40. UTR	-	Untranslated region
41. VLDL	-	Very low density lipoproteins
42. X-gal	-	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

LIST OF SYMBOLS

1. α - Alpha
2. β - Beta
3. μ - Mu

CHAPTER 1

1. Introduction

1.1 The hepatitis C virus

1.1.1 Hepatitis C epidemiology.

Hepatitis C virus (HCV) is an RNA virus that infects the liver and is strongly associated with cirrhosis, hepatocellular carcinoma and liver-related mortality (Wiese et al., 2000). Worldwide roughly 130–170 million people are chronically infected with HCV and 350 000-500 000 people die from HCV-related liver disorders every year. The prevalence rates in Africa range from 2-3% with Egypt having the highest HCV prevalence with over 10% (Averhoff et al., 2012). As many as 3–4 million new infections occur annually with a total estimate of 185 million infected individuals (acute and chronic infections) globally (W.H.O., 2014). Chronic HCV infection is a burden to health care systems as it is the leading cause for liver transplants within developed countries. HCV is the most common blood-borne disease within the United States (Boyer et al., 2002, Shepard et al., 2005). A recent economic projection estimated that the United States alone would spend up to U$ 10.7 billion from 2010-2019 on medical expenses directly related to HCV infection. The leading cause of transmission of HCV is through nonsterile therapeutic injections resulting in 2 million new infections annually. HCV can also be transmitted through intravenous drug use and unhygienic medical, dental and cosmetic procedures (Madhava et al., 2002, Shepard et al., 2005, Averhoff et al., 2012). From this it is evident that HCV is a global plight with severe consequences. Effective treatments for HCV would alleviate this burden and save patients from life-threatening illness however, the current standard of care for HCV is unsatisfactory and newer direct acting antivirals do not prevent viral escape. There

is a need for a therapeutic that has the capacity to effectively silence HCV replication and eradicate the threat of treatment resistant mutants.

1.1.2 Hepatitis C viral genome and proteins.

HCV is a positive sense RNA virus that belongs to the Flaviviridae family (Choo et al., 1989, Choo et al., 1991). The positive sense strand contains 9 379 nucleotides and a single open reading frame (ORF) that is transcribed to produce a polypeptide consisting of 3 011 amino acids (Figure 1.1) (Choo et al., 1991). The translated polypeptide is cleaved into ten proteins, consisting of structural and non-structural proteins. The core protein (C) and envelope proteins one (E1) and two (E2) are structural proteins while NS2, NS3, NS4A, NS4B, NS5A and NS5B are non-structural proteins involved in viral replication. There is currently no consensus as to the classification of p7 (Grakoui et al., 1993, Dubuisson et al., 2002). The viral genome contains 5' and 3' untranslated regions (UTRs) on either side of the single ORF (Han et al., 1991, Takamizawa et al., 1991). The 5' UTR contains an internal ribosome entry site (IRES) that is important for viral translation and attachment to the 40S ribosomal subunit at the correct start site (Takamizawa et al., 1991, Friebe et al., 2001, Spahn et al., 2001). The 5' UTR is also responsible for viral replication which occurs in the cytoplasm (Friebe et al., 2001).

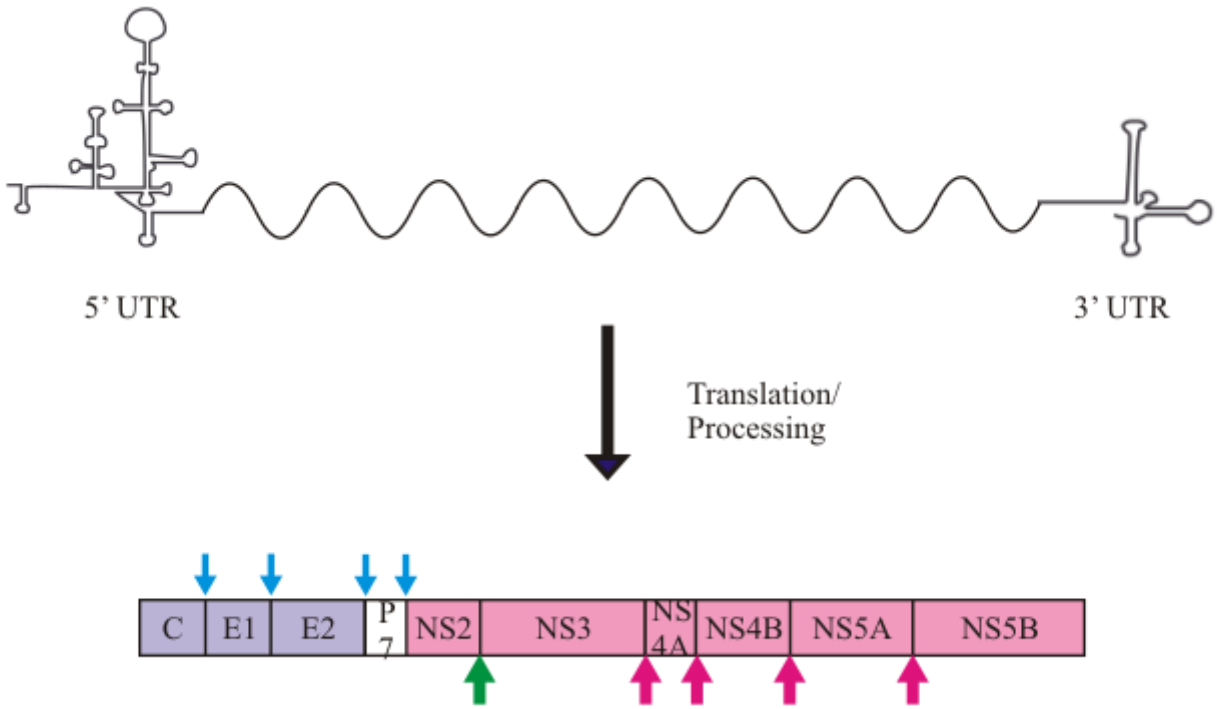

Figure 1.1: Genome organization of HCV.

5' and 3' UTRs are illustrated on either side of the viral RNA genome (top panel). The bottom panel illustrates the polyprotein. The structural proteins are illustrated in purple while the non-structural proteins are in pink. p7 does not have a classification as structural or non-structural and is indicated in white. The blue arrows illustrate cleavage sites that are mediated through host proteases. The green arrow illustrates the cleavage site of the NS2–NS3 protease while the pink arrows represent cleavage site for NS3–NS4A. Image adapted from: (Dubuisson et al., 2002).

1.1.3 Hepatitis C viral replication.

HCV infects the liver by binding receptors on the surface of hepatocytes via the envelope glycoproteins E1 and E2 (Figure 1.2). Numerous binding partners for HCV have been identified and include: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN), Liver and Lymph node-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (L-SIGN) (Gardner et al., 2003, Cormier et al., 2004a), the cholesterol absorption receptor Niemann-Pick C1-like 1 (NPC1L1) (Sainz et al., 2012), Occulin (Ploss et al., 2009), Low Density Lipoprotein Receptor (LDLR) (Agnello et al., 1999, Monazahian et al., 1999), CD81 (Pileri et al., 1998, Cormier et al., 2004b), Scavenger Receptor class B type 1 (SR-B1) (Scarselli et al., 2002), Claudin-1 (Evans et al., 2007), Asialoglycoprotein Receptors (ASGP-R) (Saunier et al., 2003) and glycosaminoglycan heparin sulphate (Barth et al., 2006). DC-SIGN and L-SIGN are capture receptors that bring the viral particles in close proximity to hepatocytes (Gardner et al., 2003, Lozach et al., 2003). As capture receptors these molecules are believed to play a role in viral tropism (Gardner et al., 2003, Lozach et al., 2003, Cormier et al., 2004a). However, neither DC-SIGN nor L-SIGN mediate viral fusion or entry. HCV particles have been shown to associate with LDL and very low density lipoproteins (VLDLs), which in turn interact with LDLR thereby facilitating viral entry. HCV can also interact directly with LDLR to initiate viral entry (Agnello et al., 1999, Monazahian et al., 1999, Germi et al., 2002). There is a second pathway utilised by HCV to enter the cell and that is through interaction with NPC1L1 or Occulin which results in viral entry upon binding to either of these receptors (Ploss et al., 2009, Sainz et al., 2012). Perhaps the most well characterised entry co-receptors of HCV are CD81 and SR-B1 both of which facilitate and are essential for viral entry into target cells (Pileri et al., 1998, Petracca et al., 2000, Scarselli et al., 2002, Bartosch et al., 2003, Cormier et al., 2004b, Catanese et al., 2007, Meuleman et al.,

2008, Meuleman et al., 2012). Once bound to receptors on the outer membrane of the cell, viral entry likely occurs by pH-dependent clathrin-mediated endocytosis (Figure 1.2) (Hsu et al., 2003). The viral particle enters the cells via endocytosis and the viral envelope then fuses with the endosome to release the nucleocapsid that contains the viral RNA. Following uncoating the viral RNA migrates to the endoplasmic reticulum (ER) where translation occurs (Figure 1.2) (Mottola et al., 2002, Serafino et al., 2003). The viral RNA does not possess a 5' cap, rather it has an internal ribosome entry site (IRES) which allows for recruitment of ribosomes and translation of the single polypeptide (Wang et al., 1993, Spahn et al., 2001). The polyprotein is proteolytically cleaved by host signal peptidases as well as viral proteases producing mature HCV proteins which associate with the ER (Hijikata et al., 1993, Suzuki et al., 2007). NS4B induces the formation of a membranous web and serves as a scaffold for the formation of a replication complex (RC) (Egger et al., 2002).Viral RNA replication occurs at the membranous webs (Egger et al., 2002, Mottola et al., 2002, Gosert et al., 2003, Aizaki et al., 2004, Gao et al., 2004b). The initial step of RNA replication begins with the generation of a negative strand RNA template and is followed by the production of a positive RNA strand (Suzuki et al., 2007). Newly synthesised positive strand RNAs may interact with core protein, initiating encapsidation and ultimately secretion of new viral particles (Tanaka et al., 2000, Boulant et al., 2008). HCV appropriates the cellular VLDL secretion system allowing for the assembly and secretion of newly formed virus (Figure 1.2) (Serafino et al., 2003, Huang et al., 2007a, Gastaminza et al., 2008). With the elucidation of the viral replication cycle novel targets for HCV therapy were discovered and subsequent treatments have been explored.

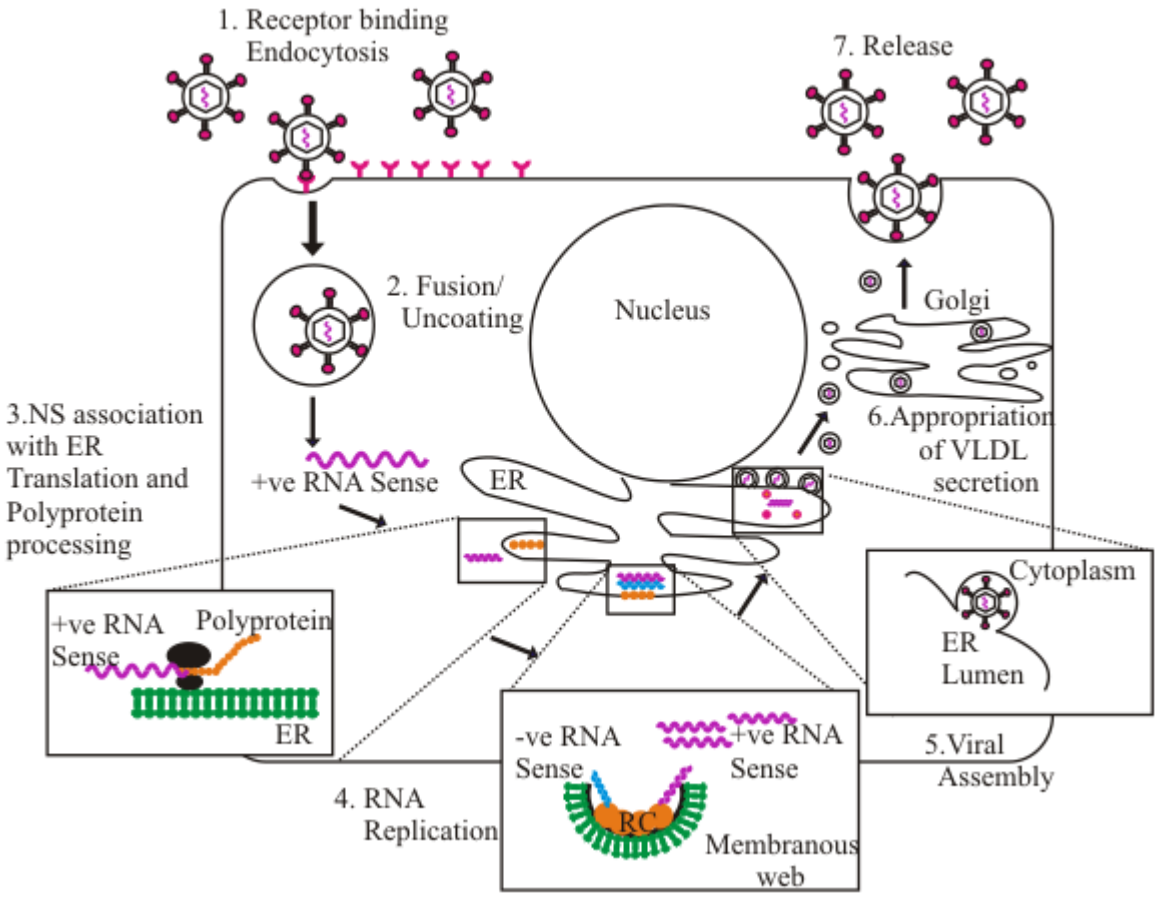

Figure 1.2: HCV replication cycle.

Once viral glycoproteins E1 or E2 (pink) bind their respective receptors on the host membrane the virus is endocytosed. Following fusion of the viral membrane with the endosomal membrane the genome containing capsid is released into the cytoplasm. The single stranded positive sense RNA genome is uncoated and migrates to the ER where translation and processing of the viral polyprotein occurs. The positive sense RNA serves as a template for the production of negative sense RNA, which in turn is used to produce more positive sense viral RNA genomes. Replication of the positive sense genomes occurs in the membranous webs. HCV co-opts the cellular VLDL secretion system allowing for the assembly and secretion of newly formed virus. Image adapted from (Scheel and Rice, 2013).

1.2 Hepatitis C treatments

The current standard treatment for chronic HCV infection is a combination of interferon-based therapies (pegylated interferon-α or simply interferon-α) and ribavirin. The main shortcoming of these treatments is that they only produce sustained viral suppression in 40-50% of patients that receive the treatment (Fried et al., 2002, Hadziyannis et al., 2004). Also, the current therapies result in various toxicities and negative side effects such as nausea, headache and depression (Fried et al., 2002, Hadziyannis et al., 2004). As interferon and ribavirin treatments are suboptimal there has been a shift in the treatment paradigm of HCV management to include at least one direct-acting antiviral or protease inhibitor in combination with interferon and ribavirin (WHO, 2014). The South African Gastroenterology Society guidelines recommend combination ribavirin and pegyated interferon for the treatment of HCV infection in South Africa (Botha, 2010). An ideal treatment for HCV would exclude the use of interferon, as interferon causes many of the unpleasant side effects associated with current therapy. An exemplary therapeutic would have high virological cure rates against all genotypes and be interferon free with minimal side effects. Many of the novel therapeutics in development have promising prospects but there are numerous challenges that still need to be met before this ideal is realised.

A number of new potential therapies for HCV have been evaluated ranging from gene therapeutics to protease inhibitors. The main treatments that have recently been approved for use include; Ezetimibe, Telaprevir, Boceprevir, Simeprevir, Sofosbuvir and Miravirsen (Tungol et al., 2011, Sainz et al., 2012, Jacobson et al., 2013, Janssen et al., 2013, Lawitz et al., 2013). Ezetimibe interacts with and blocks the NPC1L1 cholesterol uptake receptor, which is required for viral entry into the host cell (Sainz et al., 2012). Ezetimibe was only shown to delay HCV infection and was ineffective when cells were treated after infection

(Sainz et al., 2012). Further investigation is required before Ezetimibe can be implemented as a therapy (Sainz et al., 2012).

The protease inhibitors, Telaprevir and Boceprevir were approved by the FDA in May 2011 (Jacobson et al., 2011, Tungol et al., 2011). These therapies target and block HCV proteases namely the NS3 protein (Boceprevir) and the NS3/4A serine protease (Telaprevir) (Jacobson et al., 2011, Poordad et al., 2011). These protease inhibitors have an advantage over standard care medications as they reduce the duration of treatment and both treatments have been shown to produce higher rates of sustained virological responses when compared with standard therapies (Bacon et al., 2011, Jacobson et al., 2011, Poordad et al., 2011, Zeuzem et al., 2011). There are, however, some major drawbacks associated with these new therapies. Telaprevir and Boceprevir both have a range of adverse side effects that include anaemia, fatigue, headache, nausea, rash and gastrointestinal disorders (Jacobson et al., 2011, Poordad et al., 2011). The new treatments are also prohibitively expensive with Boceprevir priced at U$ 1100 per week and Telaprevir at U$ 4100 per week (Liu et al., 2012). The treatment regimens are also complex and difficult to implement (Poordad et al., 2011). Non-adherence can also result in treatment failure and development of viral resistance to both new drugs (Tungol et al., 2011). Furthermore, Telaprevir and Boceprevir were developed to only target HCV type I. Although the therapies have shown similar efficacy against HCV types 2a, 5a and 6a, there is complete resistance to these therapies by HCV type 3 (Gottwein et al., 2011, Jacobson et al., 2011, Poordad et al., 2011).

Simeprevir is yet another NS3/4A protease inhibitor, however it differs from Telaprevir and Boceprevir in that it is administered in a single daily dose (Lin et al., 2009, Fried et al.,

2013, Zeuzem et al., 2014). An added benefit of Simperevir, in comparison to Telaprevir and Boceprevir is simply that it has fewer adverse side effects. No rashes or anaemia were noted within the clinical trials and the fatigue experienced by patients was not as severe as those treated with Telaprevir and Boceprevir. (Fried et al., 2013, Bakulin et al., 2014, Scott et al., 2014, Zeuzem et al., 2014). Viral resistance developed in a large majority of patients in Phase II clinical trials and as a result viral breakthrough was seen. This often occurs when protease inhibitors are used in combination with interferon and ribavirin (Zeuzem et al., 2014) illustrating the problem with using a single target therapeutics.

Sofosbuvir is a nucleotide analogue that inhibits the activity of the HCV NS5B polymerase thereby inhibiting replication of the HCV genome. Sofosbuvir has been shown to have *in vitro* activity against all HCV genotypes (Jacobson et al., 2013, Lawitz et al., 2013). Sofosbuvir is an oral treatment for HCV and is often taken in combination with ribavarin and interferon to produce high levels of sustained virological response in patients. There are, however, differences in the efficiency of the sustained virological response in patients with HCV genotypes 2 and 3, with Sofosbuvir exhibiting a stronger inhibitory effect on HCV type 2 (Jacobson et al., 2013, Lawitz et al., 2013). Sofosbuvir received approval from the FDA on 22 November 2013 and has subsequently been approved for use in North America and Europe. There is a barrier to the accessibility of Sofosbuvir and this is its extensive cost of the medication, a single treatment (pill) costs roughly U$ 1000 and a 12 week course of treatment is required (WHO, 2014).

Miravirsen is a gene therapeutic that is slightly different to all the other treatments discussed so far in that it is designed to target an essential host molecule that HCV requires. Targeting a host factor is beneficial as it has a higher barrier to viral mutation.

HCV depends on specific host factors and this requires a greater amount of mutation to circumvent this dependence. Thus, targeting essential host factors limits the development of viral escape mutants. Miravirsen is a 15 nucleotide antisense locked nucleic acid (LNA). This LNA has a highly specific complementary sequence for the 5' region of primary microRNA-122 (pri-miRNA-122) (Janssen et al., 2013). Pri-miRNA-122 is expressed specifically within the liver and is essential for HCV replication (Lanford et al., 2010). Pri-miRNA-122 binds to the 5' UTR of HCV and protects the RNA genome from degradation by host innate immune response as well as from exonucleases. Pri-miRNA-122 also functions to stimulate internal ribosomal entry site mediated translation of the HCV genome (Jopling et al., 2005, Jangra et al., 2010, Machlin et al., 2011, Shimakami et al., 2012, Mortimer and Doudna, 2013). Administration of Miravirsen has produced dose-dependent sustained virological responses within patients with HCV genotype 1. Pri-miRNA-122 is not only involved in HCV stability but as it is a host factor it plays a role in the regulation of host genes. These genes are involved with lipid and cholesterol metabolism as well as with cancer suppression (Mortimer and Doudna, 2013). Consequently, there is the concern that suppression of pri-miRNA-122 may disrupt liver function and result in the development of hepatocellular carcinoma (HCC) (Janssen et al., 2013). The expression of pri-miRNA-122 is higher in HCV-related HCC while its expression is low in other HCC. This has led to the belief that the administration of Miravirsen is relatively safe where the induction of HCC is concerned. It is also important to note the effects of Miravirsen are reversible upon withdrawal of treatment which may negate adverse side effects. This is particularly relevant when considering restoring liver functions that are controlled by genes regulated by pri-miRNA-122. Nevertheless, no significant adverse effects were noted with administration of Miravirsen (Janssen et al., 2013).

The direct-acting antivirals currently in use target a single site to prevent HCV replication. As HCV is highly mutable, treatment with a single therapeutic is likely to result in the generation of viral escape mutants. Combination therapy may be an approach to resolving this problem but seems unlikely as the regimens are already complex and are likely to induce more off-target effects. As such there is a concern that current therapeutics may be an inadequate solution to HCV treatment, illustrating the need for efficient therapies with minimal toxicity that prevents the development of viral escape mutants. Manipulation of the RNA interference (RNAi) pathway presents an opportunity for the creation of novel therapies against HCV infection.

1.3 RNAi.

The term RNAi was coined by Fire and colleagues when they discovered that the addition of double stranded RNA (dsRNA) to *Caenorhabditis elegans* (adults) resulted in the inhibition of functional gene expression of homologous genes (Fire et al., 1998). Functional gene expression was not only repressed in the adults injected with dsRNA but also in the animals' progeny (Fire et al., 1998). Following this initial discovery the RNAi pathway was elucidated. MicroRNAs (miRNAs) are endogenous small non-coding RNAs responsible for directing the RNAi pathway for targeted gene silencing.

miRNA biogenesis begins with the transcription of primary microRNA or pri-miRNA (which contain hairpin-like structures and long flanking regions on either side) by RNA polymerase (Pol) II in the nucleus (Figure 1.3) (Lee et al., 2002, Lee et al., 2004, Arbuthnot and Thompson, 2008). The pri-miRNAs are then processed by Drosha and its co-factor DiGeorge critical region 8 (DGCR8) (Han et al., 1991, Lee et al., 2003, Han et al., 2006). Drosha processes pri-miRNAs to form a hairpin structures known as precursor

miRNA (pre-miRNA). The pre-miRNAs are released after Drosha processing and are approximately 70 nucleotides in length (Lee et al., 2002). The pre-miRNAs are exported from the nucleus via Exportin-5 and are then processed in the cytoplasm by an enzyme known as Dicer and its double stranded RNA binding partner, trans-activation response (TAR) RNA binding protein (TRBP) (Bernstein et al., 2001, Yi et al., 2003). Dicer produces dsRNA of 21-23 base pairs in length with a two nucleotide 3' overhang (miRNA duplex) (Bernstein et al., 2001, Rose et al., 2005). These dsRNAs are subsequently incorporated into an enzyme complex known as the RNA-induced silencing complex (RISC) (Hammond et al., 2000, Rose et al., 2005, Han et al., 2006). When these miRNA duplexes are loaded into RISC, the enzyme complex will then unwind the dsRNA into two single strands (Rose et al., 2005). A single strand will remain bound to RISC, the guide strand, while the anti-guide sequence is subsequently ejected from RISC and degraded (Rose et al., 2005). The guide strand will have sequence complementarity towards a specific mRNA thus facilitating the binding of RISC. RISC is responsible for subsequent gene silencing. When the guide sequence is completely complementary to target mRNA, gene silencing occurs through cleavage of the mRNA. Translational repression occurs when the guide strand has incomplete complementarity toward target mRNA and this form of gene silencing is typical for miRNA (Rose et al., 2005, Han et al., 2006). The manipulation of the RNAi pathway has become a common practice within the field of gene therapy, particularly in therapeutic applications where pathogenic genes may be silenced. RNAi activators or RNAi effectors are designed to be perfectly complementary to their targets, they enter the endogenous RNAi pathway and silence genes through targeted mRNA degradation (Figure 1.3).

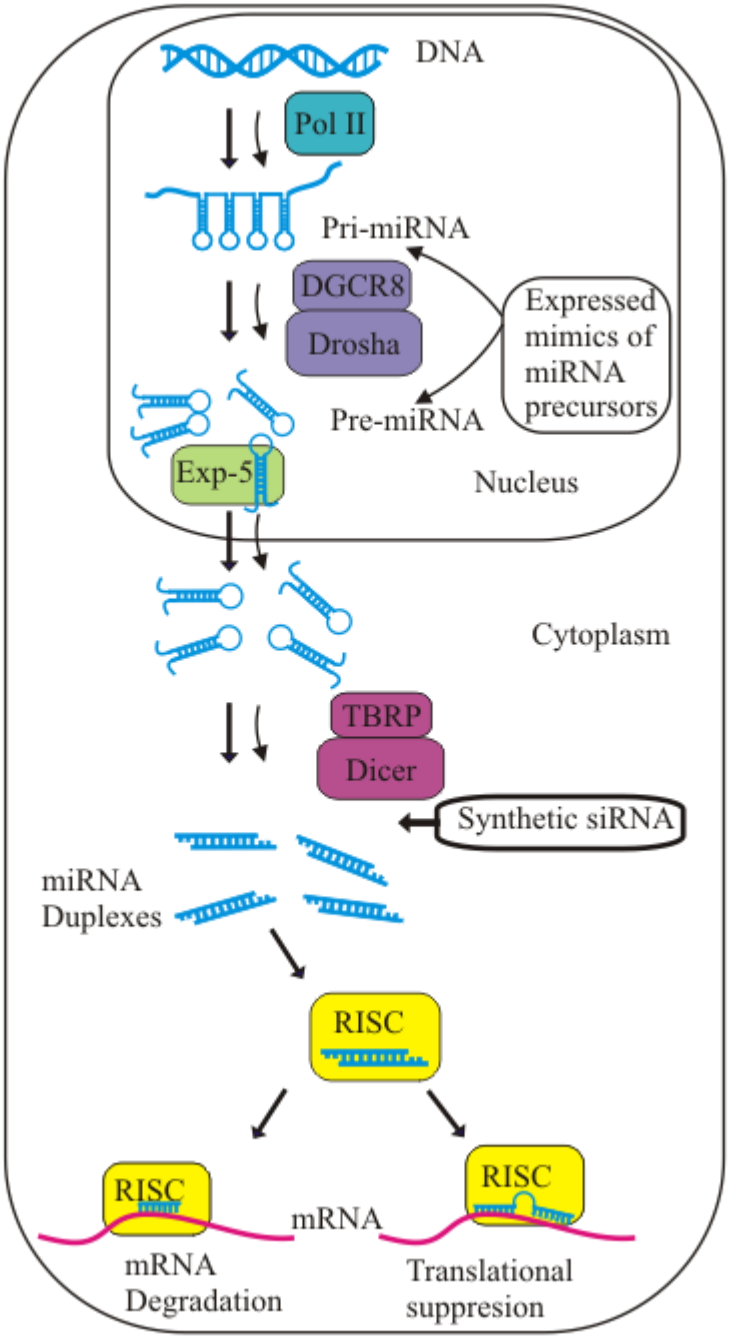

Figure 1.3: Schematic diagram illustrating the RNAi pathway.

The diagram illustrates the RNAi pathway showing essential steps and cellular compartmentalisation of the events that lead to the production of miRNA. Points at which RNAi can be induced in cells through the use of synthetic siRNA and expressed mimics are illustrated. Image adapted from: (Arbuthnot and Thompson, 2008).

1.3.1 RNAi activators.

RNAi activators are RNA sequences capable of entering the RNAi pathway and reprogramming it to effect gene silencing (Elbashir et al., 2001, Sui et al., 2002). RNAi activators are capable of entering and reprogramming the RNAi pathway because they resemble intermediates of the pathway (miRNA duplexes, pre-miRNAs or pri-miRNAs). There are two types of RNAi activators namely synthetic and expressed RNAi activators (Figure 1.4). Both synthetic and expressed RNAi activators are potent effectors of gene silencing (Elbashir et al., 2001, Brummelkamp et al., 2002). Synthetic RNAi activators as the name suggests, are chemically synthesized RNA sequences. The most common type of synthetic RNAi activator is small interfering RNAs (siRNAs) which consist of a duplexed RNA structure of 21 nucleotides and commonly resemble miRNA duplexes. Expressed RNAi activators are typically produced by transcription of a DNA vector. Numerous types of expressed RNAi activator exist and include short hairpin RNAs (shRNAs), long hairpin RNAs (lhRNAs) and miRNA mimics (also known as artificial miRNA). shRNAs comprise a 21-25 bp stem connected by a loop, which allows the shRNA to be expressed as a single sequence and mimic the hairpin-like structure of endogenous pre-miRNAs. lhRNAs have a similar structure to shRNAs but have an extended stem (60-300 bp) from which multiple guide sequences can be produced (Diallo et al., 2003, Weinberg et al., 2007, Sano et al., 2008). Although effective, shRNAs and lhRNAs have shortcomings which have been overcome through the use of expressed miRNA mimics. Using an endogenous miRNA as a base, miRNA mimics have the guide and anti-guide sequences replaced with a guide and anti-guide against a target gene. miRNA mimics based on both pre-miRNAs and pri-miRNAs (pre-miRNA and pri-miRNA mimics, respectively) have been generated and shown to be effective gene silencers (Zeng and Cullen, 2005, Grimm et al., 2006, Ely et al., 2008, McBride et al., 2008, Liu et al., 2010). Since miRNA mimics resemble

intermediates of the RNAi pathway more closely it is thought that they elicit a more natural silencing (Liu et al., 2008, Ely et al., 2009). There are various advantages and disadvantages associated with using either synthetic or expressed RNAi activators. Advantages associated with exploiting synthetic RNA sequences include: tight control of dosage, they only need to be delivered to the cytoplasm to be active, they are amenable to direct chemical modification which can improve efficacy and stability and reduce toxicity (Elbashir et al., 2001, Uprichard, 2005, Aagaard and Rossi, 2007). The disadvantages associated with synthetic RNAi activators are incompatibility with viral vectors and the expense of chemical synthesis (Elbashir et al., 2001, Uprichard, 2005, Aagaard and Rossi, 2007). Additionally, synthetic RNA activators result in transient silencing which restricts their application as a therapy for chronic disease as re-administration of the therapeutic would be necessary (Uprichard, 2005, Aagaard and Rossi, 2007). These problems can be avoided through the use of expressed RNAi systems. Expressed activators have several advantages over synthetic RNA. Viral vectors can be used to deliver expressed RNAi activators to cells that are not readily transfectable via synthetic RNA. There are lower costs associated with the production of expressed systems and the duration of silencing is longer than that noted for synthetic RNAi activators. Expressed activators also result in the continuous production of RNA sequences as long as the expression system remains active within the cell (Brummelkamp et al., 2002, Zeng et al., 2002). As with any potential therapy, off-target effects are a concern when expressed or synthetic sequences are used. Some off-target effects include silencing of unintended targets that have partial complementarity to the guide sequences or stimulation of the interferon response (Chung et al., 2006, Ely et al., 2009). Expressed and synthetic activators are capable of circumventing certain off-target effects through efficient design (Krol et al., 2004, Reynolds et al., 2004). Pol II promoters can be used to express RNAi activators that can be regulated and these

promoters can be changed to allow for tight control over the expression of the RNAi effectors (Zeng et al., 2002, Ely et al., 2009). An example of this would be the use of tissue specific promoters that reduce off-target effects by limiting where the RNAi activators are expressed (Ely et al., 2009).

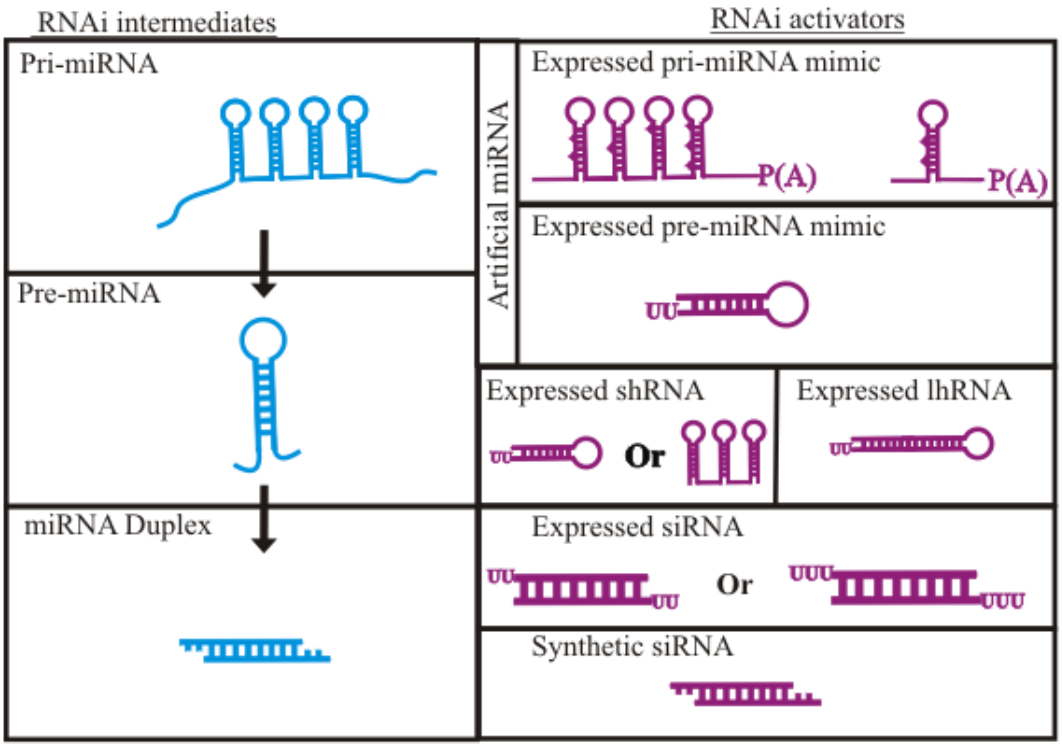

Figure 1.4: Different RNAi activators.

Various RNAi activators are illustrated in this diagram. Endogenous RNAi intermediates are indicated in blue and are on the left while various RNAi activators both synthetic and expressed are illustrated on the right in purple. Image adapted from: (Weinberg, 2014).

1.3.2 RNAi and HCV

RNAi has been evaluated as a potential therapeutic against numerous viruses such as HIV-1, HBV, rift valley fever, dengue virus and poliovirus (Kapadia et al., 2003, Randall et al., 2003, Seo et al., 2003, Ely et al., 2009, Scott et al., 2012). HCV is an RNA virus that

replicates in the cytoplasm and thus should be amenable to direct silencing by RNAi (Kapadia et al., 2003). As the HCV genome is a single stranded RNA and is crucial for both protein translation and viral replication it is a critical target for RNAi. The siRNA-mediated silencing of HCV has been investigated by a number of groups and various regions of the HCV genome such as the regions encoding core protein, E2 envelope protein, NS5B, NS3 and the 5' UTR have been targeted (Kapadia et al., 2003, Randall et al., 2003, Wilson et al., 2003, Liu et al., 2006, Kanda et al., 2007, Kim et al., 2009, Chandra et al., 2012). The 5' UTR is a popular target for siRNA-mediated gene therapy against HCV as it is a highly conserved region within the viral genome. siRNAs have been used in combination with other therapies such as interferon-based approaches. Pooling siRNAs that target different regions of HCV has also been investigated as potential therapy (Pan et al., 2009, Chandra et al., 2012). shRNAs have been frequently studied as a potential treatment for HCV with the 5' UTR also being a popular target (Kronke et al., 2004, Sakamoto et al., 2008, Pan et al., 2009, Suhy et al., 2012). Combinations of various shRNAs that target different regions on the HCV genome have been explored (Kronke et al., 2004, Kanda et al., 2007, Sakamoto et al., 2008, Suhy et al., 2012). An alternate strategy that has proved effective against HCV replication is the use of pri-miRNA mimics targeted toward HCV. Pri-miRNA mimics were used to prevent the saturation of RNAi pathway and have the added benefit of being engineered to have a single polycistronic cassette that can express multiple effector miRNAs (Yang et al., 2010). HCV is known to be a highly mutable virus with error prone RNA replication and it has been previously reported that the virus can escape RNAi-mediated silencing through various point mutations (Ogata et al., 1991, Konishi et al., 2006). A number of strategies have been developed to overcome viral escape from RNAi-mediated silencing. These include use of polycistronic pri-miRNA mimics that target different regions of the HCV genome to

strategies that involve simultaneously targeting HCV and human factors required by the virus for replication (Jopling et al., 2005, Lanford et al., 2010, Yang et al., 2010). Targeting the highly conserved 5' UTR reduces the probability that viral escape mutants will develop (Yokota et al., 2003, Wang et al., 2005, Wilson and Richardson, 2005, Watanabe et al., 2006). Targeting multiple sites on the HCV genome can potentially prevent viral escape mutants (Wilson and Richardson, 2005, Konishi et al., 2006, Ely et al., 2008, Yang et al., 2010). This approach has been shown to be effective to prevent viral escape mutants (Liu et al., 2008, ter Brake et al., 2008, Liu et al., 2009, Yang et al., 2010). A multi-targeted approach to silencing HCV with a polycistronic pri-miRNA mimic therefore offers a potentially useful addition to the arsenal of anti-HCV therapy. For this to be implemented in a clinical setting effective delivery of the anti-HCV therapeutic needs to be realised.

1.4 Vectors for the delivery of therapeutic constructs.

There are two distinct mechanisms of nucleic acid delivery; namely viral and non-viral delivery systems. Non-viral methods of delivery involve the delivery of naked DNA to target cells or DNA bound to various complexes such as polymers, liposomes and peptides. There are a variety of non-viral delivery systems such as cationic liposomes, microspheres, polymer-based matrices, biodegradable polymeric-based particles, multilayered polyelectrolyte films (MPFs) and virus-like particles. Non-viral vectors are less efficient in terms of delivery when compared to viral vectors. However, they are also less immunogenic than their viral vector counterparts and as such can be used for repeated administration of a particular therapy (Mathiowitz et al., 1997, Leong et al., 1998, Shea et al., 1999, Cohen et al., 2000, Yamada et al., 2003, Watanabe et al., 2007, Dimitrova et al., 2008, Kim et al., 2009, Wei et al., 2009, Chandra et al., 2012). Chandra *et al.* produced nanosomes that efficiently delivered siRNA to hepatocytes for the treatment of HCV. The

nanosomes repeatedly delivered effective therapeutic siRNA *in vitro* and *in vivo* (Chandra et al., 2012).

Despite recent advances in non-viral delivery, viral vectors remain more efficient at delivery and as a result they are the vectors of choice in gene therapy. Many viral vectors are used for the delivery of gene therapies. Some examples include retroviral, lentiviral, adenoviral, adeno-associated and herpes simplex viral vectors. Each viral vector has unique properties such as tropism and packaging capacity that make them useful for delivery of variety of different therapeutics (Thomas et al., 2003). There are numerous types of viral vectors used for the delivery of anti-HCV gene therapeutics such as adenoviral, adeno-associated, retroviral and lentiviral vectors. The lentiviral vector is based on human immunodeficiency virus. The major advantage of the lentiviral vector is that it allows for long-term stable expression of a transgene. Lentiviral vectors containing multiple shRNAs have been used to treat HCV infected cells with success (Henry et al., 2006). Retroviral vectors have also been used to deliver therapeutic genes to hepatocytes (Sakamoto et al., 2008). Adenoviral vectors are one of the most commonly utilised viral vectors in gene therapy and are particularly suited to HCV treatment as a consequence of its natural hepatotropism (Sakamoto et al., 2008, Waddington et al., 2008). Importantly this vector was shown to be successful in delivery of anti-HCV gene therapy which produced responses both *in vitro* and *in vivo* (Sakamoto et al., 2008, Witting et al., 2008). The disadvantage associated with this vector is that it causes inflammation and stimulates the immune system resulting in the production of neutralising antibodies. This prevents repeated administration of gene therapy. The highly immunogenic nature of the adenovirus may exclude individuals from treatment if they have had previous exposure to the virus. (Schulick et al., 1997, Witting et al., 2008). The generation of gutless adenoviral vectors

that are devoid of viral genes has reduced induction of the immune response. Adeno-associated viral vectors have become quite popular, as these vectors can produce long-term stable expression of transgenes, they are not pathogenic, it is possible to produce pure replication deficient virus and they can transfect dividing and non-diving cells (Nakai et al., 2005, Joo et al., 2010). Recently, an adeno-associated viral vector was used to co-deliver three shRNAs targeting various conserved regions of HCV into the infected livers of non-human primates (Suhy et al., 2012).

1.4.1 Adeno-associated viral vectors.

Adeno-associated viruses (AAVs) are small (25 nm), non-enveloped, single stranded DNA viruses. AAVs require the presence of a helper virus, such as adenovirus, to complete their viral replication cycle (Atchison et al., 1965, Matsushita et al., 1998, McCarty et al., 2001). The single stranded genome of AAVs contain two ORFs, for Rep and Cap proteins, flanked by inverted terminal repeats (ITRs), which are the only sequences that are required for replication of the viral genome. AAVs are non-pathogenic viruses that mainly infect primates. As AAVs are non-pathogenic they are considered safe to use as a delivery vehicles for anti-HCV therapeutics. There are over a hundred serotypes and many are used as viral vectors such as AAV2 and AAV8 (Gao et al., 2002, Xiao et al., 1999, Gao et al., 2004a). Recombinant AAVs (rAAVs) are commonly used as gene therapy vectors. The ITRs of the AAV are the only sequences necessary for copying the viral genome and as a result the *rep* and *cap* genes are dispensable for the rAAV. The Rep proteins of AAVs are responsible for integration of viral DNA into the host genome and since rAAVs lack the *rep* gene these vectors rarely undergo integration. In the absence of integration, rAAV genomes circularise and exist as episomes (DNA that does not form part of the chromosomes). These episomes persist for long periods and allow for extended transgene

expression (months to years) (Duan et al., 1998, Nakai et al., 2001, Nathwani et al., 2002, Yang et al., 2010). This is convenient as HCV is a chronic infection and prolonged expression of the therapeutic would be useful. The rate limiting step for rAAV transduction and subsequent transgene expression: is the conversion of its single stranded genome into a double stranded sequence through the synthesis of a complementary strand. Self-complementary AAVs (scAAVs) overcome this limit and exhibit increased transduction (McCarty et al., 2001, Wang et al., 2003, McCarty et al., 2003, Wu et al., 2007). The scAAV genome is replicated as a single strand, however, as the name suggests it is capable of folding back on itself to produce a double stranded sequence (Figure 1.5). scAAVs avoid the need for second strand synthesis as the double stranded genome does not require the generation of a complementary strand. As a result scAAVs allow for more efficient transduction both *in vitro* and *in vivo*. This is advantageous as it allows for quick and efficient delivery and expression of anti-HCV therapeutics within hepatocytes. The disadvantage associated with this type of vector is simply that there is a reduction in the packaging capacity of the vector from ~ 4.7 kb to roughly 2.4 kb (McCarty et al., 2001, McCarty et al., 2003, Wang et al., 2003, Wu et al., 2007). Although the scAAV vector does have a reduced packaging capacity this is sufficient for packaging the expression cassettes that encode small therapeutic molecules such as anti-HCV pri-miRNA mimics. Improved efficiency of transgene expression from rAAVs can also be achieved through the use of tissue specific promoters (Ziegler et al., 2004).

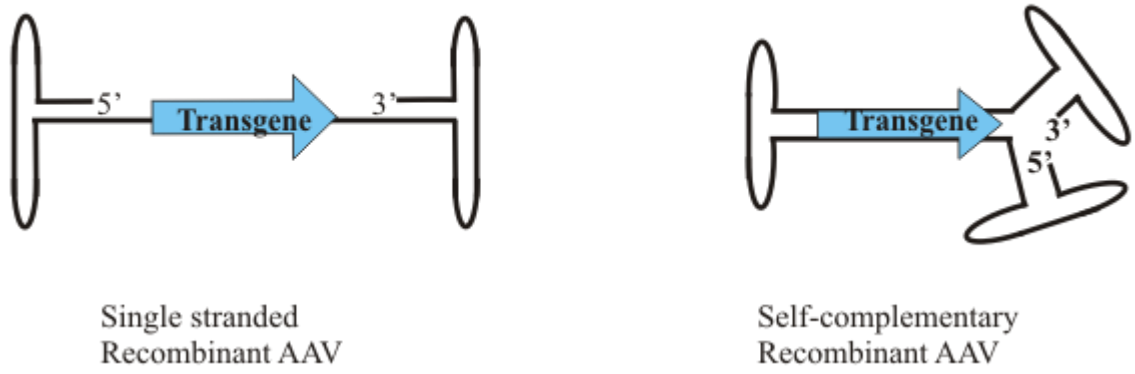

Figure 1.5: Diagram of single stranded and self-complementary AAVs.

Schematic diagram illustrating the structure of the scAAV as compared to the single stranded rAAV. Single stranded rAAVs possess a particular transgene of interest between two open ITR. This is not the case with the scAAV where the transgene is double stranded and found between a single covalently closed ITR at one end and two open ITRs at the other (McCarty, Monahan et al. 2001).

AAV vectors elicit a slight immune response through the activation of the complement system however: the response is less immunogenic than other viral vectors such as adenoviruses. This is advantageous as it prevents side effects often experienced with activation of an immune response and ensures that anti-HCV therapeutics are not cleared before they reach the liver (Rautsi et al., 2007, Zaiss et al., 2008). Serotype 2 is the most well-characterised AAV and has been extensively used as a recombinant vector. Pre-existing immunity against AAV2 can elicit an immune response to rAAV2 thereby reducing the efficiency of this vector as a gene therapy delivery vehicle. AAV2 is also inherently less effective as a vector for the delivery of transgenes. These shortcomings can be overcome through pseudotyping which entails using the capsids from one AAV

serotype in combination with the genome from a separate AAV serotype (typically AAV2). AAVs that do not commonly infect humans such as AAV8 are used for their capsid proteins which are unlikely to be recognised by the immune system (Hildinger et al., 2001, Gao et al., 2002, Grimm et al., 2003, Gao et al., 2004a, Peden et al., 2004, Gao et al., 2006, Zaiss et al., 2008). The AAV8 capsid is ideally suited to deliver anti-HCV therapeutics as its transduction efficiency of the liver is high (Gao et al., 2002, Gao et al., 2004a, Nakai et al., 2005, Gao et al., 2006). Pseudotyping is also convenient for re-administration of a rAAVs as it can be used to prevent immune recognition of an already administered viral vector. This is beneficial when considering HCV infections are chronic and are likely to need more than one treatment. rAAV vectors have been utilised successfully in numerous large animal models and clinical trials for diseases such as haemophilia, which is promising for their application to HCV (Monahan et al., 1998, Nathwani et al., 2002, Manno et al., 2006). Importantly, rAAV vectors have successfully been used to deliver shRNA and pri-miRNA mimic expression cassettes that target HCV (Yang et al., 2010, Lavender et al., 2012). rAAV vectors are therefore ideally suited for the delivery of the pri-miRNA polycistronic cassettes targeted towards the 5' UTR of HCV.

1.5 Aims

The purpose of the investigation was to develop an anti-HCV gene therapy that utilised rAAVs to deliver therapeutic constructs to HCV infected hapatocytes. The study aimed to exploit the characteristics of a naturally occurring pri-miRNA to create a multi-targeting system capable of inhibiting HCV replication. The endogenous polycistronic clusters of pri-miRNA were mimicked as multiple pri-miRNAs are expressed and can target numerous sites within the HCV genome simultaneously. Specifically, the production of pri-miRNA mimics that were targeted to the 5' UTR of HCV were assessed in human hepatoma cells. The 5' UTR was targeted as it is a highly conserved region within the

HCV genome and would result in a therapeutic agent that has broad coverage of all HCV genotypes with a high barrier to HCV resistance. rAAVs are showing promise as a gene therapeutic delivery vector as they are non-pathogenic and importantly produce long term stable expression of transgenes. Additionally, pure replication deficient virus can be produced preventing off-target effects. rAAVs also transduce dividing and non-dividing cells allowing for efficient delivery of their therapeutic payloads. Thus, rAAVs were selected for the delivery of the therapeutic sequences. It was the objective of this study to develop rAAVs that could effectively deliver an anti-HCV therapeutic that had broad inhibition of HCV replication and had the potential to prevent the emergence viral escape mutants. Ultimately the aim of the investigation was to develop a novel gene therapeutic and provide proof of its utility against HCV. Thus, illustrating that it is a good candidate for further investigation both *in vivo* and in a clinical setting.

CHAPTER 2

2 Materials and Methods.

2.1 Generation of pri-miRNA expression plasmids

2.1.1. Design of pri-miRNA sequences targeting 5' UTR of HCV

The genomes of HCV 1b (Genbank: AJ238799.1) and HCV 2a (Genbank: AB047639.1) were aligned and conserved regions in the 5' UTR of the virus were chosen as target sites. These genotypes were chosen as they are the most common HCV genotypes and have global distribution (W.H.O., 2014). A collection of five anti-HCV pri-miRNA mimics were designed. The pri-miRNA mimics were based on the naturally occurring pri-miRNA-31. The naturally occurring guide sequence on the 5' arm of the hsa-miRNA-31 (*Homo sapiens* miRNA-31) was replaced with sequences complementary to regions of the 5'UTR of the HCV. The anti-HCV pri-miRNA mimics contain a hairpin structure of 102 nucleotides flanked by a region of 51 nucleotides (Figure 2.1). This was to ensure correct processing by Drosha. The guide sequence of each pri-miRNA mimic was designed to target a unique sequence in the highly conserved region of the HCV 5' UTR (Figure 2.1). Nucleotide mismatches (GU, CA, UU, and CU) were introduced into the stem structure of the pri-miRNA mimic to maintain secondary structure of the pri-miRNA. The mismatches were placed in the anti-guide in order to maintain complete complementarity between the guide and its target site in the 5' UTR of HCV.

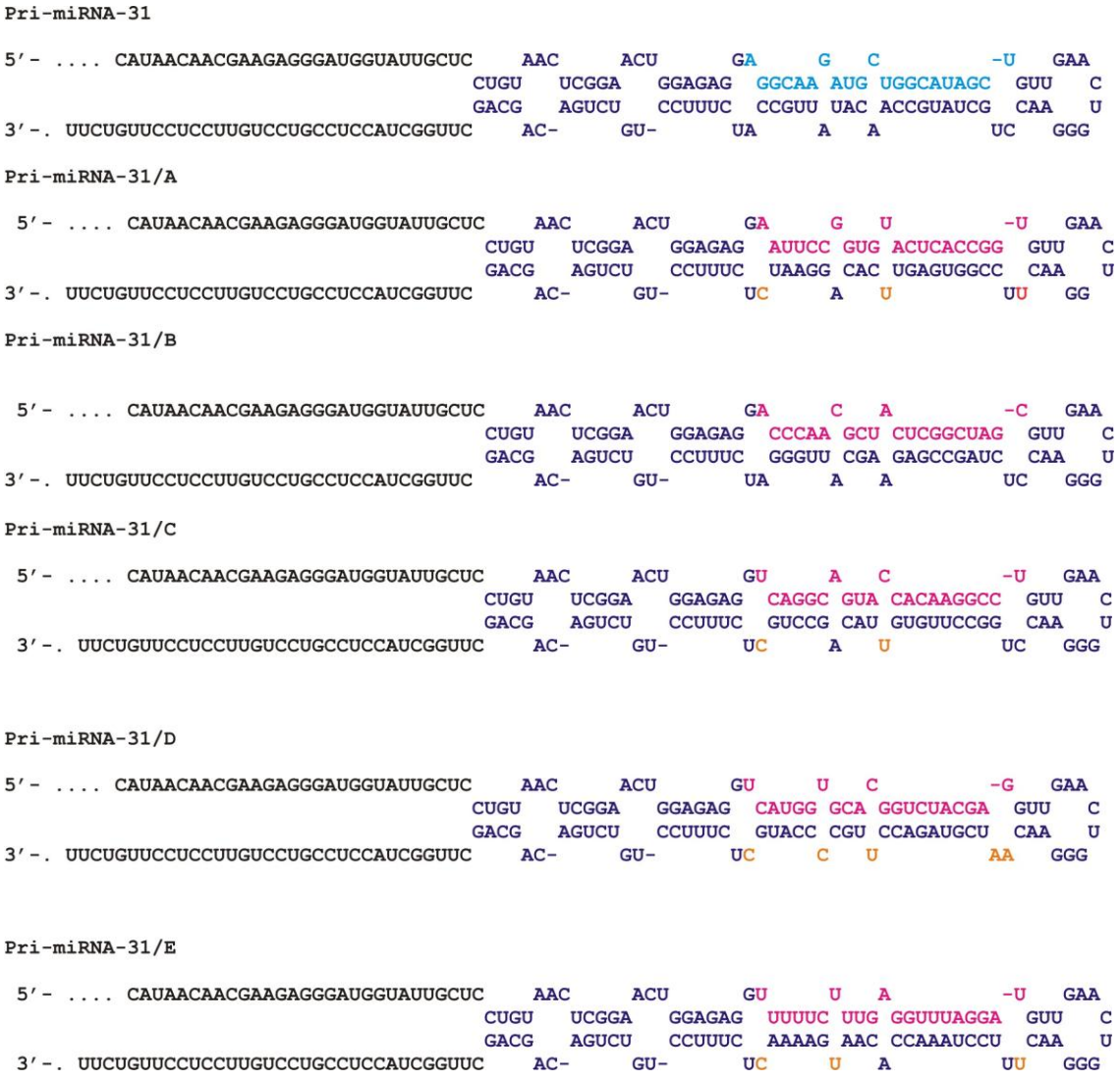

Figure 2.1: Design of pri-miRNA mimics targeting 5' UTR of HCV.

The naturally occurring guide sequence on the 5' arm of the hsa-miRNA-31 (indicated by light blue colour) was replaced with an anti-HCV sequence (pink sequences). The secondary structure of the pri-miRNA was maintained through the use of nucleotide mismatches (GU, CA, UU, and CU, indicated in orange). The pre-miRNA sequences (dark blue) are flanked by 51 nucleotide sequence (wild type sequence).

2.1.2.1 Construction of pri-miRNA expression vectors targeted to the 5' UTR of HCV.

Anti-HCV pri-miRNA mimics were constructed through a two-step PCR. The first PCR was a primer extension and required annealing of partially complementary forward (F) and reverse (R) oligonucleotide primers. The oligonucleotides were synthesised through the use of phosphoramidite chemistry (IDT, IA, USA). The sequences generated from the first round of PCR code for the pre-miRNA mimics. The oligonucleotide sequences are indicated in Table 2.1. PCR products were subjected to 1% agarose gel electrophoresis and subsequently extracted and purified using the MinElute® Gel extraction kit (Qiagen, CA, USA) (Appendix 5.2.1.1). The second PCR step was used to introduce the 51 nucleotide flanking regions containing *Nhe* I (5' end) and *Bcu* I (3' end) restriction sites. Universal forward and reverse primers (see Table 2.1) that were complementary to the 5' and 3' ends of all the pre-miRNA sequences were used to incorporate flanking regions onto pre-miRNA template sequences. This resulted in the production of a dsDNA sequence encoding the entire pri-miRNA mimic. The first round of PCR was carried out using 100 pmol of each primer 1.25 U DreamTaq™ DNA, 2.5ul of 10× DreamTaq™ and each deoxynueclotide at a final concentration of 0.2 mM in a total reaction volume 25 µl. In the second round of PCR, 0.09-0.2 µg of template was used with 100 pmol of forward and reverse universal primers. The rest of the reaction conditions remained the same.

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Table 2.1: Oligonucleotide sequences used for the creation of primiRNA expression constructs.

Pri-miRNA/A F:	5′-GTAACTCGGAACTGGAGAGGAATTCCGGTGTACTCAC**CGGTGTTGAACTGGGAACTTCC**-3′
Pri-miRNA/A R:	5′**-CTGCTGTCAGACAGGAAAGAGA**TTCCTGTGAACTCACCGGAAGTTCCCAGTTCAACACCG-3′
Pri-miRNA/B F:	5′-GTAACTCGGAACTGGAGAGGACCCAACGCTACTCGGC**TAGCGTTGAACTGGGAACCTCT**-3′
Pri-miRNA/B R:	5′-**CTGCTGTCAGACAGGAAAGATC**CCAATGCTTCTCGGCTAGAGGTTCCCAGTTCAACGCTA-3′
Pri-miRNA/C F:	5′-GTAACTCGGAACTGGAGAGGTCAGGCAGTACCACAAG**GCCTGTTGAACTGGGAACCTGG**-3′
Pri-miRNA/C R:	5′**-CTGCTGTCAGACAGGAAAGAGC**AGGCTGTAACACAAGGCCAGGTTCCCAGTTCAACAGGC-3′
Pri-miRNA/D F:	5′-GTAACTCGGAACTGGAGAGGTCATGGTGCACGGTCTA**CGAGGTTGAACTGGGAACAATC**-3′
Pri-miRNA/D R:	5′- **CTGCTGTCAGACAGGAAAGAGC**ATGGGGCAAGGTCTACGATTGTTCCCAGTTCAACCTCG-3′
Pri-miRNA/E F:	5′-GTAACTCGGAACTGGAGAGGTTTTTCTTTGAGGTTTA**GGATGTTGAACTGGGAACTTTC**-3′
Pri-miRNA/E R:	5′**-CTGCTGTCAGACAGGAAAGAGT**TTTCATTGTGGTTTAGGAAAGTTCCCAGTTCAACATCC-3′
Universal primers	
Forward:	5′-GCTAGCCATAACAACGAAGAGGGATGGTATTGCTCCTGTAACTCGGAACTGGAGAGG-3′
Reverse:	5′-ACTAGTAAGACAAGGAGGAACAGGACGGAGGTAGCCAAGCTGCTGTCAGACAGGAAAGA-3′

The complementary sequences are indicated in bold. The HCV target sites are underlined.

The amplicons were then subjected to electrophoresis on a 1.5 % agarose gel followed by gel extraction and phenol:chloroform purification (Appendix 5.2.1.2) (Figure 5.1). Purified fragments were then ligated into the pTZ57R/T vector according to the specifications of the manufacturer (InsTAclone™ PCR Cloning Kit, Thermo Fischer Scientific, MA, USA). The ligation reaction was then used to transform chemically competent *Escherichia coli (E. coli)*. The transformed *E. coli* were plated onto Luria Bertani (LB) agar plates containing ampicillin, 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal), and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Appendix 5.2.3). The plates were incubated overnight at 37°C. Colonies containing the plasmids with an insert (white colonies) were selected (Appendix 5.2.5). The colonies were grown overnight in LB media at 37°C. Plasmid was prepared from the bacterial cultures using the High Pure plasmid isolation kit (Roche Applied Science, Germany) (Appendix 5.2.6.1). Plasmids were screened using *Pvu* II as well as *Nhe* I and *Bcu* I digest (Figure 5.2). Orientation of the insert was confirmed with a *Pvu* II and *Nhe* I screen. Positive clones were sequenced (inqaba biotec™, South Africa) to ensure the correct sequence was maintained (Figure 5.3). Successful cloning yielded pTZ-pri-miRNA-B, pTZ-pri-miRNA-C, pTZ-pri-miRNA-D and pTZ-pri-miRNA-E.

2.1.2.2 Construction of polycistronic pri-miRNA mimics.

To generate polycistronic cassettes 2-mers first had to be generated. The conjugation of two pri-miRNA mimic sequences within a single vector constitutes a 2-mer, thus pri-miRNA-B together with the pri-miRNA-C generates the 2-mer pTZ-pri-miRNA-BC. To generate pTZ-pri-miRNA-BC, pTZ-pri-miRNA-B was digested with *Sca* I and *Bcu* I while pTZ-pri-miRNA-C was digested with *Sca* I and *Xba* I. The digested plasmids were subjected to 1% agarose gel electrophoresis. Each digest creates two fragments. The smaller fragment (1 256 bp) generated from the pTZ-pri-miRNA-B digest contains the pri-miRNA-B monomer while the larger fragment (1 980 bp) generated by the pTZ-pri-

miRNA-C digest contains the pri-miRNA-C monomer (Figure 2.2). The small fragment (pri-miRNA-B) and the large fragment (pri-miRNA-C) were subjected to gel extraction and purification using the MinElute® Gel Extraction kit (Qiagen, CA, USA) (Appendix 5.2.1.1). Fragments from pTZ-pri-miRNA-B were then ligated to fragments from pTZ-pri-miRNA-C. The ligation was transformed into *E. coli*, colonies were selected and plasmids preparations were conducted. *Pvu* II was used to identify positive clones while *Bcu* I and *Xba* I were used to identify clones that retained these restriction sites (Figure 5.4). To create pTZ-pri-miRNA-CE; pTZ-pri-miRNA-DE and pTZ-pri-miRNA-BD 2-mers a similar procedure was followed.

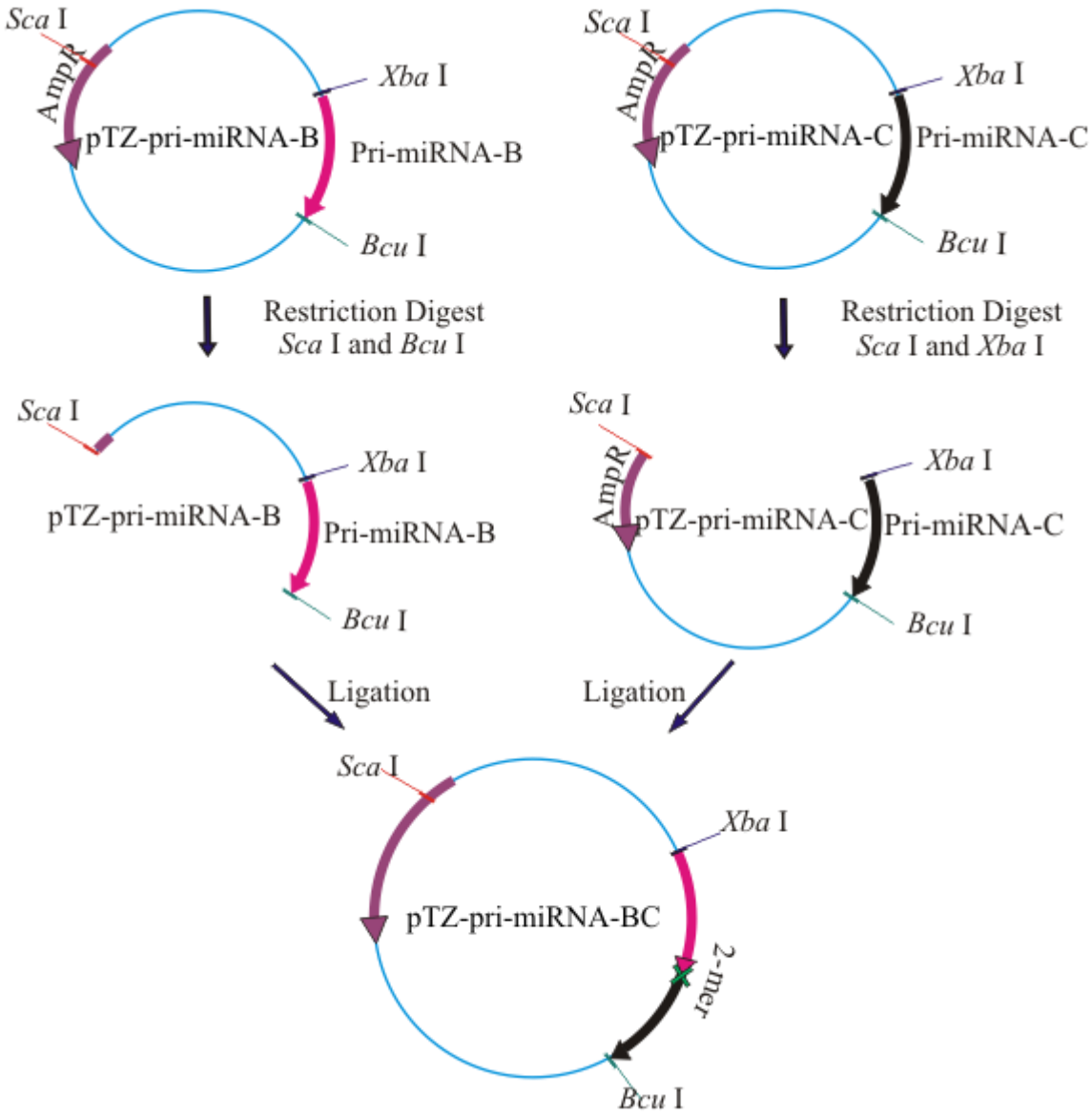

Figure 2.2: Cloning strategy for the generation of 2-mers.

pTZ-pri-miRNA-B was digested using *Sca* I and *Bcu* I while pTZ-pri-miRNA-C was digested using *Sca* I and *Xba* I. Each digest creates two fragments. The smaller fragment generated from the pTZ-pri-miRNA-B digest contains the pri-miRNA-B monomer while the larger fragment generated by the pTZ-pri-miRNA-C digest contains the pri-miRNA-C monomer. The sites generated but *Xba* I and *Bcu* I are complementary and allows for ligation and generation of the 2-mer. Ligation of *Nhe* I/*Xba* I overhang to *Bcu* I overhang abolishes both sites (as indicated by the green cross on pTZ-pri-miRNA-BC).

The 2-mer constructs were subsequently used to generate 4-mers. pTZ-pri-miRNA-BC was digested with *Pst* I and *Bcu* I while *Pst* I and *Xba* I were used to digest pTZ-pri-miRNA-DE. Digests were then subjected to 1% agarose gel electrophoresis and extraction. The large fragment would have a size of 3 185 bp and the smaller fragments would be 363 bp (Figure 2.3). The two fragments were subsequently ligated together. Plasmids were screened using restriction digest analysis using *Pvu* II as well as *Bcu* I and *Xba* I (Figure 5.5). A similar procedure was followed for the generation of pTZ-pri-miRNA-BDCE from pTZ-pri-miRNA-BD and pTZ-pri-miRNA-CE.

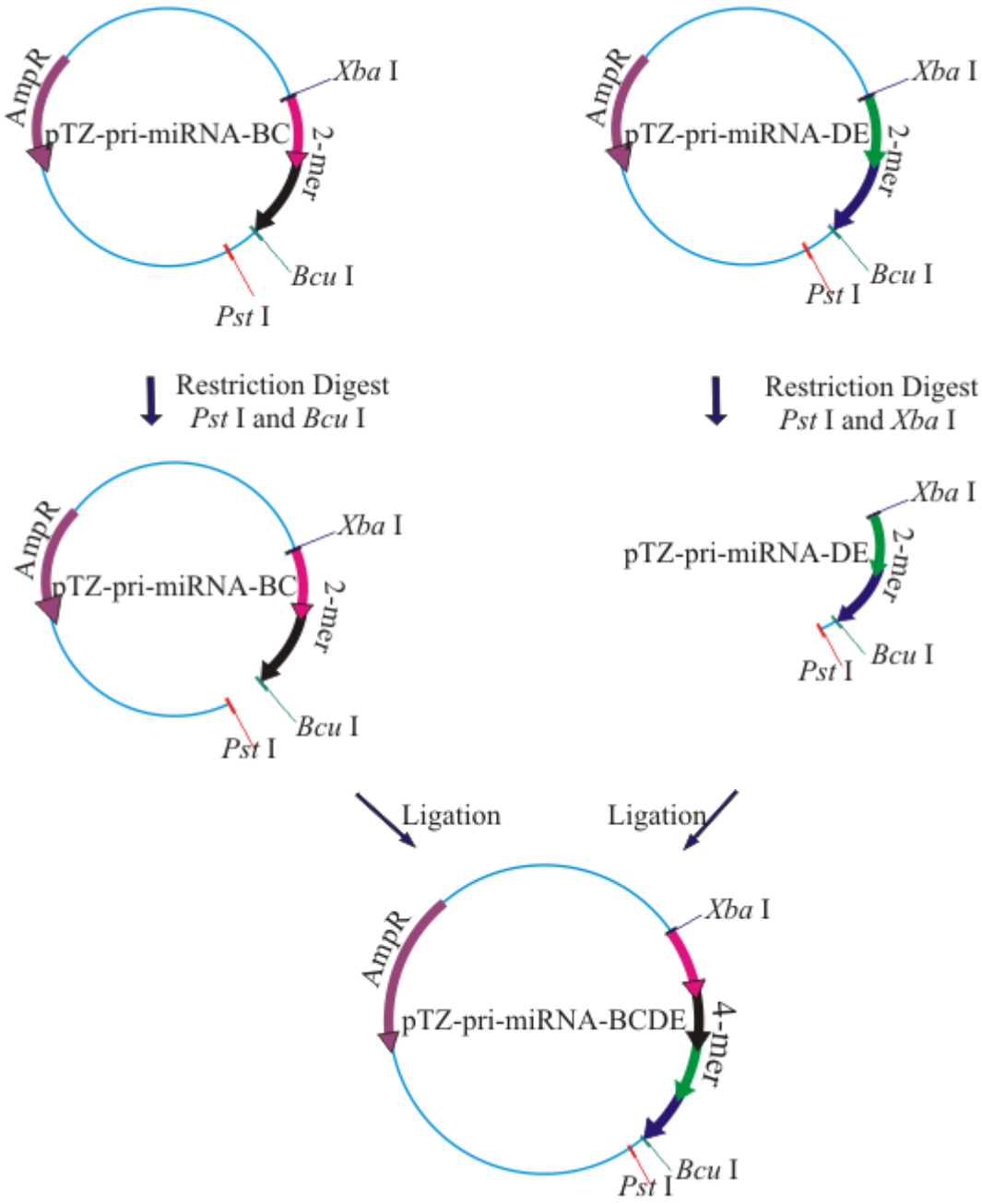

Figure 2.3: Cloning strategy for the generation of 4-mers.

pTZ-pri-miRNA-BC is digested using *Pst* I and *Bcu* I while pTZ-pri-miRNA-DE was digested using *Pst* I and *Xba* I. The smaller fragment generated from the pTZ-pri-miRNA-BC digest contains the pri-miRNA-BC 2-mer while the larger fragment generated by the pTZ-pri-miRNA-DE digest contains the pri-miRNA-DE 2-mer. Ligation of the two fragments allows for the generation of the 4-mer.

2.1.2.3 Construction of CMV-driven polycistronic pri-miRNA expression cassettes.

The polycistronic cassettes were subsequently cloned into a mammalian expression vector, pCI-neo (Promega, WI, USA). pTZ-pri-miRNA-BCDE, pTZ-pri-miRNA-BDCE and pCI-neo were digested using *Xba* I and *Sma* I and subsequently subjected to agarose gel electrophoresis. The 681 bp fragments from the pTZ-pri-miRNA-BCDE, pTZ-pri-miRNA-BDCE digests and the 5 459 bp fragment from the pCI-neo digest were extracted. Fragments from pTZ-pri-miRNA-BCDE and pTZ-pri-miRNA-BDCE were each ligated into the pCI-neo backbone separately. *Hind* III as well as *Xba* I and *Sma* I digestions were used to screen for positive clones. The resultant plasmids were named pCI-CMV-BCDE and pCI-CMV-BDCE.

2.1.2.4 Construction of mTTR-driven polycistronic pri-miRNA expression cassettes.

The polycistronic cassettes were then cloned into a vector containing a liver-specific promoter. pCI-CMV-BCDE, pCI-CMV-BDCE and pCI-mTTR were digested with *Sca* I and subsequently subjected to agarose gel electrophoresis. The 3 688 bp fragment from the pCI-CMV-BCDE and pCI-CMV-BDCE and the 2 376 bp fragment from the pCI-mTTR were gel extracted. The small fragment was ligated into both the pCI-CMV-BCDE and pCI-CMV-BDCE. *Hind* III and *Pst* I single restriction digestions were used to identify positive clones. The resultant plasmids were named pCI-mTTR-BCDE and pCI-mTTR-BDCE.

2.2 Generation of rAAV packaging plasmids.

A piecemeal cloning strategy was employed to generate a packaging plasmid that contained CMV- and mTTR-driven pri-miRNA cassettes. pBS-RSV-GFP, a packaging plasmid containing the inverted terminal repeat sequences of AAV-2 was the vector used (Grimm et al., 2006). The pBS-RSV-GFP plasmid expresses green fluorescent protein and

is driven by a Rous sarcoma virus (RSV) promoter. The promoter, pri-miRNA and polyadenylation sequences of the CMV- and mTTR-driven expression cassettes were each separately subcloned into pBS-RSV-GFP.

2.2.1 Generation of CMV, mTTR and polyadenylation sequences.

To facilitate sub-cloning of the CMV and mTTR promoters and the polyadenylation sequence into pBS-RSV-GFP, PCR was used to introduce convenient restriction sites upstream and downstream of these sequences. The primers were synthesised through the use of phosphoramidite chemistry (IDT, IA, USA) (Table 2.2). *Bgl* II and *Asc* I sites were included in the forward primers for CMV and mTTR promoter while *Xho* I and *Asc* I sites were included in the reverse primers. *Xho* I and *Not* I were incorporated into the forward primer while an *Asc* I site was integrated into the reverse primer for the polyadenylation sequence. For each PCR the conditions were as follows: initial denaturation at 94°C for three minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for one minute, followed by a final extension at 72°C for ten minutes. The reaction was set up using High Fidelity PCR Enzyme Mix ™ (Thermo Fischer Scientific, MA, USA). PCR was carried out using 100 pmol of each primer, 200 ng of plasmid DNA, 2.5 U of High Fidelity PCR Enzyme Mix™, 5 ul of 10× High Fidelity PCR Buffer™ and each deoxynucleotide at a final concentration of 0.2 mM in a total reaction volume 50 µl. PCR products were subjected to 1% agarose gel electrophoresis and subsequently extracted and purified using the MinElute® Gel extraction kit (Qiagen, CA, USA) (Appendix 5.2.1.1). Purified fragments were ligated into the pTZ57R/T vector according to the specifications of the InsTAclone™ PCR Cloning Kit (Thermo Fischer Scientific, MA, USA). The ligation was performed at 22°C overnight using 381 ng of purified mTTR, CMV and polyadenylation sequences, 5 U T4 DNA Ligase , 6 µl of 5×

Ligation buffer and 165 ng of the pTZ57R/T vector (Thermo Fischer Scientific, MA, USA). Ten microlitres of the ligation reaction was then used to transform 100 µl chemically competent *E. coli*. The transformed *E. coli* were plated on LB agar plates containing ampicillin, X-gal and IPTG (Appendix 5.2.3). The plates were incubated overnight at 37°C. Colonies able to grow on plates which contain plasmids with insert (white colonies) were selected (Appendix 5.2.5). Ten millilitres of LB media was inoculated with a single colony and cultured overnight at 37°C. Plasmids were prepared from bacterial cultures using the alkaline lysis method (Appendix 5.2.6.2). Purified plasmids were screened using restriction digest analysis using *Pvu* II and *Sal* I (Figure 5.6). Subsequent restriction digests used to screen isolated clones utilised double digestions that included *Bgl* II and *Xho* I digests for pTZ-CMV and pTZ-mTTR plasmids, while *Xho* I and *Hind* III were used to screen the pTZ-PolyA plasmids. All digests were subjected to 1% agarose gel electrophoresis. Positive clones were sequenced (inqaba biotec™, South Africa Biotechnology, South Africa) to ensure the correct sequence was maintained.

Table 2.2: Oligonucleotide sequences used for the generation of promoter and polyadenlyation sequences.

pCI-neo-mTTR F:	5′- GATCGGCTCGAC**AGATCT**GTCGAC -3′
pCI-neo-mTTR R:	5′- GATC*GGCGCGCCC*CTCGAGGCTAGCCTATAGTGA -3′
pCI-neo-CMV F:	5′- GATC**AGATCT**GTCGACTCAATATTGGCCATTAGCCATATT -3′.
pCI-neo-CMV R:	5′- GATC*GGCGCGCCC*CTCGAGGCTAGCCTATAGTGA -3′
pCI-neo-polyA F:	5′- GATCCTCGAGGTGTCGACCCGG**GCGGCCGC** -3′
pCI-neo-polyA R:	5′- GATC*GGCGCGCCC*GCTATTACGCCAGCCCGGAT -3′.

Bgl II is indicated in bold, *Sal* I is double-underlined, *Asc* I is italicised, *Xho* I is underlined and *Not* I is underlined and in bold.

2.2.2 Subcloning promoter sequences into rAAV packaging plasmids.

pTZ-CMV, pTZ-mTTR and the vector pBS-RSV-GFP were then digested using *Sal* I and *Asc* I (Figure 2.4). For this digest 4 000 ng of pTZ-CMV, pTZ-mTTR and pBS-RSV-GFP were each individually digested with *Asc* I and *Sal* I. The promoter sequences (1 500 bp fragment for CMV and mTTR) as well as linearised pBS-RSV-GFP (3 200 bp fragment) were extracted using EconoSpin™ pre-packed silica Membrane spin columns (Epoch Life Sciences, TX, USA). Extractions were performed according to manufacturer's specifications (Appendix 5.2.1.3) followed by precipitation using 3 M sodium acetate (pH 7) and 100% ethanol and washed using 70% ethanol. The promoter sequences and pBS backbone DNA were ligated in a 20 µl reaction volume containing 5 U T4 DNA Ligase. The ligation was performed at 4°C overnight using 102 ng of purified promoter encoding DNA, 2 µl of 10× Ligation buffer and 100 ng of the pBS vector (Thermo Fischer Scientific, MA, USA). Ten microlitres of each ligation was used to transform 100 µl of chemically competent *E. coli*. The *E. coli* were plated onto LB agar plates containing ampicillin and incubated overnight at 37°C. Colonies were selected and used to inoculate ten millilitres of LB media, inocula were incubated overnight and plasmids were subsequently prepared from cultures using the alkaline lysis method (Appendix 5.2.6.2). *Sac* I was used to screen for the presence of the insert within the vector (Figure 5.7). *Pvu* II restriction analysis was used as confirmation for the presence of the insert. Subsequent restriction analysis using *Spe* I digestion for pBS-CMV plasmids and *Tat* I for pBS-mTTR plasmids was performed. All restriction digests were then subjected to agarose electrophoresis within a 1% gel.

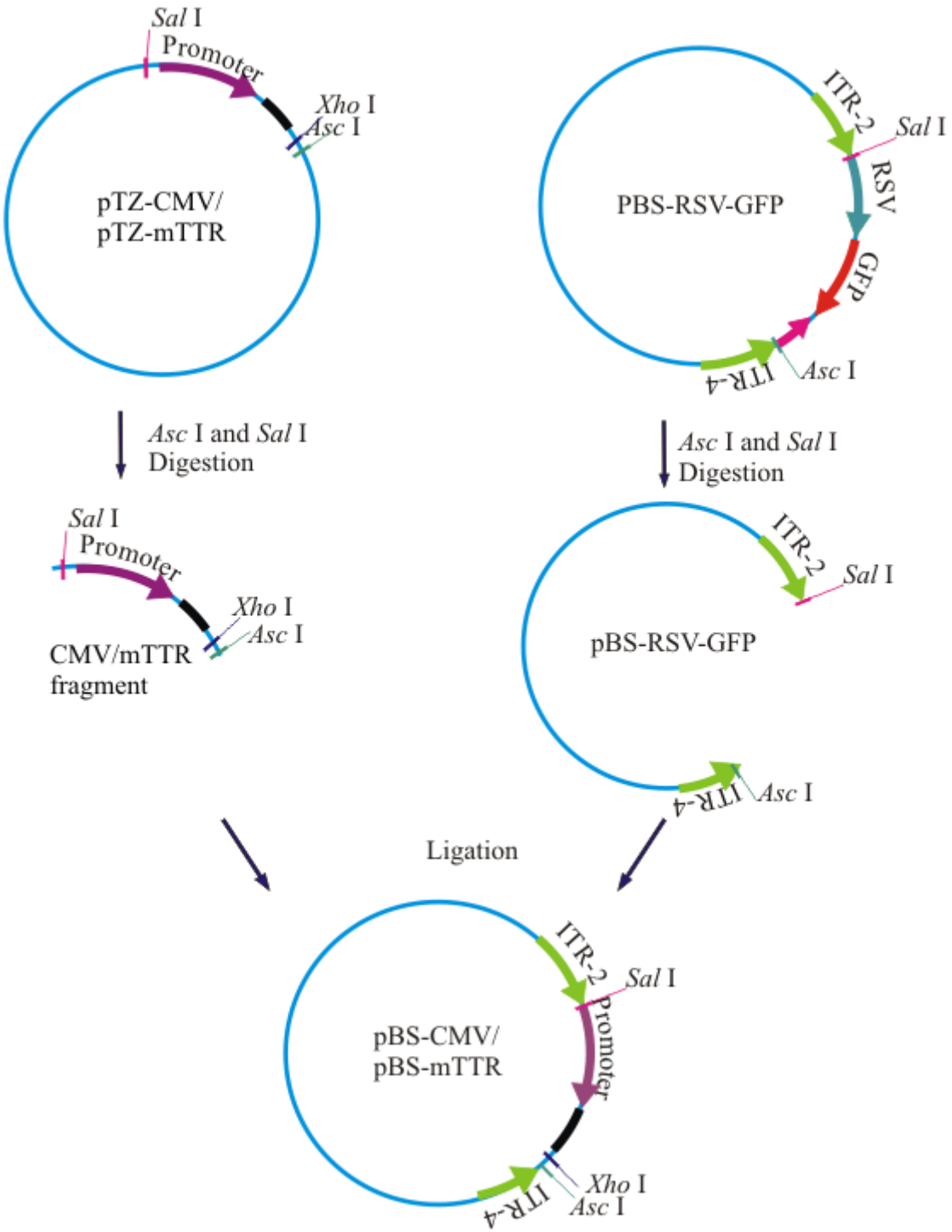

Figure 2.4: Cloning strategy to insert promoter sequences into AAV packaging plasmids.

pTZ-CMV, pTZ-mTTR and pBS-RSV-GFP were digested using *Sal* I and *Asc* I. The CMV and mTTR fragments were ligated separately into the AAV packaging plasmid to produce pBS-CMV and pBS-mTTR.

2.2.3 Subcloning the polyadenylation signal into rAAV packaging plasmids.

pTZ-pA, pBS-CMV and pBS-mTTR vectors were digested with *Xho* I and *Asc* I (Figure 2.5). For this digestion 3 410 ng of the pTZ-pA and 3 000 ng of pBS-mTTR and pBS-CMV were each individually digested using, 4 U of *Asc* I and 5 U of *Xho* I, 2 µl of 10× Red Buffer and 15 µl of distilled water was used in a total reaction volume of 20 µl. Restriction digests were subsequently incubated at 37°C for 1 hour. The pBS-CMV and pBS-mTTR restriction digests was subsequently treated with 5 U of Fast Alkaline phosphatase for 30 minutes at 37°C. Digested fragments were then subjected to 1% agarose gel electrophoresis. The polyadenylation sequence (222 bp fragment) and the pBS-CMV and pBS-mTTR fragments (4 300 bp) were extracted using EconoSpin™ pre-packed silica Membrane spin columns (Epoch Life Sciences, TX, USA) (Appendix 5.2.1.3). The polyadenylation sequence was ligated into each of the pBS-CMV and pBS-mTTR sequences. The ligation reaction included, 5 U T4 DNA ligase, 110 ng of each of the pBS-CMV and pBS-mTTR DNA, 75 ng of the polyadenylation sequence, 2 µl of 10× T4 ligation buffer and 15 µl of distilled water. The ligation was incubated at 22°C overnight. One hundred microlitres of chemically competent *E. coli* were transformed with 10 µl of each ligation. The transformed *E. coli* were plated and allowed to grow on LB agar plates containing ampicillin overnight at 37°C. Colonies growing on the plate were used to inoculate ten millilitres of LB media. The cultures were allowed to grow at 37°C overnight. The plasmids were then prepared through the use of the alkaline lysis method (Appendix 5.2.6.2). Isolated and purified plasmids were then screened with *Sal* I as well as *Dra* I and *Pst* I restriction digest. All restriction digests were then subjected to a 1% agarose gel electrophoresis and correct clones identified (Figure 5.8).

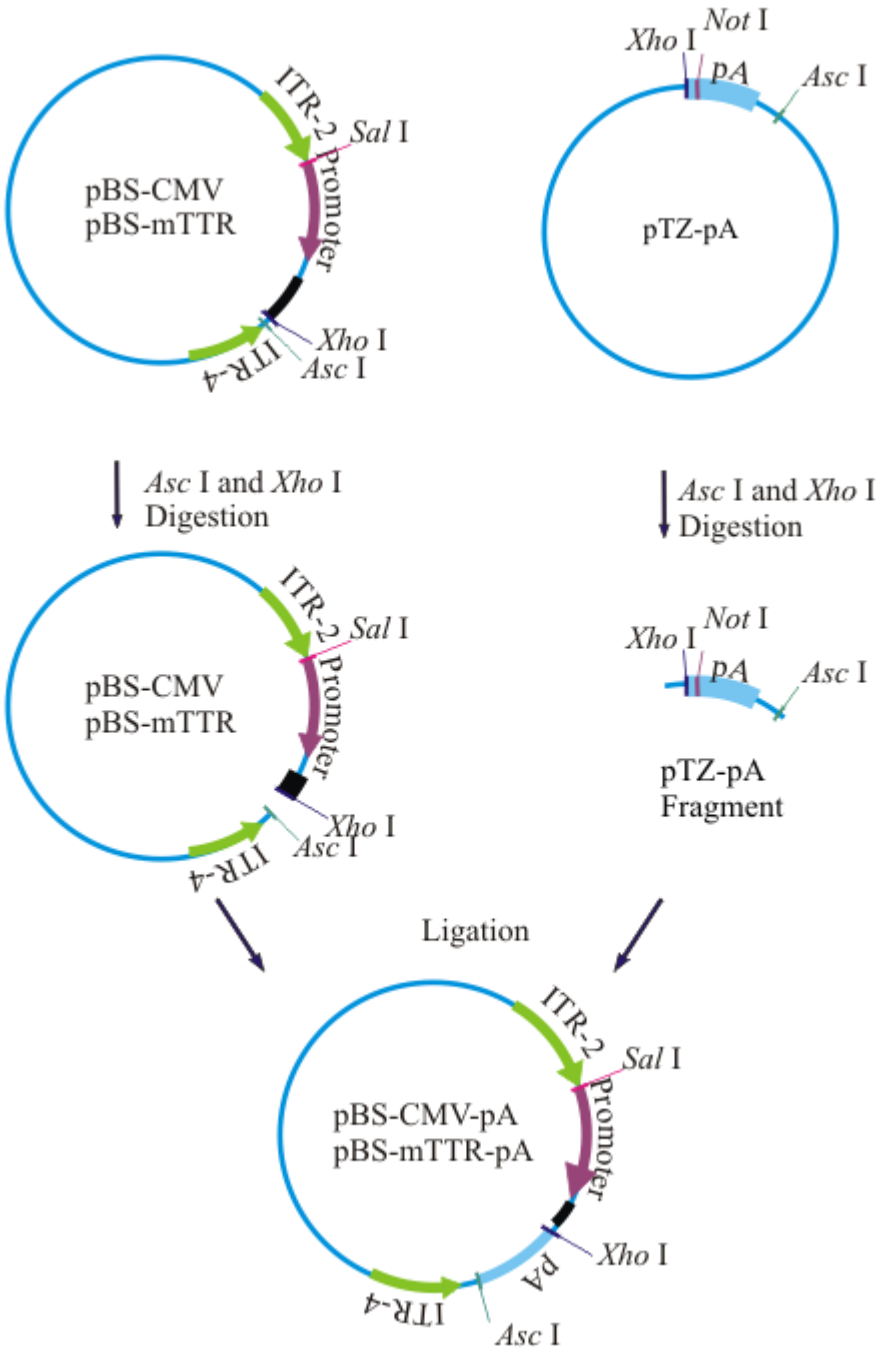

Figure 2.5: Subcloning the polyadenylation signal into the promoter containing AAV packaging plasmids.

pTZ-pA, pBS-CMV and pBS-mTTR were all separately digested using *Asc* I and *Xho* I. The polyadenylation sequence was ligated separately into pBS-CMV and pBS-mTTR to yield pBS-CMV-pA and pBS-mTTR-pA.

2.2.4 Subcloning pri-miRNA encoding sequences.

During the final round of cloning plasmids pBS-CMV-pA and pBS-mTTR-pA as well as pCI-pri-miRNA-BCDE and pCI-pri-miRNA-BDCE were subjected to a double restriction digestion by *Xho* I and *Not* I (Figure 2.6). Each plasmid was digested separately as follows; five units of each restriction enzyme *Xho* I and *Not* I, 2 000 ng of the plasmid, 2 µl of 10× Orange buffer and 14 µl of distilled water were used in each digestion. Restriction digests were subjected to 1% agarose gel electrophoresis and appropriate bands (4 500 bp backbone fragments and 670 bp for pri-miRNA fragments) were then excised. EconoSpin™ pre-packed silica Membrane spin columns were used to extract DNA from the gel (Epoch Life Science, Inc., TX, USA) (Appendix 5.2.1.3). Polycistronic sequences were ligated into the pBS-mTTR-pA and pBS-CMV-pA fragments. The ligation reactions had a total volume of 20 µl and consisted of the following, 200 ng of pBS-CMV-pA or 150 ng of pBS-mTTR-pA backbone, 70 ng polycistronic cassette BCDE or 60 ng polycistrinic cassette BDCE, 2 µl of 10× T4 ligation buffer, 15 µl of distilled water and 5 U of T4 ligase. Ligation reactions were incubated at 22°C for an hour. Chemically competent *E. coli* were transformed and plated onto LB agar plates containing ampicillin. The *E. coli* were incubated overnight at 37°C. Colonies were picked and used to inoculate 10 ml of LB media and incubated overnight at 37°C. The cultures were subjected to alkaline lysis method (Appendix 5.2.6.2) to extract plasmids. Screening of the purified plasmids was performed via restriction digest analysis using *Bgl* II, *Sal* I and double digestion with *Xho* I and *Not* I. All restriction digests were subjected to 1% agarose gel electrophoresis (Figure 5.9). The resultant plasmids were named pBS-CMV-BCDE-pA, pBS-CMV-BDCE-pA, pBS-mTTR-BCDE-pA and pBS-mTTR-BDCE-pA.

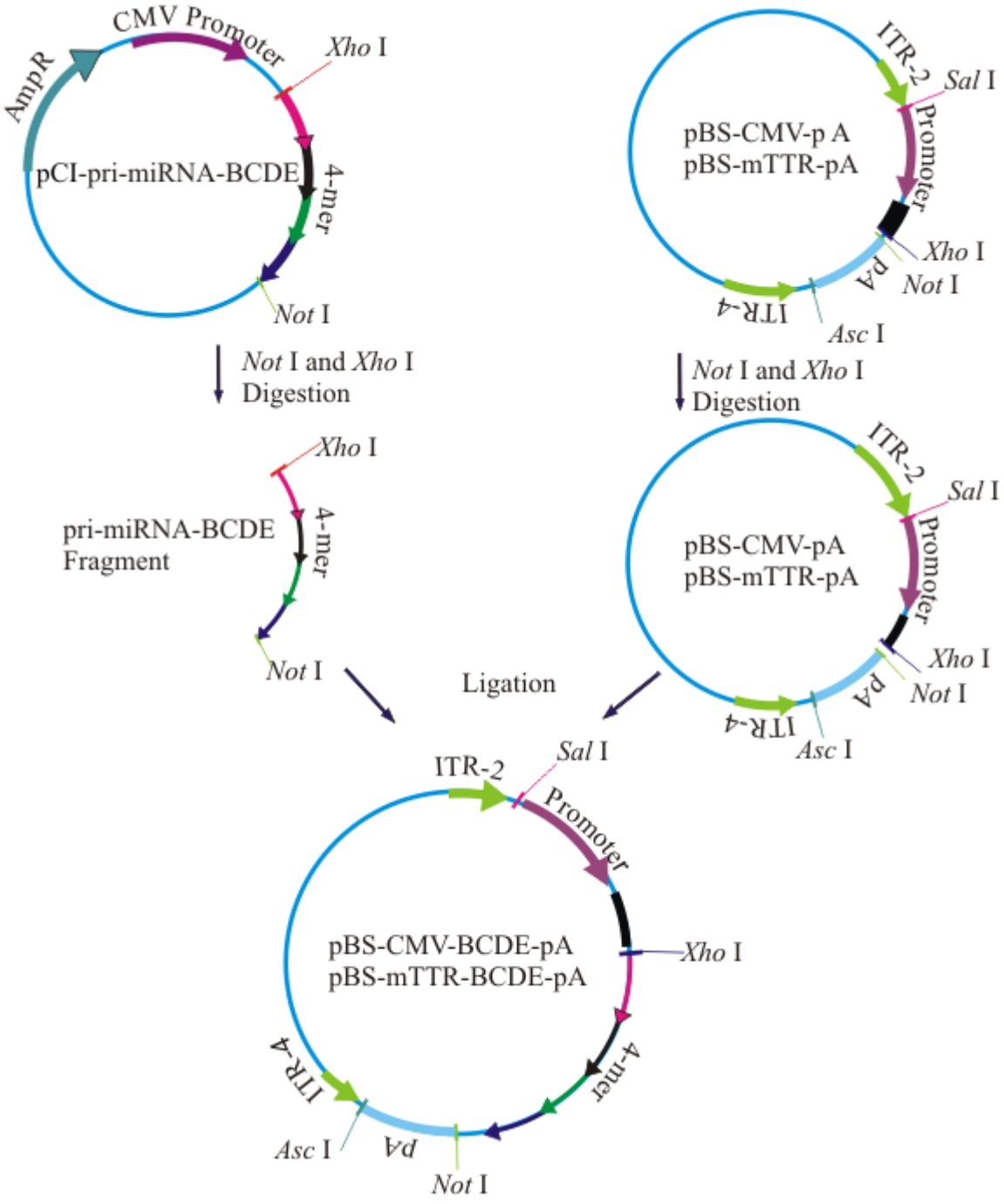

Figure 2.6: Generation of rAAV packaging plasmids expressing polycistronic cassettes.

pBS-CMV-pA and pCI-CMV-BCDE were digested with *Not* I and *Xho* I. The pri-miRNA-BCDE sequence was ligated into the pBS fragment to generate pBS-CMV-BCDE-pA. A similar strategy was employed to generate pBS-CMV-BDCE-pA, pBS-mTTR-BCDE-pA and pBS-mTTR-BDCE-pA.

2.3 rAAV propagation.

2.3.1 Plasmid preparation.

rAAV packaging plasmids, pBS-H1-GFP, Helper and AAV2 capsid plasmids were prepared using the Plasmid Maxi Kit (Appendix 5.2.6.3) (Qiagen, CA, USA) (Matsushita et al., 1998, Kienle, 2014).

2.3.2 rAAV propagation.

The human kidney cell line (HEK293T) was maintained as described in Appendix 5.2.7. One day before transfection cells were seeded into 15 cm dishes at 70-80% confluency (Corning Inc, NY, USA) in 25 ml DMEM growth medium. Twelve plates were seeded for each viral preparation. Two hours before transfection spent media was replaced with 25ml of fresh DMEM media with antibiotics.

Each transfection was prepared as follows: 14 µg of AAV2 Capsid plasmids, 14 µg of helper plasmid and 14 µg of packaging plasmid (one of the five packaging plasmids; pBS-CMV-BCDE-pA, pBS-CMV-BDCE-pA, pBS-mTTR-BCDE-pA, pBS-mTTR-BDCE-pA or pBS-RSV-GFP) was made up to 500 µl with distilled water. The plasmids were thoroughly mixed with 500 µl of 300 mM Sodium chloride. One hundred microlitres of Polyethylenimine (PEI: 1 mg/ml in water) (Polysciences Inc., PA, USA) was mixed with 400 µl of water, 500 µl of 300 mM NaCl and the solution added drop-wise to the DNA:sodium chloride mixture followed by vortexing. The mixture was then allowed to incubate for 8 minutes at room temperature and 2 000 µl of transfection mixture was then added to the plate. This was done in 10 replicates. Two control plates were included in the preparation the first used the pBS-RSV-GFP packaging plasmid and the second was control transfection lacking the helper plasmid. After transfection cells were left to

incubate at 37°C with 5% CO_2 for 24 hours. The following day spent media was replaced with fresh media and the cells were left to incubate for a further 72 hours at 37°C with 5% CO_2.

A cell scraper was used to detach all the cells from the dish for each of the plates, the cells were re-suspended within the spent media and transferred into a 50 ml conical centrifuge tube. Conical centrifuge tubes were subjected to centrifugation at 4°C for 15 minutes at 392× g. The supernatant was discarded followed by re-suspension of the cell pellet in 10 ml of sterile phosphate buffered saline (PBS). The re-suspension was again subjected to centrifugation at 4°C for 10 minutes at 392× g. The supernatant was discarded and 1 ml of PBS was used to re-suspend all but the control samples. The 1 ml re-suspended cells were subsequently pooled and a single millilitre of each sample (experimental as well as the control re-suspensions) was removed for Hirt analysis (see section 2.3.3 Confirmation of the presence of viral genomes). Control samples were re-suspended in 10 ml PBS and centrifuged for a second time with the pooled experimental and control samples for 10 minutes at 392× g at 4°C. The supernatant was discarded and the cells were re-suspended in 5 ml of viral lysis solution (150 mM NaCl, 50 mM Tris-HCl, pH 8.5). Once re-suspended the samples were placed in liquid nitrogen (-196°C) for 5 minutes followed by thawing in a water bath at 37°C for 5 minutes. The freeze-thaw cycle was repeated five times. Once samples had thawed for the final time, they were sonicated within a sonication bath for 1 minute and 20 seconds. Benzonase® (Sigma-Aldrich, MO, USA) was added to a final concentration of 50 U/ml and the samples were incubated at 37°C for an hour and vortexed every 20 minutes. The cell lysates were centrifuged at 3 270× g for 15 minutes at room temperature and the supernatant was transferred into a new 50 ml tube.

An iodixanol gradient was then set up in a Beckman Quick-Seal®, polypropylene centrifuge tube (Beckman and Coulter, CA, USA). The gradient was made using 60%, 40%, 25% and 15% iodixanol solutions, with the 60% at the bottom of the gradient and the cell lysate on the top of the gradient. The 50 ml 60% iodixanol solution was made by mixing 60% iodixanol (Sigma-Aldrich Co., MO, USA) with 125 µl of 2.5 µl/ml phenol red solution. The remaining iodixanol solutions were made by diluting the 60% ioixanol with PBS, $MgCl_2$, NaCl solution to the appropriate concentration. Beckman Quick-Seal® centrifuge tubes were sealed and subjected to ultracentrifugation at 4°C for 2 hours at 50 000 rpm. The rAAV particles accumulate within the 40% iodixanol phase. To collect the viral particles, first a 22G needle was used to pierce the top of the sealed Beckman Quick-Seal® tube. Following this a 22G needle attached to a 1 ml syringe was used to remove the 40% iodixanol solution from the gradient, a maximum of 1.2 ml of this phase was removed. This procedure was followed for the production of the 5 rAAVs namely; AAV-CMV-BCDE-pA, AAV-CMV-BDCE-pA, AAV-mTTR-BCDE-pA, AAV-mTTR-BDCE-pA and AAV-GFP. The GFP virus was used a control.

2.3.3 Confirmation of the presence of viral genomes.

The presence of viral genomes was confirmed through the use of Hirt extractions. One millilitre of cells suspended in PBS was taken from the viral propagation protocol. These samples were immediately centrifuged at 2 000× g at 4°C for 10 minutes. The supernatant was discarded and the pelleted cells were re-suspended in 1 ml lysis buffer (5 mM Tris-HCl (pH 8); 5 mM EDTA (pH 8); 1% SDS; 250 µg/ml Proteinase K). The lysate was incubated at 55°C for 3 hours then placed on ice for 10 minutes. Two hundred and fifty microlitres of 5 M sodium chloride was added to the lysate drop-wise and the samples inverted several times. Cell lysates were then incubated at 4°C overnight. Following this,

cell lysates were centrifuged at 15 700× g for 60 minutes at 4°C. The supernatant was removed and placed in a clean microcentrifuge tube and centrifuged for a further 15 minutes at 15 700× g and 4°C. Seven hundred microlitres of the supernatant was transferred to a clean microcentrifuge tube, 700 µl of phenol:chloroform was added and the samples mixed. The mixture was then centrifuged at 15 700× g for 5 minutes at 4°C. Six hundred microlitres of the aqueous phase was removed and placed in a clean microcentrifuge tube and 420 µl of isopropanol was added. The samples were centrifuged for 30 minutes at 15 700× g and 4° C. The supernatant was discarded and 500 µl of 70% ethanol was used to wash the pellet, followed by centrifuging at 15 700× g for 10 minutes. The supernatant was again discarded and the pellet was re-suspended in a solution containing 0.1 µl *Dpn* I, 2 µl of 10× Tango Buffer and 17.9 µl of distilled water and incubated at 37°C overnight. Four microlitres of 10× loading buffer with 1 µl RNAse A was added and the samples were allowed to incubate for 15 minutes at room temperature. The samples were subjected to electrophoresis using a 1% agarose gel (Figure 5.10).

2.3.4 rAAV titration.

To quantify the number of viral particles isolated from cells real-time quantative PCR (qPCR) was performed. To establish a standard curve for this quantification method three standards were developed, namely a standard for AAV-GFP, a standard for AAV-mTTR and finally a standard used to quantify AAV-CMV viral particles. The standards were developed through the restriction digestion and linearisation of plasmid DNA followed by dilution and purification. The standards were made by restricting the sequence from ITR-2 to ITR-4 of the plasmids, which would be a close approximation of the viruses' genomes. pBS-RSV-GFP was subject to restriction digestion with *Bgl* II while pBS-mTTR-BDCE-pA and pBS-CMV-BCDE-pA were digested with *Pvu* II. Linearised sequences were diluted in series to generate a standard curve with a range of 10^3-10^9 copies of DNA.

Specific primers were designed and subsequently generated through of phosphoramidite chemistry (inqaba biotec™, South Africa) (Table 2.3).

To quantify the amount of viral genomes isolated after iodixanol purification 20 µl of viral samples were treated with 30 µl of DNAse (1 U/µl) in 150 µl of distilled water for 30 minutes at 37°C. This was followed by incubation at 65°C for 10 minutes and purified through the use of QIAamp DNA mini kit (Qiagen, CA, USA) according to manufacturer's specifications (Appendix 5.2.2). A dilution series was made ranging from undiluted to 1:1000. Each dilution series together with its respective standard curve was quantified by qPCR. qPCR conditions were as follows; initial denaturation at 94°C for 30 seconds, 40 cycles of denaturation at 94°C for 5 seconds, annealing at 60°C for 5 seconds, extension at 72°C for 5 seconds. A melt curve analysis that begins at 65°C and steadily increases in increments of 0.5 degrees with 5 second intervals was performed until 95°C was reached. The qPCR reaction was set up using SsoFast™ EvaGreen® supermix (Bio-Rad, CA, USA). PCR was carried out using 100 pmol of each primer, template DNA, 10 µl of ssoFast™ EvaGreen® supermix and 6 µl sterile water in a total reaction volume 20 µl.

Table 2.3: Oligonucleotide sequences used for the titration of viral genomes.

CMV/mTTR intron F:	5′- TTG GTC GTG AGG CAC TGG GC -3′
CMV/mTTR intron R:	5′- GGG AGT GGA CAC CTG TGG AG -3′
RSV F:	5′- ACG CGG TTA GGA GTC CCC TC -3′.
RSV R:	5′- CCA ATC GGC ATG CAC GGT GC -3′

2.4 Characterisation of anti-HCV rAAV vectors.

2.4.1 HCV target plasmid.

To generate a luciferase-based reporter plasmid for the use in quantifying anti-HCV pri-miRNA mimic mediated knockdown the 5' UTR of HCV (subtype 2a) was cloned downstream of the Renilla *luciferase* ORF from pTZ-HCV-2a. pTZ-HCV-2a was generated by cloning a PCR amplicon of the 5' UTR of HCV containing *Sal* I and *Not* I sites at its 5'- and 3'-ends, respectively. This plasmid was digested and the 5' UTR ligated into psiCHECK2.2 (Ely et al., 2009). pTZ-HCV-2a was digested using *Sal* I and *Not* I while psiCHECK2.2 was digested using *Not* I and *Xho* I. Five units of FastAP (Thermo Fischer Scientific, MA, USA) was subsequently added to the psi-CHECK2.2 digestion and incubated at 37°C for 30 minutes. The restriction digests were then subjected to 1% gel electrophoresis. The psiCHECK2.2 backbone (6 242 bp) and pTZ-HCV-2a insert (473 bp) were extracted and purified from the gel (Appendix 5.2.1.1) and ligated together. After transformation of *E. coli*, inoculation and growth of the cultures the plasmids were purified through the use of the alkaline lysis method (Appendix 5.2.6.2). The plasmids were screened using *Pst* I restriction digest. The resultant plasmid was named psiCHECK-HCV.

2.4.2 Plasmid preparation.

The pCI-eGFP plasmid expresses green fluorescent protein and is driven by a CMV promoter as described previously (Passman et al., 2000). Both pCI-eGFP and psi-CHECK-HCV were prepared using the Plasmid Maxi Kit (Appendix 5.2.6.3) (Qiagen, CA, USA).

2.4.3 Infection of cultured human hepatocytes with rAAVs.

The human hepatocyte (Huh 7) cells were maintained as described in Appendix 5.2.7. On the day of infection, 5×10^4 cells were seeded per well in a Costar® 24-well plate (Corning Inc, NY, USA) in 0.5 ml DMEM growth medium. Cells were infected with rAAVs at MOI

of 10 000. Cells were then incubated at 37°C for 24 hours with 5% CO_2. Infections with each AAV-CMV-BCDE-pA, AAV-CMV-BDCE-pA, AAV-mTTR-BCDE-pA and AAV-mTTR-BDCE-pA, the mock control (AAV-GFP) were all carried out in triplicate. One hour before transfection (23 hours after infection) spent media was replaced with 0.5 ml of fresh DMEM media antibiotics.

Each transfection was prepared as follows: 100 ng of pCI-eGFP and 100 ng psiCHECK-HCV were thoroughly mixed with Opti-MEM® I (Thermo Fischer Scientific, MA, USA). Fifty microlitres of Opti-MEM® was used to dilute 0.2 µl Lipofectamine™ 2000 (Thermo Fischer Scientific, MA, USA). Transfection mixes were incubated at room temperature for five minutes. After the five minute incubation, the Lipofectamine:Opti-MEM was mixed with the DNA:Opti-MEM and incubated at room temperature for 20 minutes. This was done to ensure that formation of Lipofectamine:DNA complexes. Following this, 100 µl of the transfection samples were added to the Huh 7 cells seeded into a 24-well plate. The cells were subsequently incubated at 37°C for 72 hours in a humidified incubator with 5% CO_2. Further controls included cells that were not infected but transfected with psiCHECK-HCV alone as well as cells transfected with psiCHECK-HCV and pCI-eGFP.

All cells transfected had their DMEM medium replenished 24 hours post transfection and incubated for a further 72 hours in a humidified incubator with 5% CO_2 at 37°C. Infection efficiency for cells infected with AAV-GFP was used as a proxy for infection efficiency of the other viruses. Ninety-six hours post infection, cells were analysed for the expression of GFP, through Fluorescence Microscopy. The cells were analysed using a confocal microscope (Axiovert 100M, Zeiss, Germany) with a FITC filter to identify green fluorescent cells.

Luciferase assays were carried out on the lysates of infected cells using the Dual-Luciferase® Reporter Assay system (Promega, WI, USA) according to the manufacturer's recommendations. The media was removed from the cells 72 hours post transfection. One hundred microlitres of 1× Passive Lysis Buffer was added and the cells were incubated at room temperature on an orbital shaker for 15 minutes. Luciferase Assay Reagent II (LAR II) was prepared by dissolving Luciferase Assay Substrate in Luciferase Assay Buffer II. Stop & Glo® Substrate was mixed with Stop & Glo® Buffer to make a 1× Stop & Glo® reagent. The lysates were placed in a microcentrifuge tube and 10 µl of each sample was added per well of a Costar® 96-well assay plate. The plate was placed into a luminometer (Veritas™ Microplate Luminometer) 50 µl of LAR II (substrate for Firefly luciferase) was injected into each well and luminescence was measured and recorded. Subsequently 50 µl of Stop & Glo® reagent (stops the activity of Firefly luciferase and introduces the substrate for *Renilla* luciferase) was added to each well and luminescence was measured and recorded.

2.4.5 Infection of HCV replicon model with rAAVs.

Huh 7-11-7 and Huh 7-luc-ET cell lines were maintained as described in Appendix 5.2.7. The Huh 7-11-7 cells are human hepatocyte cells that are stably transfected with HCV replicon, while Huh 7-luc-ET cells are stably transfected with an HCV replicon that expresses Firefly luciferase. Fifty thousand Huh 7-11-7 cells and 140 000 Huh 7-luc-ET cells were seeded into Costar® 24-well plate (Corning Inc, NY, USA) in 0.5 ml DMEM growth medium. The cells were infected upon seeding with rAAV at an MOI of 10 000. Cells were then incubated at 37°C for 24 hours with 5% CO_2. Infections were carried out in triplicate for all five rAAVs; AAV-CMV-BCDE-pA, AAV-CMV-BDCE-pA, AAV-mTTR-BCDE-pA, AAV-mTTR-BDCE-pA and AAV-GFP. The spent media was replaced

with fresh media and infected cells were allowed to incubate for a further 72 hours at 37°C with 5% CO_2

2.4.6 qRT-PCR of replicon model after rAAVs infection.

Seventy-two hours post infection, the media on the Huh 7-11-7 cells was removed and RNA extracted (Appendix. 5.2.8). The RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, CA, USA). Five hundred nanograms of RNA was mixed with 2 µl gDNA wipeout Buffer, the reaction volume was made up with RNase free water to total reaction volume of 14 µl. The sample was incubated at 42°C for 2 minutes and then placed on ice. One microlitre of Quantiscript® reverse transcriptase, 4 µl of 5× Quantiscript® RT Buffer and 1 µl of the RT Primer Mix were then added to the solution. Solutions were incubated at 42°C for 15 minutes followed by incubation at 95°C for 3 minutes. qPCR was then carried out on the cDNA generated. Primers targeted to the neomycin resistance gene present in the HCV replicon genome were computationally designed and subsequently generated through the use of phosphoramidite chemistry (inqaba biotec™, South Africa). The primer sequences were as follows; neomycin forward primer 5'- CGT TGG CTA CCC GTG ATA TT -3', neomycin reverse primer 5'- CTC GTC AAG AAG GCG ATA GAA G -3', GAPDH forward 5'- AGG GGT CAT TGA TGG CAA CAA TAT CCA -3' and GAPDH reverse 5'- TTT ACC AGA GTT AAA AGC AGC CCT GGT G -3'. The conditions were as follows; initial denaturation at 95°C for ten minutes, 60 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 15 seconds, extension at 72°C for 15 seconds. A melt curve analysis was performed beginning at 65°C and steadily increasing in increments of 0.5 degrees at 30 seconds intervals up to 95°C. Real time PCR was carried out with 2 µl cDNA, 100 pmol of each primer, 10 µl of ssoFast™ EvaGreen® supermix (Bio-Rad, CA, USA) and 6 µl distilled water in a total reaction volume 20 µl.

2.4.7. Luciferase assay performed on HCV replicon after rAAV infection.

Luciferase assays were carried out on the lysates of infected Huh 7-luc-ET cells using the Dual-Luciferase® Reporter Assay system. The luciferase assays were carried out as described in Section 2.4.3. Ten microlitres of each sample was diluted in 90 µl saline and was used in a BCA assay to determine total protein concentration within the cell. The BCA assay was performed using Pierce™ BCA Protein assay kit (Thermo Fischer Scientific, MA, USA) and the protocol was followed according to the manufacturer's specifications (Appendix 5.2.9).

2.4.8 Immune stimulation by anti-HCV rAAV vectors.

HEK293T cells were seeded in a Costar® 6-well plate (Corning, NY, USA) at 25% confluency (3×10^{5} cell/well). Each well was infected with one of the five rAAVs at the time of seeding with an MOI of 10 000. After 24 hours incubation at 37°C with 5% CO_2, media was removed from cells and an RNA extraction performed (Appendix 5.2.8). The RNA was reverse transcribed by using the QuantiTect Reverse Transcription Kit (Qiagen, CA, USA) according to manufacturer's specifications (refer to Section 2.4.6). qPCR was performed on the cDNA generated as follows: 95°C for ten minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 15 seconds, extension at 72°C for 15 seconds. A melt curve analysis was performed at the end of the PCR reaction beginning at 65°C and increasing increments of 0.5 degrees for 30 seconds until 95°C was reached. The qPCR reaction was set up in a total reaction volume of 20 µl and included: 100 pmol of each primer, 2 µl cDNA, 10 µl of ssoFast™ EvaGreen® supermix (Bio-Rad, CA, USA) and 6 µl distilled water. Primers were designed to target the *Interferon-β* gene as well as housekeeping gene *GAPDH*. The sequences for the interferon primers was as follows: interferon forward 5'- TCC AAA TTG CTC TCC TGT TGT GCT -3',

interferon reverse 5’- CCA CAG GAG CTT CTG ACA CTG AAA A -3’. The sequences for the GAPDH forward and reverse primers are described in Section 2.4.6.

2.4.9 Saturation of endogenous RNAi pathway by rAAV vectors.

Three hundred thousand HEK293T cells were seeded in a Costar® 6-well plates (Corning, NY, USA) in 2 ml DMEM containing antibiotics. The cells were subsequently infected with rAAVs at an MOI of 10 000. After an incubation of 24 hours at 37°C with 5% CO_2, the media was replaced and transfection carried out. Transfections were performed on all infections as follows: 100 ng psi-miRNA-16 T×7, 800 ng pGEM®-T easy (Promega, WI, USA), 100 ng pCMV-GFP. The sponge plasmid pU6-miRNA-16 S×7 was used as a positive control for saturation, the plasmids used for this transfection were as follows: 100 ng pU6-miRNA-16 S×7, 800 ng pGEM®-T easy, 100 ng pCMV-GFP. Plasmid DNA was mixed with Opti-MEM® I (Thermo Fischer Scientific, MA, USA). One microlitre of Lipofectamine™ 2000 was mixed with 49 µl of Opti-MEM® I and allowed to incubate at room temperature for 5 minutes. The Lipofectamine:Opti-MEM was mixed with the DNA:Opti-MEM and incubated at room temperature for 20 minutes. After the incubation, 100 µl of the transfection mixture was added to each well. The cells were then incubated at 37°C for 48 hours with 5% CO_2. Forty eight hours after transfection the Dual-Luciferase® Reporter Assay (Promega, WI, USA) was performed as described in Section 2.4.3.

2.4.10 Analysis of rAAV pri-miRNA processing by Northern Blot.

HEK293T cells were seeded into 10 cm dishes in 10 ml DMEM with antibiotics at a confluency of 75% (6.6×10^{6} cells/plate). Cells were infected at the time of seeding with one of the five rAAV at an MOI of 10 000. The plates were incubated for 48 hours at 37°C with 5% CO_2. After 48 hours the media from the cells was removed and RNA extraction performed on cells from each of the plates (Appendix 5.2.8). The RNA extracted from

each infection was then quantified by spectrophotometric analysis. Approximately 30 µg of total RNA was run on a 15% denaturing polyacrylamide gel. A molecular weight marker was labelled with 20 µCi of [γ-^{32}P]-ATP with a T4 polynucleotide kinase (Thermo Scientific, MA, USA), 10 pmol of the molecular weight marker was run on the polyacrylamide gel. The gel was allowed to run for 6 hours at 150 V. The gel was stained with 4 µg/ml ethidium bromide in a 0.5× TBE buffer for 5 minutes with shaking. The gel was placed in a UV transilluminator to visualise and confirm RNA was present and equal volumes were loaded correctly. The RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham, NJ, USA), at 3.3 mA/cm^2 for an hour at 4°C in 0.5× TBE. This was done through the use of a Semi-Dry Electroblotting Unit Z34,0502 (Sigma-Aldrich, MO, USA). The RNA was cross-linked to the nylon membrane with 200 000 µJ/cm^2 of energy using a UV cross-linker (UVP, Inc., CA, USA).

The nylon membranes were pre-hybridised in 10 ml of Rapid-hyb buffer (Amersham, NJ, USA) at 42°C for 20 minutes. Probes that recognise each pri-miRNA and U6 snRNA sequence (Table 2.4) were labelled with 20 µCi of [γ-^{32}P]-ATP using a T4 polynucleotide kinase (Thermo Scientific, MA, USA). The membranes were hybridised at 42°C with appropriate probes (10 ng/ml) overnight with rotation. After overnight incubation the membranes were subjected to a low stringency wash with 50 ml of 5× SSC (Appendix 5.2.10) with 0.1% SDS followed by two high stringency washes with 1× SSC (Appendix 5.2.10) with 0.01% SDS at 42°C. The hybridised membranes were then subjected to autoradiography for 4 or 14 days. The membranes were stripped and re-probed with a ^{32}P-labelled primer that detects U6 snRNA.

Table 2.4: Oligonucleotides used to detect pri-miRNA guide sequences.

Pri-miRNA-B	5′-GCT AGC CGA GTA GCG TTG GGT -3′
Pri-miRNA-C	5′-AGG CCT TGT GGT ACT GCC TGA- 3′
Pri-miRNA-D	5′-CTC GTA GAC CGT GCA CCA TGA- 3′
Pri-miRNA-E	5′-ATC CTA AAC CTC AAA GAA AAA - 3′
U6 snRNA guide	5′-TAG TAT ATG TGC TGC CGA AGC GAG CA - 3′

CHAPTER 3

3 Results

3.1 rAAV-delivered anti-HCV pri-miRNA mimics mediate efficient silencing of viral gene expression.

3.1.1 Monomeric anti-HCV pri-miRNA mimic expression plasmids efficiently silence 5' UTR expression.

Plasmids expressing each of the monomeric pri-miRNA mimics driven by the CMV-promoter were simultaneously transfected into hepatocytes. The silencing efficiency the pri-miRNA mimics was determined through dual luciferase assays. A luciferase-based reporter plasmid for quantifying pri-miRNA mimic-mediated knockdown was constructed. The 5' UTR of HCV is expressed downstream of the Renilla *luciferase* gene (Figure 5.10a). Significant knockdown of *Renilla* luciferase expression was seen for each of the monomeric pri-miRNA constructs with pri-miRNA-E having the most potent silencing of 79.82% (Figure 5.10). Simultaneously targeting multiple sites within the 5' UTR can potentially prevent viral escape mutants. Four pri-miRNA mimics were selected to generate polycistronic pri-miRNA mimic cassettes. Polycistronic constructs consisting of four mimics were generated as Liu et al, 2008 showed that four siRNAs was sufficient to prevent viral escape of HIV-1 (Liu et al., 2008). The constructs with the best knockdown were chosen for the generation of the polycistronic constructs namely, pri-miRNA-B (62.89%), pri-miRNA-C (75.53%), pri-miRNA-D (45.52%) and pri-mRNA-E (79.82%)

3.1.2 Infection of cultured human hepatocytes with rAAVs.

To determine the silencing efficiency of the polycistronic pri-miRNA mimic payloads of the rAAVs a dual luciferase assay was conducted. The 5' UTR of HCV was cloned into the multiple cloning site of psiCHECK2.2. The 5' UTR is expressed downstream of the Renilla *luciferase* gene. Firefly *luciferase* is expressed independently of Renilla *luciferase* as it has its own promoter (Figure 3.1). If the polycistronic constructs target and silence the

5’ UTR effectively, Renilla mRNA will be silenced and this prevents the translation of *Renilla* luciferase thus no luminescence will be detected. The Firefly luciferase will be translated irrespective of the presence of polycistonic cassettes. Huh 7 cells were infected with rAAVs at an MOI 10 000. Following a 24 hour incubation, the target plasmid was transfected into cells. Cells were incubated for a further 72 hours to allow for expression of polycistronic cassettes. Luciferase assays were conducted to determine the efficacy of each construct targeted towards the 5’ UTR of HCV. Significant knockdown of the expression of the 5’ UTR was seen for all four of the rAAVs while AAV-GFP, as expected had no effect of 5’ UTR expression (Figure 3.1). The rAAVs driven by the liver-specific promoter had the best silencing efficiency with rAAV-mTTR-BCDE at 76.73% knockdown and rAAV-mTTRBDCE at 71.92% knockdown. This was not unexpected as the liver-specific promoter expresses well in hepatocytes resulting in better knockdown. The CMV promoter-driven rAAVs also produced significant knockdown but to a lesser degree than the mTTR driven rAAVs. The rAAV-CMV-BCDE and rAAV-CMV-BDCE silenced reporter gene expression by 51.9% and 44.63%, respectively. Negative controls included cells transfected with either target plasmid psiCHECK-HCV only or target plasmid and a marker plasmid pCI-eGFP without infection of rAAVs (Figure 3.1).

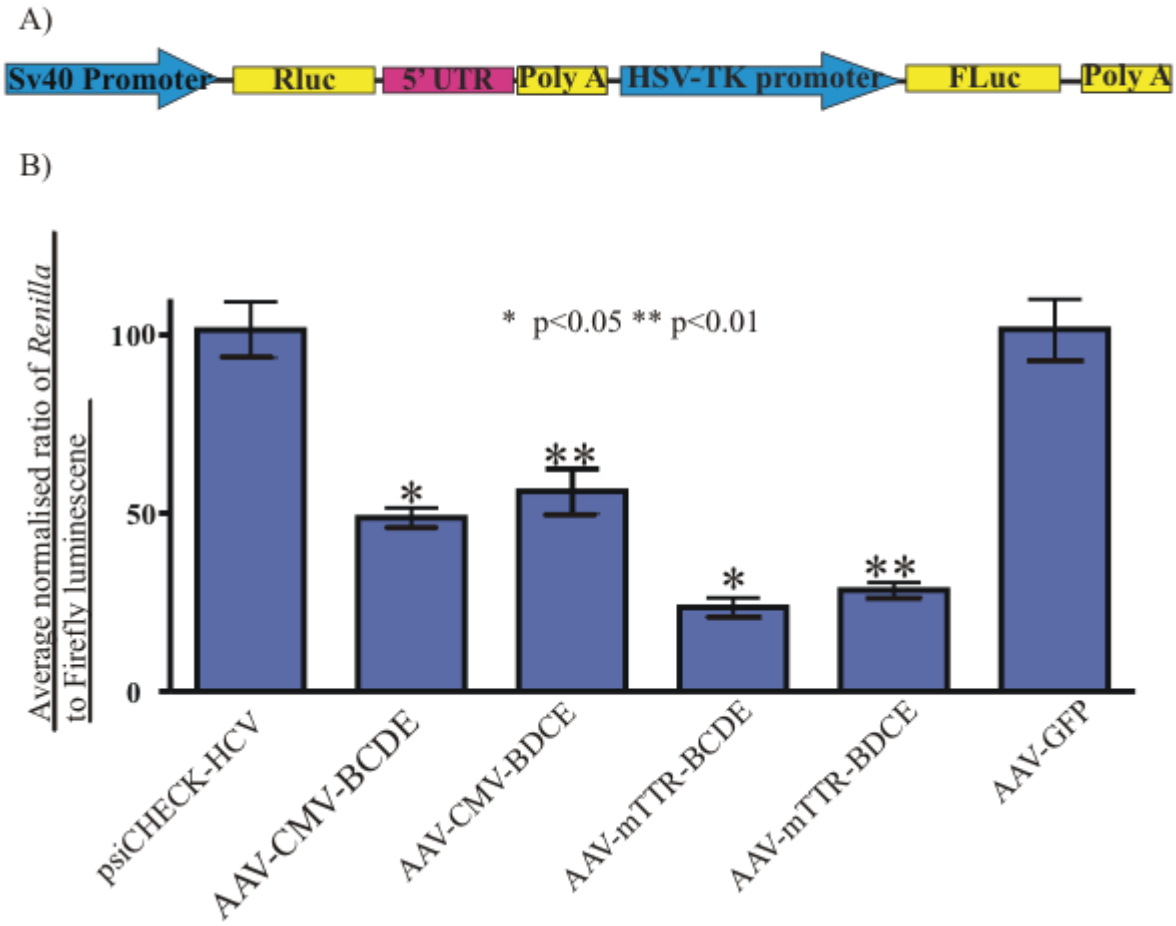

Figure 3.1: Silencing efficacy of rAAVs expressing anti-HCV polycistronic expression cassettes.

A) The 5' UTR region of HCV was cloned into psiCHECK2.2 and is expressed as part of the Renilla *luciferase* (Rluc) mRNA. Firefly *luciferase* (Fluc) is expressed independently of Renilla *luciferase* as it has its own HSV-TK promoter. B) To determine the efficacy of each construct targeted towards the 5' UTR of HCV, a dual luciferase assay was performed. Luciferase assays were performed on lysates from cells infected with rAAVs followed by transfection of the target plasmid. The data represents the average ratio of *Renilla*/Firefly luciferase activity from transfections carried out in triplicate (error bars indicate standard error of the mean). Data was normalised to the negative control (target plasmid, psiCHECK-HCV).

3.1.3 Infection of HCV replicon model with rAAVs.

Transfection of Huh 7 cells with target plasmid is a fairly artificial system in which to test the efficacy of anti-HCV rAAVs. It does have an advantage in that it is convenient for initial testing of rAAVs. A model system of HCV was subsequently employed to test the silencing efficiency of the rAAVs. The HCV replicon system is a model in which transfection of an HCV RNA sequence mimics the wild type HCV genome and allows viral replication to be recapitulated in cultured cells (Lohmann et al., 1999). The replicon is produced from a DNA sequence that consists of T7 RNA polymerase promoter, HCV 5' UTR, a *neomycin resistance* gene followed by the IRES from encephalomyocarditis virus (EMCV), the non-structural proteins of HCV and finally the HCV 3' UTR (Figure 3.2). The T7 RNA polymerase promoter allows the RNA replicon to be transcribed *in vitro* which is subsequently transfected into cells. The 5' UTR is important for replication and allows the replicon to replicate autonomously. The IRES of EMCV directs the translation of sequences encoding the non-structural proteins required for replication and the 3' UTR encodes a polyadenylation sequence (Lohmann et al., 1999, Blight et al., 2003, Evans et al., 2004). The *neomycin resistance* gene is a selectable marker that replaces the structural proteins of HCV and allows the selection of cells that maintain the replicon (Figure 3.2). The HCV replicon system mimics HCV replication but does not imitate other aspects of the viral life cycle. As the neomycin selectable marker replaces the structural proteins viral particle assembly as well as viral release from liver-derived cells is not possible. The replicon model does not produce infectious viral particles and is thus safe to use when determining the efficiency of the rAAV-mediated inhibition of HCV (Lohmann et al., 1999, Blight et al., 2000, Pietschmann et al., 2002, Lindenbach et al., 2005). The vector allows stable expression of viral RNA that encodes all the non-structural proteins required for viral replication (Lohmann et al., 1999).

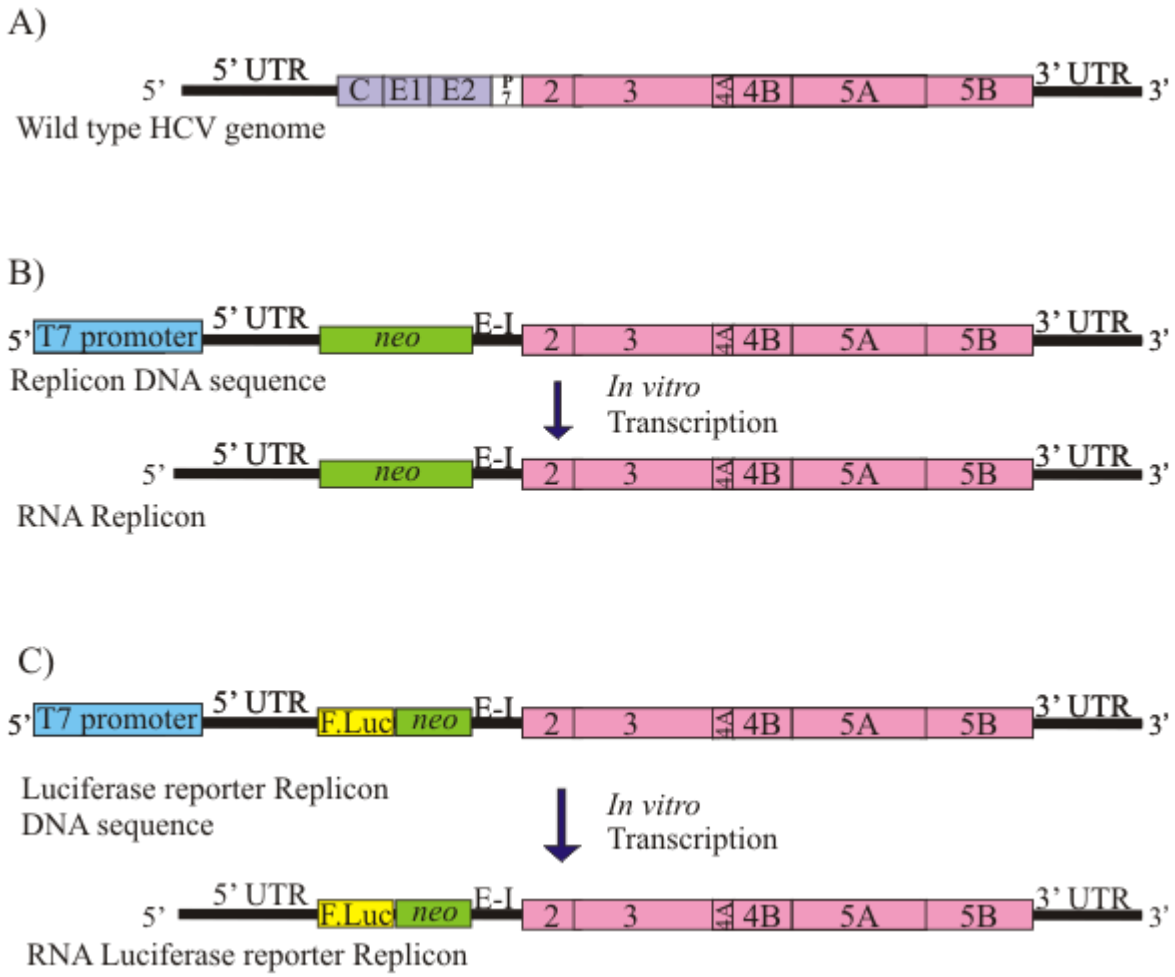

Figure 3.2: Diagram illustrating the DNA structure of replicon model systems.

Schematic diagram illustrating the structure of the wild type HCV genome structure (A), the DNA sequence encoding the replicon (B) and the sequence encoding the luciferase replicon model (C). The DNA sequence encoding the replicon contains the T7 RNA polymerase promoter (blue) 5' UTR, the *neomycin resistance* gene (green), the EMCV-IRES (E-I), and HCV sequences for non-structural proteins NS2 to NS5B (purple) up to the authentic 3' UTR end (Lohmann et al., 1999, Krieger et al., 2010). The DNA sequence encoding the luciferase reporter replicon model contains a Firefly *luciferase* gene (yellow) between the *neomycin resistance* gene and the 5' UTR. *In vitro* transcription of DNA sequences yields the RNA of replicon or luciferase reporter replicon which, upon transfection of liver-derived cells, is capable of establishing the viral replication cycle.

After infection of the replicon cells with each of the rAAVs quantitative reverse transcriptase PCR (qRT-PCR) was carried out on extracted RNA to measure intracellular HCV replicon RNA levels. Significant knockdown of the expression of the 5' UTR was seen for all four of the rAAVs while AAV-GFP had no effect on viral replication as expected (Figure 3.3). A similar trend in efficacy was noted between the rAAVs used to prevent expression of the 5' UTR in target plasmids and the replication of the replicon model. The greatest reduction in replicon mRNA expression was noted for rAAV-mTTR-BCDE with a knockdown of 89.66% followed by rAAV-mTTR-BDCE with knockdown of 82.90%. This was the same trend noted for knockdown of the 5' UTR in target plasmids. The CMV-driven rAAVs also showed significant knock down of replicon expression, as rAAV-CMV-BCDE showed a 60.15% knockdown efficiency and rAAV-CMV-BDCE had a 62.42% knockdown. The rAAV-GFP did not indicate any significant knock down of replicon mRNA as was expected. Interestingly, the mTTR-driven rAAVs are more effective at silencing expression than their CMV driven counter-parts in both model systems.

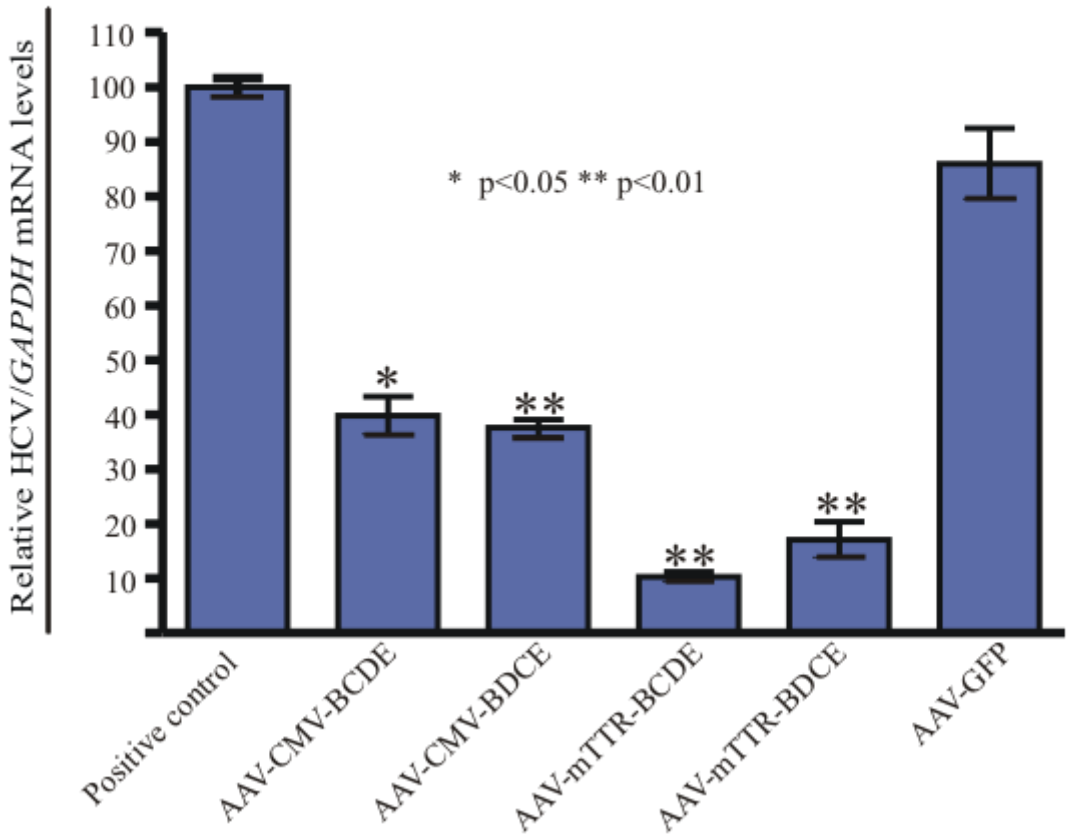

Figure 3.3: Silencing efficiency of rAAVs expressing anti-HCV polycistronic expression cassettes within replicon model.

A qRT-PCR assay was performed to determine the efficacy of each construct targeted towards the 5' UTR of HCV. The *neomycin resistance* gene was used to quantify the presence of HCV replicons within infected cells. *Neomycin resistance* gene expression was relativised to the presence of housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) expression. The qRT-PCR was performed in triplicate with the indicated constructs and data plotted as the average ratio of *neo* to *GAPDH* expression (error bars indicate standard error of the mean). Expression was normalised to the positive control.

3.1.4 Infection of cellular replicon luciferase model with rAAVs.

To corroborate the findings from the replicon model, a second replicon model that utilises Firefly luciferase expression was used. The Firefly *luciferase* gene is found between the 5' UTR and the *neomycin resistance* gene within the replicon mRNA (Figure 3.2). The construct expresses luciferase and allows for the measurement of luminescence as a marker for viral replication. The rAAVs were successful in disrupting 5' UTR expression and thus clear knock down in luminescence is noted for all the constructs and significant knockdown was noted in 3 of the 4 constructs (Figure 3.4). Again the liver-specific promoter-driven rAAVs show the most significant knockdown of this model as with the replicon and transfection models. The rAAV-mTTR-BCDE was once again the most efficient at silencing with a knockdown of 85.77% while rAAV-mTTR-BDCE had a similar silencing efficiency of 84.35% knockdown. The rAAV-CMV-BCDE did not show significant knockdown but this is likely due to variation within the sample, and is intrinsic to the luciferase reporter replicon model system. rAAV-CMV-BDCE did however show significant knockdown of 61.52%.

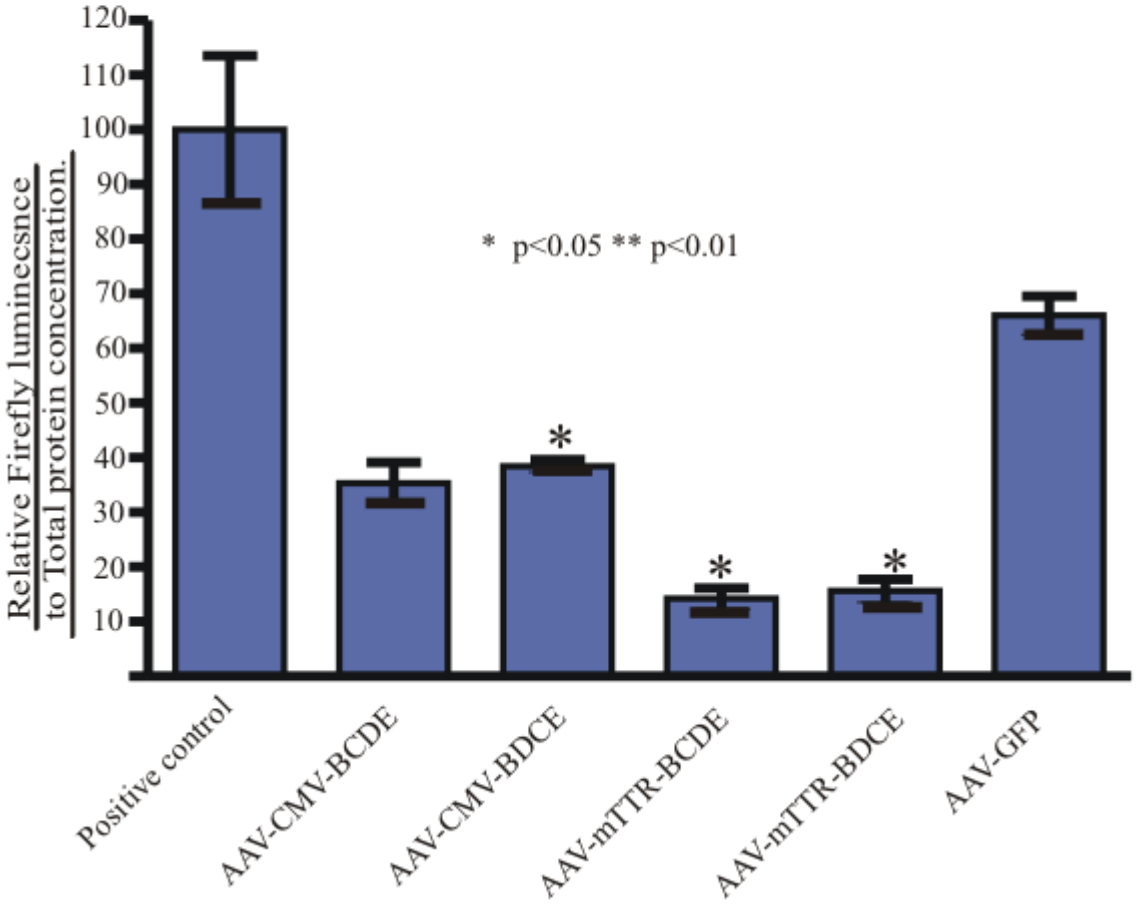

Figure 3.4: Silencing efficiency of rAAVs expressing anti-HCV polycistronic expression cassettes within luciferase replicon model.

A luciferase assay was performed to determine the efficiency of each construct targeted towards the 5' UTR of HCV. Luminescence readings taken 96 hours post-infection with rAAVs and were relativised to total protein content within the sample analysed (error bars indicate standard error or the mean). Expression was normalised to the positive control.

3.2 Assessing the safety of anti-HCV pri-miRNA mimics.

3.2.1 Pri-miRNA expression cassettes do not stimulate the innate immune response.

Activation of the interferon response results in unwanted cytotoxic and off-target effects. Induction of the interferon response is a defence mechanism against viral infection and as such raises concern when dsRNA gene therapy is administered as dsRNA is a molecular pattern that is associated with viral infections (Alexopoulou et al., 2001). Toll-like receptors 3, 7 and 8, RNA helicase retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated protein-5 (MDA-5) are responsible for the recognition of dsRNA. Recognition of dsRNA results in the production of inflammatory cytokines such as interleukin (IL)-6, tumour necrosis factor (TNF) and interferon-α (IFN-α) (Alexopoulou et al., 2001, Kim et al., 2004, Hornung et al., 2005, Judge et al., 2005, Kato et al., 2006, Marques et al., 2006, Judge and MacLachlan, 2008). These pathways are important when considering the impact of administering RNAi activators. To test the safety of expressed pri-miRNA mimics, the expression of interferon response genes were measured 24 hours post infection to determine the degree of stimulation of the immune response. IFN-β is the downstream effecter activated by stimulation of many of the aforementioned interferon genes. Measuring IFN-β activation is therefore a convenient method of assaying whether any interferon stimulation has occurred. *IFN-β* mRNA was measured using qRT-PCR in the kidney-derived cell line (HEK293T) after infection with rAAVs. The liver-derived Huh 7 cell line is known to have a weak interferon response and as such the kidney-derived cell line was used to measure interferon response (Zhong et al., 2005). None of the rAAV-delivered pri-miRNA expression cassettes elicited a significant immune response within these cells (Figure 3.5). Transfection of polyinosinic:polycytidylic acid (Poly I:C) was used as a positive control. Each of the rAAVs as well as the negative control were

significantly different from the Poly I:C stimulated cells. A negative control in which cells were not infected or transfected was included. There was no significant difference between this control and the cells infected with rAAVs with polycistronic constructs. Importantly the polycistronic cassettes did not elicit an immune response. This may be due to the fact that the cassettes mimic endogenous pri-miRNAs which do not stimulate the immune response and are readily incorporated into the RNAi pathway.

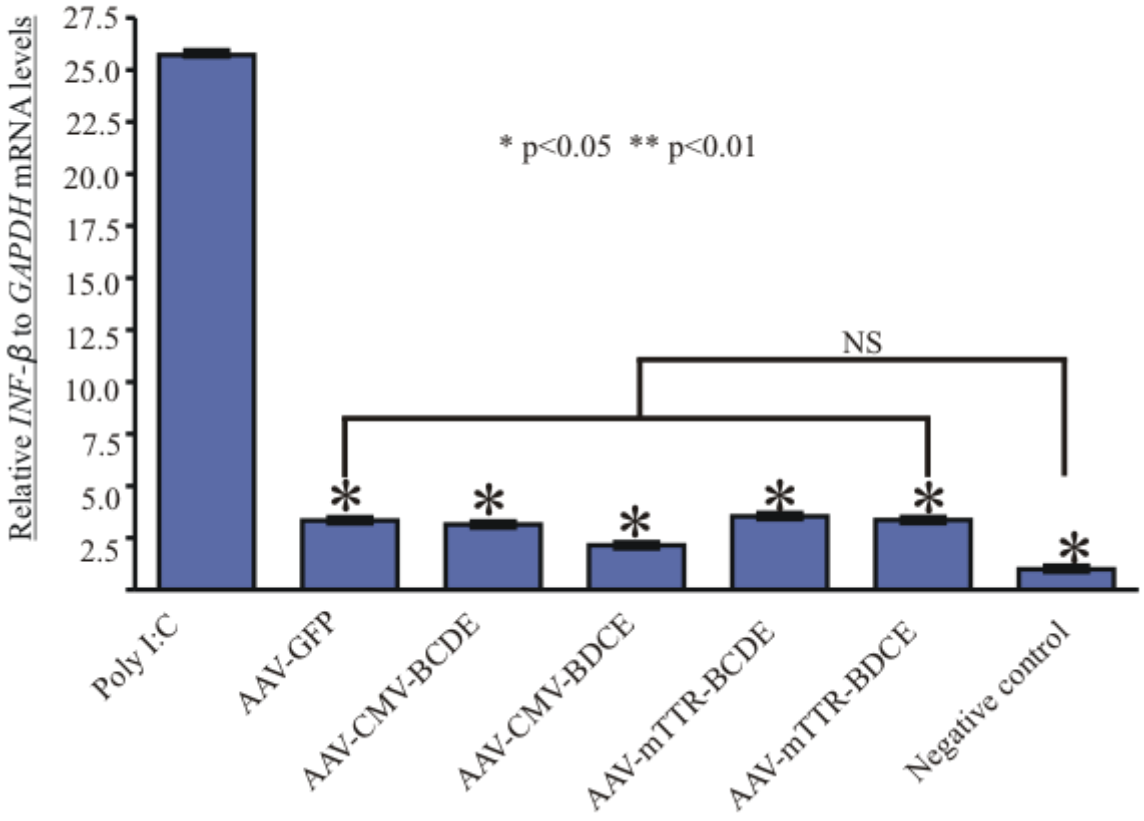

Figure 3.5: Assessing immunostimulatory potential of pri-miRNA expression cassettes.

A qRT-PCR assay was performed on RNA extracted from HEK293T infected with rAAVs to determine the extent of immune stimulation. *INF-β* mRNA quantified and relativised to *GADPH* mRNA levels. The qRT-PCR was performed in triplicate with the indicated constructs and data plotted as the average ratio of *IFN- β* mRNA expression to

GAPDH expression (error bars indicate standard error of the mean). Expression was normalised to the negative control.

3.2.2 Pri-miRNA expression cassettes do not saturate endogenous RNAi pathway.

Saturation of the RNAi pathway occurs when exogenous activators compete with endogenous miRNA for the intracellular processing machinery, which leads to dysregulaton of cellular miRNA and potentially causes cell death (Grimm et al., 2006). Saturation of the endogenous RNAi pathway results in severe cytotoxic effects as was the case in a study presented by Grimm *et al* where saturation of the natural RNAi pathway in mice resulted in organ failure and mortality (Grimm et al., 2006). To test whether expression of the polycistronic cassettes disrupts the endogenous RNAi pathway, a saturation assay was carried out. The saturation assay entails the use of a luciferase reporter with a target site for miR-16 downstream of the Renilla *luciferase* ORF (Figure 3.6). Saturation of the RNAi pathway will prevent the processing of endogenous miR-16, which will not be able to silence *Renilla* luciferase expression. Derepression of *Renilla* luciferase expression can therefore be used as a measure of saturation of the RNAi pathway (Ebert et al., 2007). A sponge plasmid was included as a positive control. This plasmid expresses a sequence containing multiple miR-16 target sites which sequester miR-16 thereby mimicking the effects of RNAi saturation. All of the rAAVs were significantly different to the positive control (sponge) and thus the rAAV polycistronic payload does not result in saturation of the RNAi pathway (Figure 3.6). Negative control cells only received *Renilla* luciferase target and shows normal silencing by miR-16. When compared to the negative and positive controls it is clear that none of the rAAVs, irrespective of promoter and organisation of polycistronic cassette, caused the saturation of the RNAi pathway. This is not unexpected as miRNA mimics have been shown to have reduced saturation of RNAi when compared to shRNA and siRNA (Boden et al., 2004, McBride et al., 2008, Boudreau et al., 2009). Reduced expression of miRNA, as compared to shRNA, contributes to the absence of saturation (Boudreau et al., 2009, Ely et al., 2009).

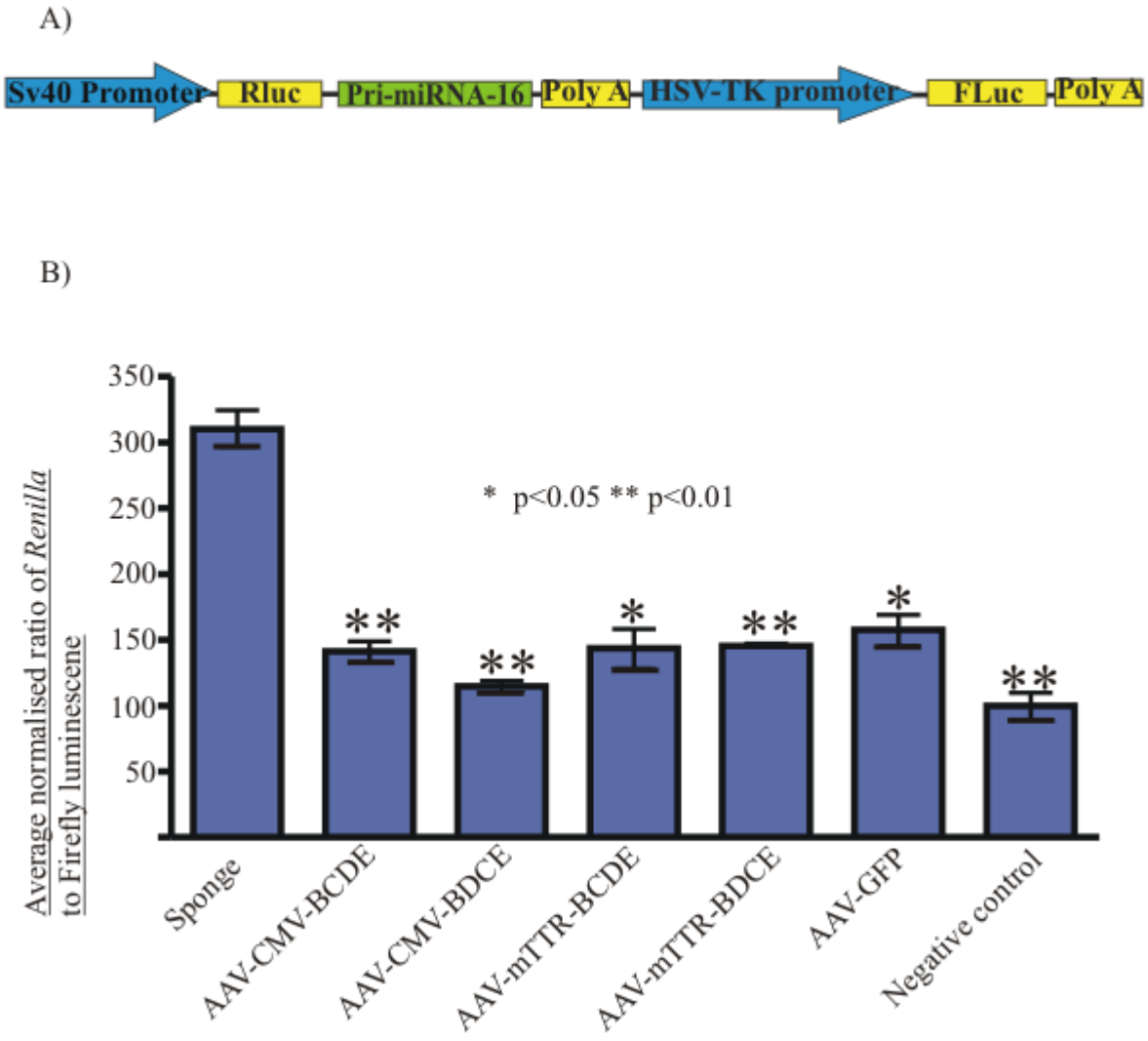

Figure 3.6: Saturation of the RNAi pathway by anti-HCV rAAVs.

A) Luciferase reporter plasmid with a target site for miR-16 downstream of the Renilla *luciferase* ORF. B) A luciferase assay was performed on Huh 7 cells infected with rAAVs and transfected with plasmid expressing miR-16 target sites downstream of *Renilla* luciferase. *Renilla* luciferase expression was quantified through dual luciferase assay and relativised to Firefly luminescence. The assay was performed in triplicate with the indicated constructs and error bars indicate standard error of the mean. Expression was normalised to the negative control.

3.3 pri-miRNA within polycitronic cassettes are effectively processed.

Northern blot analysis was carried out to detect pri-miRNA mimic guide sequences to ensure that all monomeric constructs are processed according to intended design. The putative guide sequences were detected with a complementary radiolabelled oligonucleotide probe. Infection of the Huh 7 cells resulted in poor yields of RNA as a result of low infection efficiency as compared to infection within HEK293T cells (Figure 3.7). Consequently, Northern blot analysis was carried out in HEK293T cells infected with various rAAVs. The RNA was subjected to PAGE followed by transfer onto positively charged nylon membranes, which were probed with radiolabelled oligonucleotides targeted to each of the individual pri-miRNA mimics. The membranes were exposed for a period of 4 and 14 days. After 4 days, a signal can be seen for each of the pri-miRNA mimics within the CMV-driven rAAVs. No signal for the mTTR-driven promoters could be discerned at this time point (Figure 3.8 and Figure 3.9). After 14 days of exposure faint signals could be seen for the mTTR-driven rAAVs. This was not unexpected as the mTTR promoter is liver-specific and is not expected to express at the same levels as the CMV promoter. Nevertheless, the presence of a faint signal indicates that the putative guide sequences are produced from the mTTR-driven pri-miRNA constructs. There was a strong signal for each pri-miRNA mimic driven by the CMV promoters. Bands for each of the pri-miRNA mimic appeared at the expected size of approximately 20 bp. All the pri-miRNAs produced a clear signal for both rAAV-CMV-BCDE and rAAV-CMV-BDCE indicating that the order of the pri-miRNAs within the polycistronic cassettes does not affect how these pri-miRNAs are processed and thus it is expected that each pri-miRNA silences its target effectively.

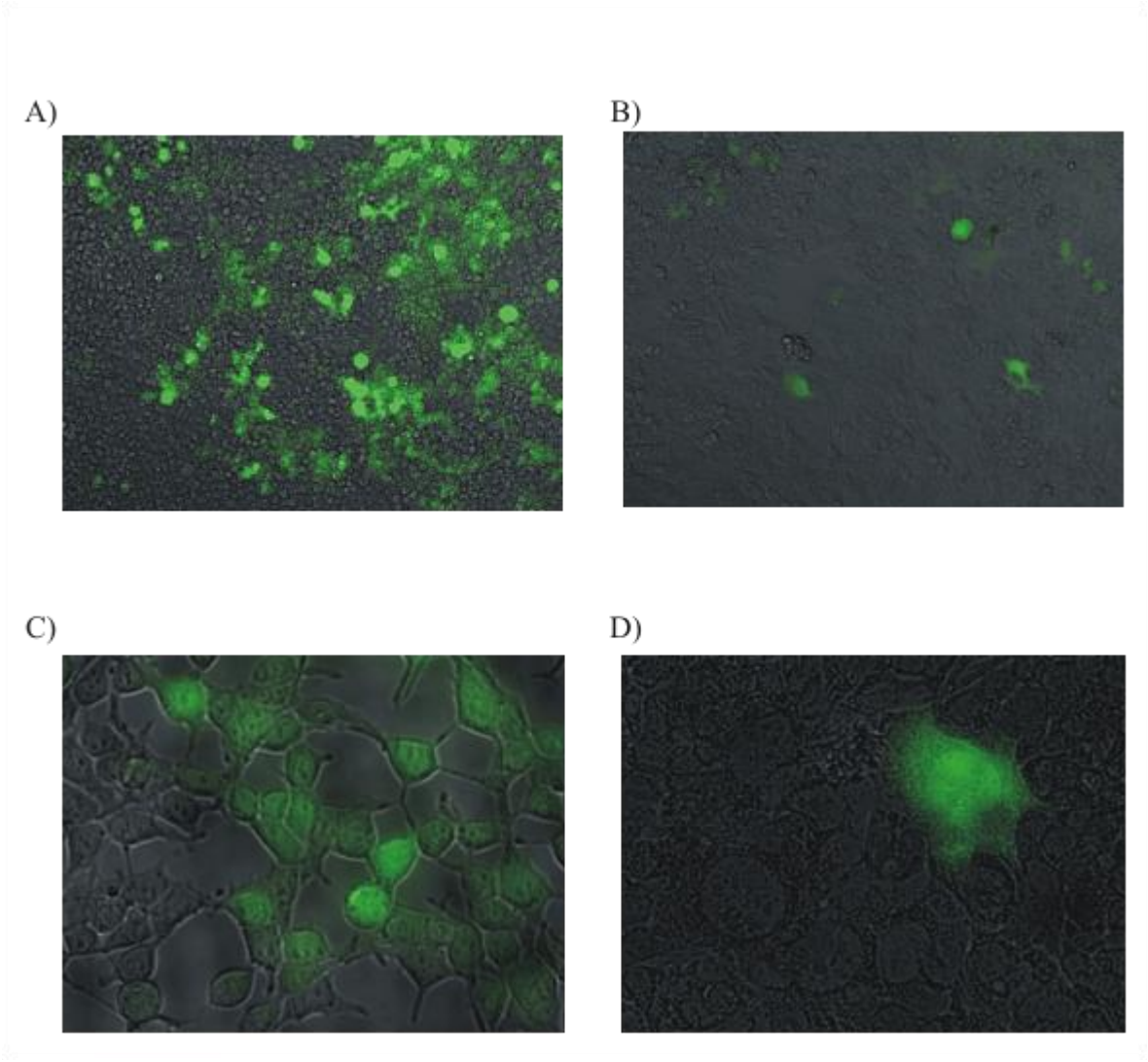

Figure 3.7: Infection efficiency of rAAVs within HEK293T and Huh 7 cells.

A) HEK293T and B) Huh 7 cells were infected with AAV-GFP at an MOI 10 000. The cells were left to incubate for 72 hours and GFP expression was measured as means to determine the efficiency with which the AAVs infect the respective cell lines. It is clear the HEK293T cells are more susceptible to infection than the Huh 7 cell line. A) and B) images taken at 100× magnification. C) HEK293T and D) Huh 7 cells expressing GFP taken at 400× magnification.

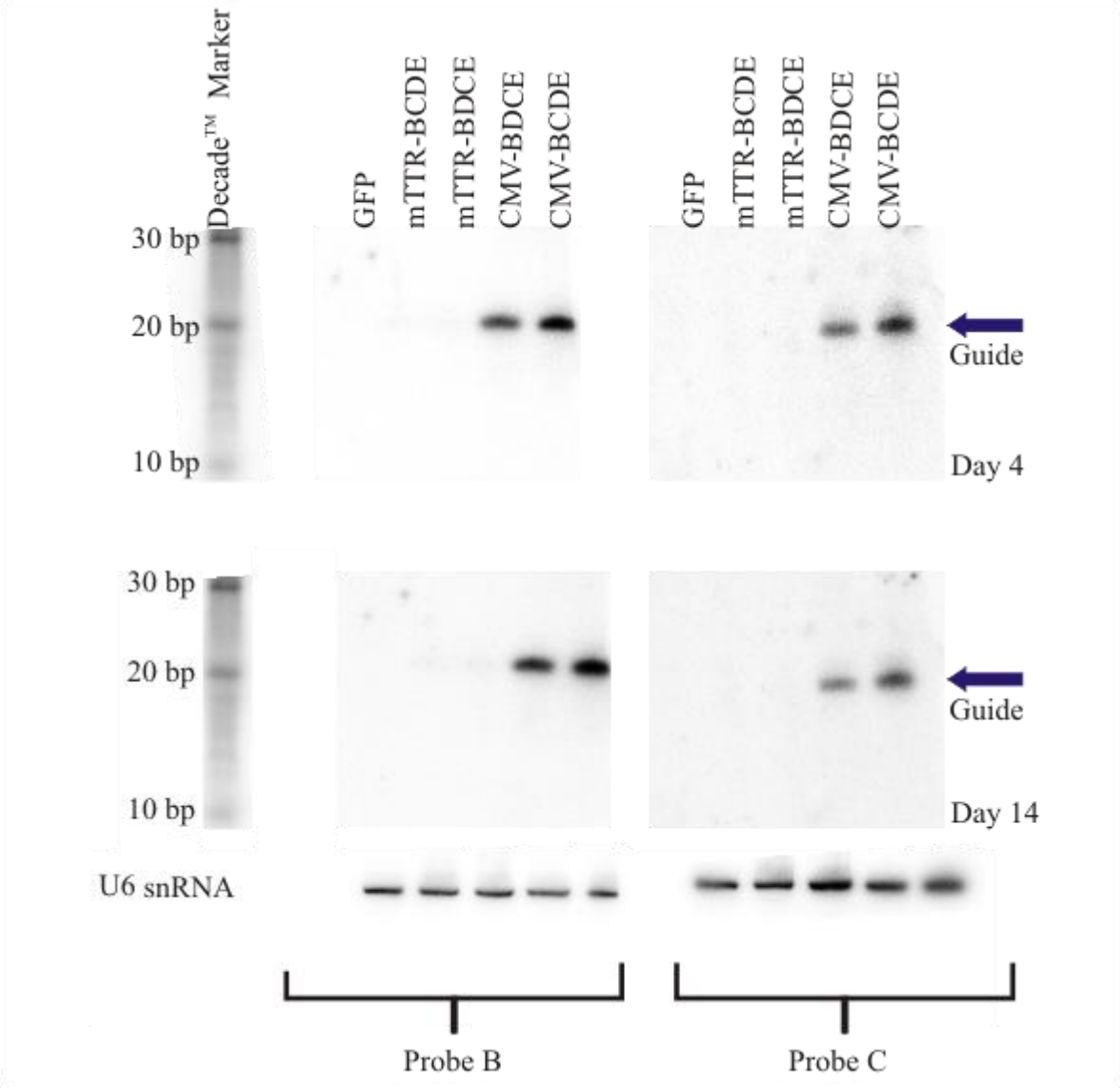

Figure 3.8: Northern Blot analysis to demonstrate processing of pri-miRNA-B and pri-miRNA-C.

Autoradiograph produced from Northern blot analysis illustrates the presence of pri-miRNA-B and pri-miRNA-C guide sequences produced from the polycistronic expression cassettes. Northern blots were exposed for periods of 4 and 14 days. Position of the guide sequences with an expected size of approximately 20 bp is indicated by the blue arrow. Decade ™ Marker (Thermo Fischer Scientific, MA, USA) was used to determine size of each band. Blots were re-hybridised with probes directed against U6 snRNA to illustrate that equivalent amounts of RNA loaded into each sample.

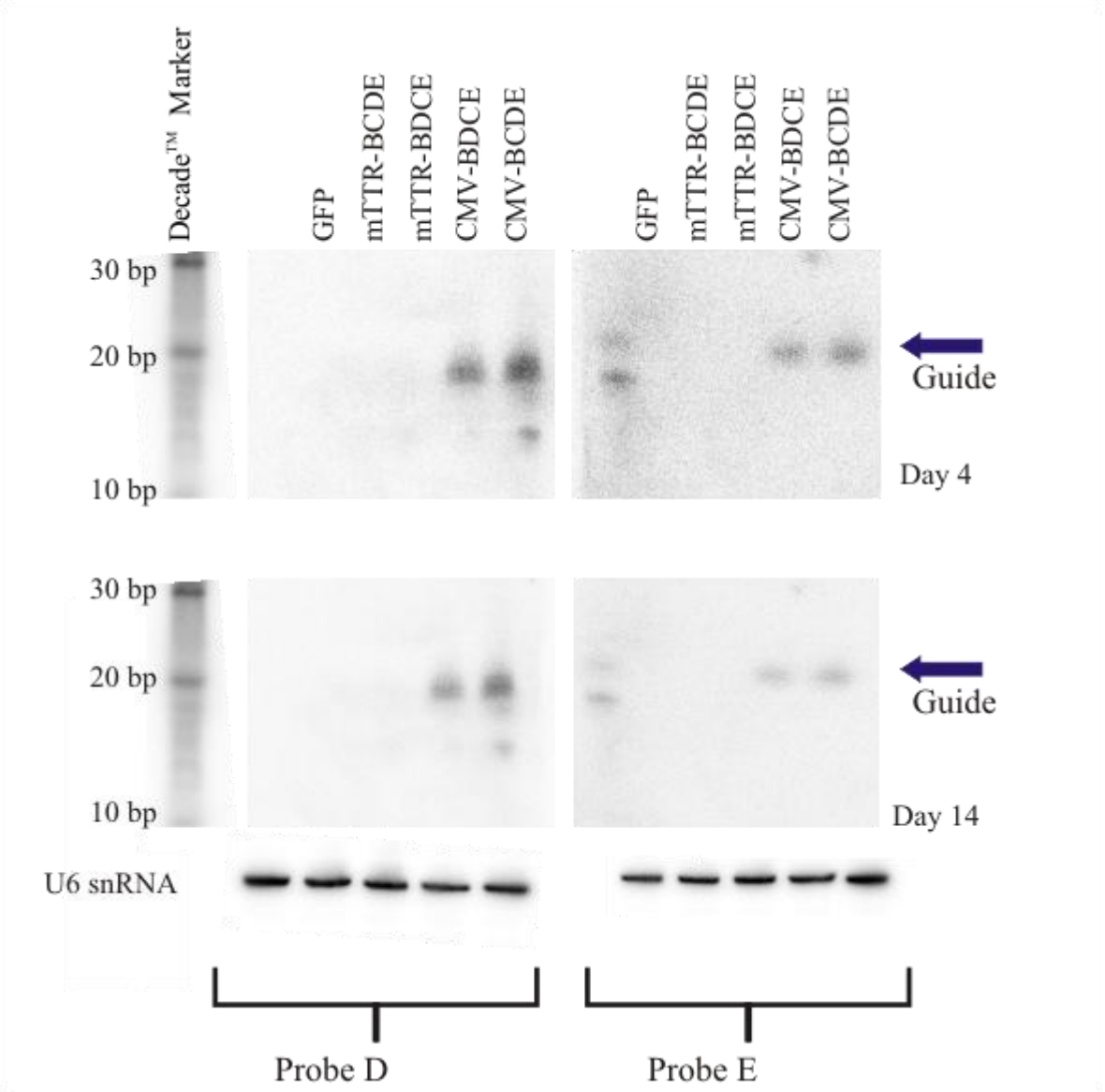

Figure 3.9: Northern Blot analysis to demonstrate processing pri-miRNA-D and pri-miRNA-E.

Autoradiograph produced from Northern blot analysis illustrates the presence of pri-miRNA-D and pri-miRNA-E guide sequences produced from the polycistronic expression cassettes. Guide sequences with an expected size of approximately 20 bp are indicated by the blue arrow. Decade ™ Marker (Thermo Fischer Scientific, MA, USA) was used to determine size of each band. Blots were re-hybridised with probes directed against U6 snRNA as a loading control.

CHAPTER 4

4. Discussion

Four different rAAVs that expressed polycistronic pri-miRNA cassettes were generated. Polycistronic pri-miRNA mimics expressed from these rAAVs targeted to the 5' UTR of HCV were capable of inhibiting the expression of a reporter gene as well as preventing viral replication within two HCV replicon models. The rAAVs that were developed had two different polycistronic cassette payloads; the order of the effectors within the polycistronic cassette was altered to create two different constructs. This was done as processing of each pri-miRNA mimic within a polycistronic cassette can vary and thus silencing efficacy may be altered by the position of each monomeric cassette (Ely et al., 2009). The position of the pri-miRNA mimics within the polycistronic cassette did not alter their silencing capacity. There is a notable difference in the efficacy between the CMV- and mTTR-driven cassettes. The mTTR-driven rAAVs exhibited greater knockdown capacity than their CMV-driven counterparts and this is likely due to the fact that mTTR is a liver-specific promoter and HCV silencing was tested in human hepatocytes. Although the infection efficiency of the rAAVs seen in hepatocytes was lower than that of the HEK293T cell line the pri-miRNA polycistronic cassettes are potent activators of RNAi accounting for the potent silencing noted within the hepatocytes. The infection of rAAVs showed specific and potent inhibition of viral replication as well as knockdown of reporter genes.

The construction of pri-miRNA mimics allows for the exploitation of natural features of miRNA. First, pri-miRNA occurs naturally as polycistronic sequences. This allows for the expression of multiple pri-miRNA mimics from a single expression cassette, which is

beneficial in the prevention of viral escape mutants (Liu et al., 2008). It has been shown that expression of pri-miRNA constructs within a polycistronic cassette are not always equivalent (Ely et al., 2008). The expression of each pri-miRNA mimic within all the polycistronic cassette was shown to be equivalent when Northern blot analysis was conducted. Development of the cassettes with differing promoter sequences presented here is required to find the balance between reduced concentration of expressed pri-miRNA to prevent cytotoxcity and sufficient production of pri-miRNA to induce silencing.

The delivery of RNAi activators and expression of shRNA and miRNA can result in cellular toxicity from a variety of causes such as induction of the interferon response as well as saturation of the endogenous RNAi pathway. As expected, the polycistronic pri-miRNA mimics produced in this study did not saturate the endogenous RNAi pathway. Unwanted cytotoxic and off-target effects may be caused as a result of consistent activation of the interferon response. None of the rAAVs with polycistronic cassettes described in this study induced the interferon response.

Wild type AAV2 commonly infects humans and as such pre-existing immunity against AAV2 can elicit an immune response to this vector thereby reducing the efficiency as a gene therapy delivery vehicle. rAAVs have had success in a number of clinical trials ranging in the treatment of various genetic diseases that include haemophilia, Leber's Congenital Amaurosis, lipoprotein lipase deficiency, cystic fibrosis, limb girdle muscular dystrophy and Duchenne Muscular dystrophy to name a few. Although the packaging capacity of rAAVs is limited, its application as a delivery vehicle is not. This is exemplified through the delivery of a minigene to treat Duchenne Muscular dystrophy (Mendell et al., 2010). AAV2 has been used as the delivery vehicle for many gene

therapeutics however there are two major hindrances associated with the use of this vector. First, roughly 25% of the human population has been exposed to the wild type AAV2 by the time they reach adulthood. As a result 80% of population has antibodies directed toward AAV2 and 30% have neutralising antibodies directed towards AAV2. This primary immunity to AAV2 results in rapid clearance of the viral vectors and prevents delivery of therapeutics (Chirmule et al., 1999, Hildinger et al., 2001, Gao et al., 2004a, Peden et al., 2004, Zaiss et al., 2008). The second major hurdle in using this vector for gene therapy targeted towards the liver is that AAV2 only infects roughly 10% of all hepatocytes and the transduction is slow (Nakai et al., 2005). Numerous strategies have been developed to circumvent these issues such as mutation of key amino acids within the capsid protein to allow for efficient transduction into hepatocytes (Sen et al., 2013, Gabriel et al., 2013). A short course of immuno-suppressants have been used in conjunction with AAV2 treatment to avoid immune response to the vector (Mingozzi et al., 2007, Wang et al., 2007, Karman et al., 2012). The most prominent strategy employed is pseudotyping, which involves using the capsid proteins from one serotype of AAV in combination with a genome from a different serotype. For example, AAVs that do not commonly infect humans, such as AAV8, are used for their capsid proteins and are typically used in combination with the genome from AAV2 serotype. (Hildinger et al., 2001, Gao et al., 2002, Grimm et al., 2003, Gao et al., 2004a, Peden et al., 2004, Gao et al., 2006, Zaiss et al., 2008). The AAV8 capsid is ideally suited to deliver anti-HCV therapeutics as its transduction efficiency of the liver is higher than that of AAV2 and is unlikely to be recognised by the immune system (Gao et al., 2002, Gao et al., 2004a, Nakai et al., 2005, Gao et al., 2006). AAV8 can transduce the liver much more efficiently than AAV2 as a consequence of the rapid transduction into all hepatocytes via clathrin coated pits and trafficking to the nucleus. The added benefit of higher transduction efficiency is of course that lower vector doses can be

used. As AAV8 is derived from a virus that infects non-human primates (rhesus monkeys) there is low pre-existing immunity within the human population against this capsid (Gao et al., 2002, Mount et al., 2002, Grimm et al., 2003, Thomas et al., 2004, Gao et al., 2006, Liu et al., 2013, Louis Jeune et al., 2013). Pseudotyping is commonly used to allow for repeat administration of gene therapy particularly when combating chronic diseases such as haemophilia (Xiao et al., 1999, Halbert et al., 2000, Riviere et al., 2006). This is done to allow for sustained and prolonged expression of therapeutic sequences. As AAVs allow prolonged expression of therapeutic sequences, from months to years, there may be no need for repeated administration of the anti-HCV therapeutic sequences. This period of time should be sufficient to ensure clearance of HCV infection and thus further expression of anti-HCV sequences may not be required. Future work could use rAAVs pseudotyped with AAV8 capsid to deliver the polycistronic cassettes described here to an animal model as this vector is well characterised and is known to be effective when used in animals (Monahan et al., 1998, Gao et al., 2002, Mount et al., 2002, Davidoff et al., 2005, Nakai et al., 2005, Gao et al., 2006, Wang et al., 2007, Nathwani et al., 2011).

The development of effective anti-viral treatments towards HCV has been hindered by the lack of an appropriate animal model. Humans and chimpanzees are the only known species that can be infected with HCV. While chimpanzees have been utilised in many pre-clinical studies of potential therapeutics, their use as an animal model is complicated by high costs, limited number of animals and low chronic HCV infection rates (Carroll et al., 2009, Carroll et al., 2011, Olsen et al., 2011). A number of mouse models have been developed that are amenable to HCV infection. Trimeric mouse models where human liver fragments that are infected with HCV are transplanted into the ear pinna or under the kidney capsule of the mouse permits HCV replication. Trimeric mice have been utilised for validation of

anti-HCV therapeutics (Ilan et al., 2002, Eren et al., 2006). Humanised mice that are genetically engineered to express HCV entry co-factors (CD81, occludin, SCARB-I, and CLDN1) were developed by Dorner *et al.* for the study of viral entry and immunisation (Dorner et al., 2011). Chimaeric mouse models where human hepatocytes are engrafted into SCID (severe combined immunodeficiency) mice have been used to validate anti-HCV therapeutic approaches (Mercer et al., 2001, Kneteman et al., 2006, Chayama et al., 2011). Washburn *et al.* developed a mouse model in which human hepatocyte progenitors as well as human $CD34^+$ human haematopoietic stem cells are engrafted into mice resulting in the expression human T cells and human hepatocytes (Washburn et al., 2011). These small animal models provide a platform for efficacy testing of potential HCV treatments and as such the rAAVs developed in this study could be used to demonstrate their efficacy *in vivo* in pre-clinical and clinical applications.

Another challenge associated with use of rAAVs for therapeutic application is the requirement for large scale production of vectors of clinical grade purity. There are numerous methods used to produce rAAV vectors for clinical applications however, each is associated with its own unique challenges. There are three main methods used in the upstream production of rAAVs and all involve cell culture. The predominant method employed involves transient transfection, where HEK293T cells are transiently transfected with the plasmids encoding the transgene, *rep* and *cap* genes and a helper plasmid that express genes required to generate rAAVs (Li et al., 1997, Matsushita et al., 1998, Xiao et al., 1998, Lock et al., 2010). This method is favoured as it allows for rapid modification of vectors, allows for the validation of rAAVs of diverse serotypes and allows for the validation of rAAVs with variations in expression cassettes. It does however present a problem with regard to scalability. Baculovirus systems are the second main upstream

method for production of rAAVs. This procedure involves infecting cells grown in suspension with Baculovirus and is suitable for large scale production. Difficulties associated with this production method include the need for multiple recombinant viruses and their associated instability (Huang et al., 2007b). A less common technique used for AAV production involves the use of producer cell lines which have integrated *rep* and *cap* genes and allow for efficient production of rAAVs. Producer cell lines are maintained in serum free cell suspensions which is attractive for scalability. These cell lines require the use of helper virus which is of concern as contamination of the rAAV vector preparation with helper virus may induce immune responses in patients (Yuan et al., 2011, Martin et al., 2013). Thus, this method requires robust downstream purification methods and screening. There are numerous well known techniques used for downstream purification of rAAVs. The main protocols employed here involve ultra high speed gradient centrifugation (caesium chloride and iodixonal) which are not amenable to large scale production as it requires specialised skills and is costly (Lock et al., 2010). There have also been investigations into chromatographic techniques which include ion-exchange, affinity, gravity flow and high resolution chromatography. These are convenient for large scale production but do not produce rAAVs of high purity (Zolotukhin et al., 1999, Auricchio et al., 2001, Kaludov et al., 2002, Zolotukhin et al., 2002, Urabe et al., 2006, Qu et al., 2007). Promising advances in large scale production include techniques that isolate rAAVs from media rather than cell lysates have the potential to significantly simplify downstream processing. Additionally, techniques such as tangential flow filtration have been combined with gradient centrifugation to purify rAAVs in a quick scalable manner (Vandenberghe et al., 2010, Doria et al., 2013). Although there are numerous challenges associated with large scale production new advances in technology promise to streamline the development of rAAVs for clinical use.

Numerous treatment options have recently been made available for HCV and include Ezetimibe, Telaprevir and Boceprevir, Simeprevir, Sofosbuvir and Miravirsen. All the current therapies developed have single targets within the HCV replication cycle and many of the currently developed treatments only target specific serotypes of HCV (Tungol et al., 2011, Sainz et al., 2012, Jacobson et al., 2013, Janssen et al., 2013, Lawitz et al., 2013). This presents a partial solution to the treatment of HCV as the virus is highly mutable. The emergence of treatment resistant virus or viral escape mutants is highly likely as HCV has mutation rates of 10^{-3} to 10^{-4} base substitutions per genome site per year, which is higher than the mutation rate of HIV (Ogata et al., 1991, Martell et al., 1992, Konishi et al., 2006). Many of the newly approved medications have not been tested in combination with other drugs. These medications have complicated regimens required to implement treatment for a single therapeutic making combination therapy difficult. Mimics of a naturally occurring polycistronic miRNA cluster have been employed to effect the knockdown of multiple targets of HIV-1 and prevent emergence of viral escape mutants (Liu et al., 2008). This principle can be applied to the therapeutic presented here with four pri-miRNA mimics targeting different regions in the highly conserved 5' UTR (Yokota et al., 2003, Wang et al., 2005, Wilson and Richardson, 2005, Watanabe et al., 2006). The highly mutable nature of HCV has led to the development of novel therapeutic approaches to limit the emergence of escape mutants. Various strategies have been developed from multiple siRNAs targeting different regions of the HCV genome to strategies that involve simultaneously targeting HCV and human receptors for the virus (Kronke et al., 2004, Wang et al., 2005, Henry et al., 2006, Sun et al., 2006, Shin et al., 2009). Targeting multiple sites of a virus' genome can potentially prevent emergence of viral escape mutants (Wilson and Richardson, 2005, Konishi et al., 2006, Ely et al., 2008). The

therapeutic modality described here would also be administered as a single intervention preventing the use of cumbersome therapeutic regimens.

The treatment of HCV has entered a new era of direct-acting antivirals, with small molecules leading the charge against HCV infection. The small molecule inhibitors are vastly improving therapeutics, showing much higher rates of sustained viral suppression than standard care. Some may speculate that advances in the field of direct-acting antivirals may render gene therapeutics against HCV obsolete. The future of HCV therapeutics will involve progression towards treatments that are potent, have short durations, are interferon free and have fewer side effects. Small molecule inhibitors are close to this ideal therapeutic modality but are not yet perfect. Many of the direct-acting antivirals target a single site within the HCV replication cycle and have incomplete coverage of HCV genotypes. Thus, a major hurdle in developing an ideal cure for HCV is the prevention of treatment resistant HCV. Combinational therapies with direct-acting antivirals have shown success in the past as was seen with ribavirin and Gilead's sofosbuvir which produced sustained viral suppression in 90% of patients (Lawitz and Gane, 2013). Although the combinational therapeutics can produce an impressive sustained viral suppression rate, this approach does have its own obstacles. Combinatorial approaches may increase treatment duration, drug-drug interactions require classification and increased side effects are a concern. This seems to be a regression in the treatment ideal for HCV and thus gene therapy still has a significant role to play in HCV treatment. Advantages conferred by the use of gene therapeutics include high barriers to viral resistance, simultaneous delivery of different therapeutic sequences, broad genome coverage, reduced off-target effects and a shorter duration of treatment. The RNAi pathway can be used to effect knock down of highly conserved regions within HCV, thus

providing a high barrier to resistance. Simultaneous targeting of the HCV genome has a twofold benefit as it allows for the inhibition of replication and provides a high barrier to HCV mutation. Gene therapeutics are highly specific which may prevent off-target effects resulting in less side effects. Finally, gene therapeutics can be delivered by vectors such as AAVs that allow for long term stable expression of transgenes resulting in durable expression of the therapeutic while reducing the number of treatments required to one or two administrations. The techniques presented here have not been investigated for their application to HCV treatment, and have predominantly been used in the study of HBV. In comparison to clustered expression cassettes, the technology developed here has numerous advantages (Yang et al., 2010). The pri-miRNA mimics developed here represent a modular technology, the number and order of pri-mRNA within a polycistronic cassette can be altered with relative ease thus allowing quick and efficient alteration of potential therapeutics. As monomeric pri-miRNAs are mimicked, maintaining the structural rigidity found within clustered pri-miRNA cassettes is of little consequence. An additional advantage to this modular system is that more than four pri-miRNAs may be included within a polycistronic cassette which is not the case with clustered expression cassettes. Rapid generation of different polycistronic cassette sequences with differing regulator elements such as promoters aids in the development of tailored therapeutics. The flexible nature of the modular multi-targeting system developed here allows for rapid alteration of therapeutic sequences and thus the development of potent, specific and safe gene therapeutics targeted toward HCV is possible. The anti-HCV rAAVs developed here demonstrate significant knock down of the expression of 5’ UTR and show dramatic inhibition of HCV replicon replication. This illustrates their utility as a potential therapeutic. A logical progression would be to pseudotype the rAAVs with capsids from AAV8. Pseudotyped rAAVs would allow the efficient delivery of the therapeutic

polycistronic constructs to small animal models. Humanised or chimeric mice would be the best models in which to determine efficacy and safety the pseudotyped rAAVs (Kneteman et al., 2006, Washburn et al., 2011, Chayama et al., 2011). Following this, investigations within a large animal model may be necessary. Considerations should be made for the production of sufficient quantities of the therapeutic rAAV for large animal studies, clinical trials and ultimately medical intervention. The multi-targeting gene therapeutic developed here has the capacity to prevent viral escape mutants and effectively eradicate HCV. These rAAVs successfully target and silence the 5' UTR and as such they represent a potential and efficient cure for HCV infection.

CHAPTER 5

5. APPENDIX.

5.1 Additional Figures.

5.1.1 Generation of pri-miRNA constructs.

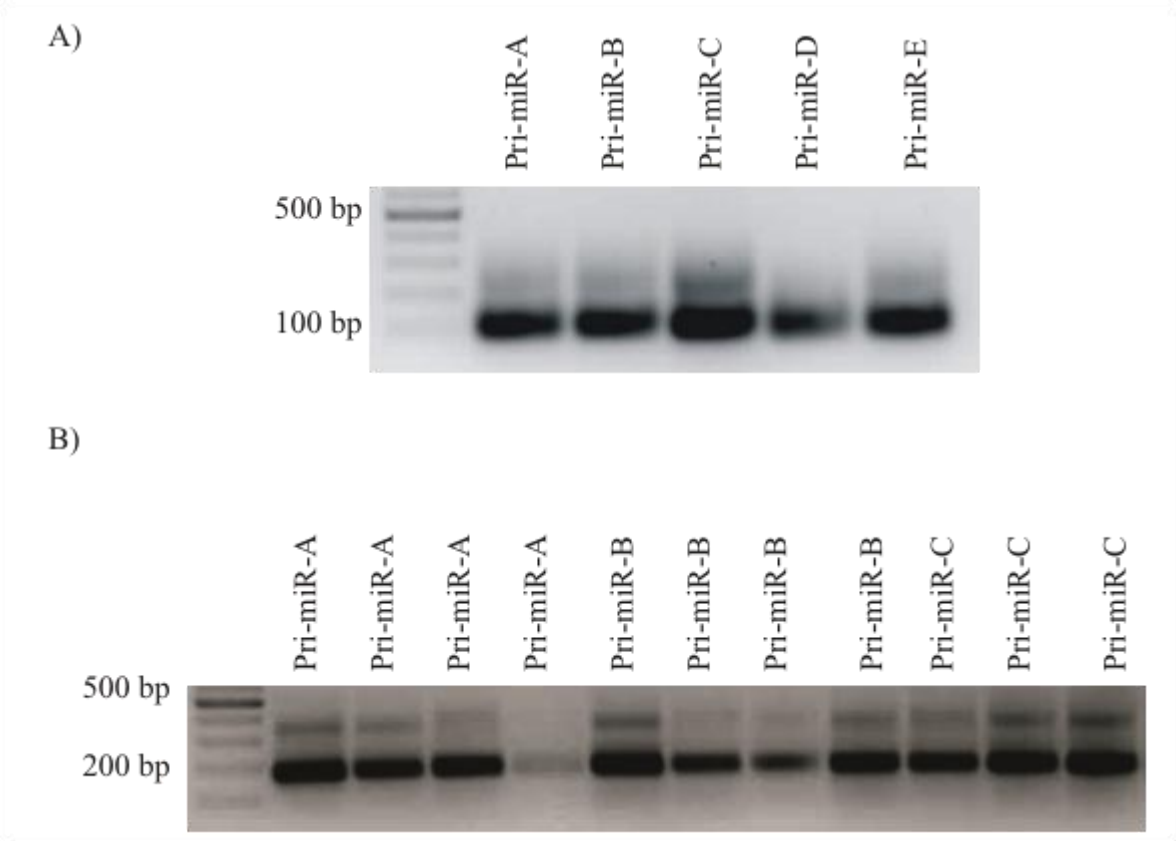

Figure 5.1: Gel electrophoresis of PCR products.

A) PCR (primer extension) amplification of the DNA required for the expression of pre-miRNA-31 constructs. PCR products were subjected to electrophoresis and fragment sizes were determined to be accurate at 100 bp. B) Amplification to create DNA constructs that encode complete pri-miRNA mimic sequence. PCR products were subject to electrophoresis and have accurate size of 200 bp. PCR products were attained for all 5 constructs.

5.1.2 Generation and selection of monomeric pTZ-pri-miRNA cassettes.

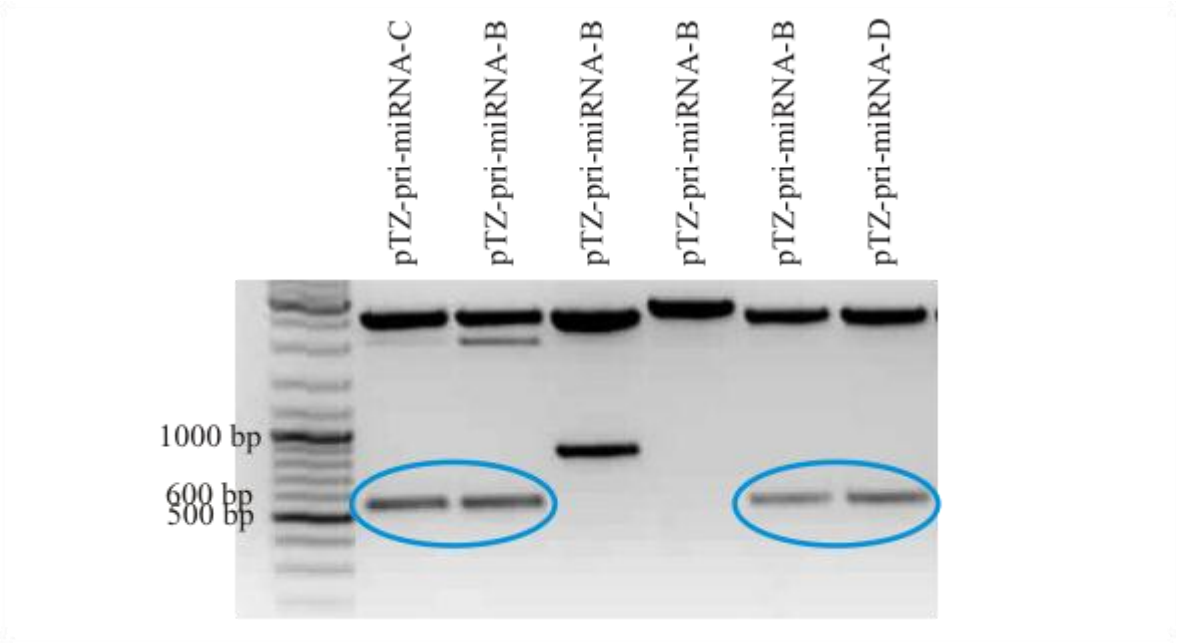

Figure 5.2: *Pvu* II screen of pTZ57R pri-miRNA mimic expression cassettes.

Fragments encoding the pri-miRNA where cloned into pTZ57R/T. The clones created were subsequently screened using *Pvu* II digest. The expected banding pattern for positive clones was 2 513 bp and 548 bp (indicated by blue circles). Postive clones were identified for all five constructs. Lane 1 O'GeneRuler™ DNA ladder mix (Thermo Fischer Scientific, MA, USA), 2- pTZ-pri-miRNA-C, 3-6 pTZ-pri-miRNA-B, 7 pTZ-pri-miRNA-D.

5.1.3 Sequencing pTZ-pri-miRNA momeric cassettes.

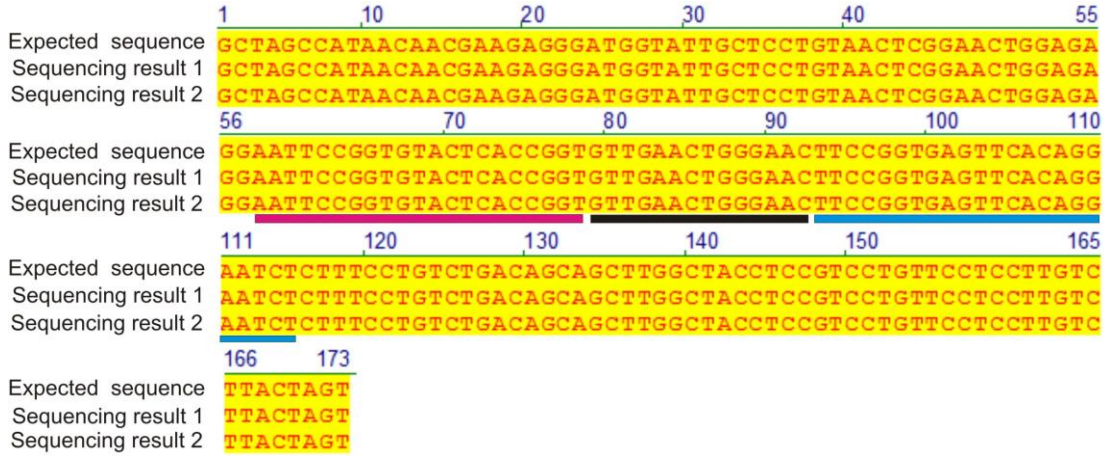

Figure 5.3: Sequencing of plasmid vectors carrying pri-miRNA mimics.

Sequencing on each clone was performed to ensure that the correct PCR product was cloned into pTZ57R/T. The sequencing results (pTZ-pri-miRNA-A is illustrated here) were subsequently aligned to the expected sequences and all constructs were found to possess the correct sequence. The guide sequence (indicated by pink bar), loop (black bar) and anti-guide sequence (blue bar) are also illustrated. Sequence alignments were similar for the other monomeric cassettes.

5.1.4 Selection of Two-mer pTZ-pri-miRNA cassettes.

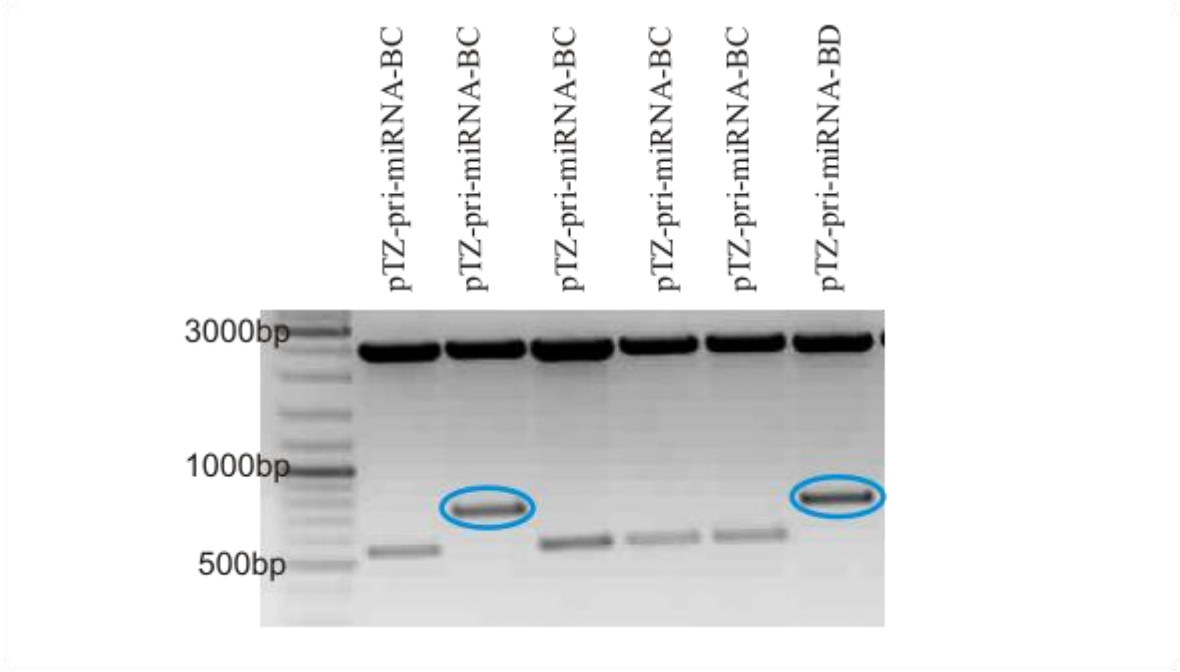

Figure 5.4: *Pvu* II screen of 2-mer expression cassettes.

Two separate fragments encoding separate pri-miRNA mimics were cloned into pTZ57R. The clones created were subsequently screened using *Pvu* II digest. The expected banding pattern for positive clones was 2 513 bp and 704 bp (indicated by blue circles). Lane 1- O'GeneRuler™ DNA ladder mix (Thermo Fischer Scientific, MA, USA),2-6 pTZ-pri-miRNA-BC, 7- pTZ-pri-miRNA-BD.

5.1.5 Selection of polycistronic pTZ-pri-miRNA cassettes.

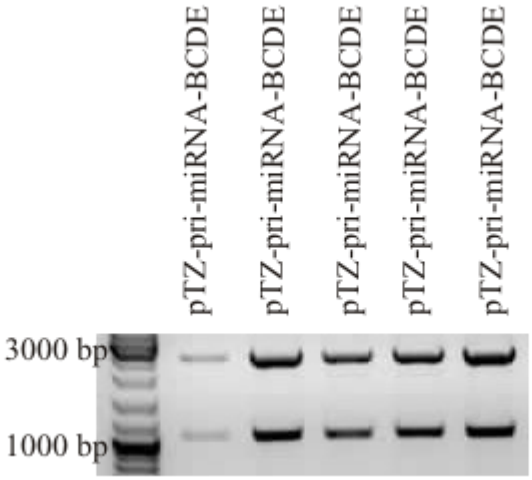

Figure 5.5: *Pvu* II screen of polycistronic cassettes.

Four separate fragments encoding the pri-miRNA mimics were cloned into pTZ57R. The clones created were subsequently screened using *Pvu* II digest. The expected banding pattern for positive clones was 2 513 bp and 1 038 bp. Lane 1- O'GeneRuler™ DNA ladder mix (Thermo Fischer Scientific, MA, USA), 2-6 pTZ-pri-miRNA-BCDE.

5.1.6 Generation of rAAV packaging plasmids containing polycistronic cassettes.

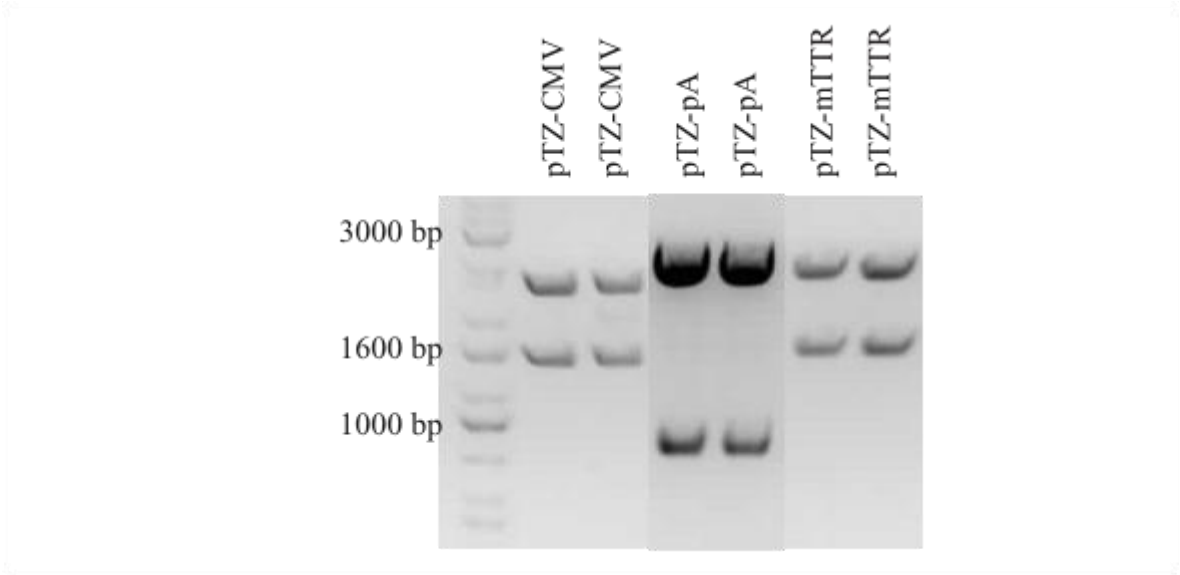

Figure 5.6: *Pvu* II screen of pTZ57R promoter and polyadenylation signal cassettes.

Fragments encoding the promoter sequences and polyadenylation sequences were cloned into pTZ57R/T. The clones created were subsequently screened using *Pvu* II digest. The expected banding pattern for positive clones was 2 513 bp and 1 499 bp for the promoter sequences and 2 513 bp and 705 bp for the polyadenylation signal. Lane 1 O'GeneRuler™ DNA ladder mix (Thermo Fischer Scientific, MA, USA), 2-3: pTZ-CMV, 3-4: pTZ-PolyA, 5-6: pTZ-mTTR.

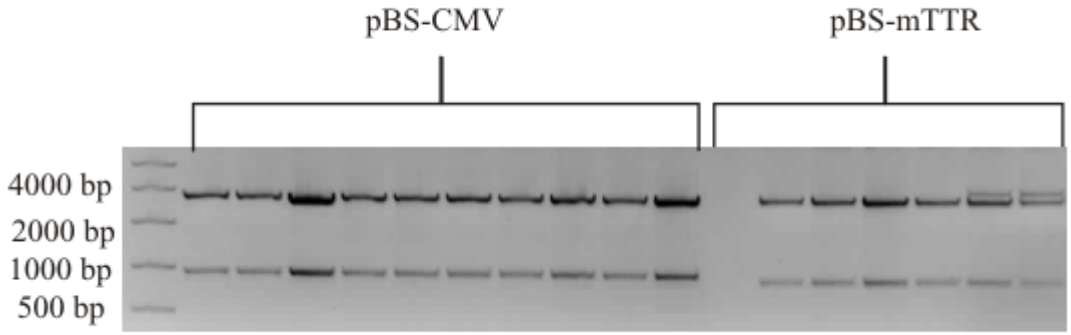

Figure 5.7: *Sac* I analysis of pBS-CMV and pBS-mTTR.

Fragments encoding the promoters were digested out of pTZ57R and ligated into pBS-H1-GFP. Screen for pBS-mTTR and pBS-CMV clones digested with *Sac* I, expected banding pattern for positive clones includes; 3 423bp and 914 bp for pBS-CMV and 3 423 bp and 835 bp for pBS-mTTR. Lane 1: FastRuler™ High range DNA ladder (Thermo Fischer Scientific, MA, USA). Lane: 2-11 pBS-CMV. Lane 13-18: pBS-mTTR.

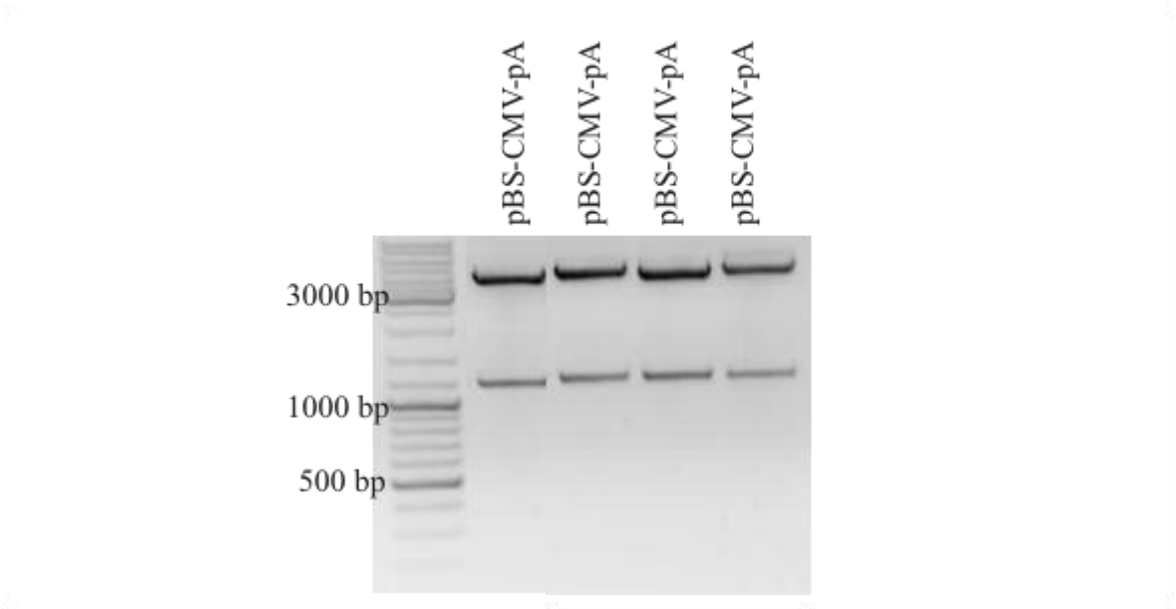

Figure 5.8: *Sal* I analysis of pBS-CMV-pA and pBS-mTTR-pA.

Fragments encoding the polyadenylation signal were digested out of pTZ57R and ligated into either pBS-CMV or pBS-mTTR. The clones created were subsequently screened using restriction digestion. Screen for pBS-mTTR-pA and pBS-CMV-pA clones digested with *Sal* I, expected banding pattern for positive clones includes; 3 540 bp and 1 105 bp for pBS-CMV-pA. Positive clones were also identified for pBS-CMV-pA. Lane 1: O'GeneRuler™ DNA ladder mix (Thermo Fischer Scientific, MA, USA) 2-4 pBS-CMV-pA.

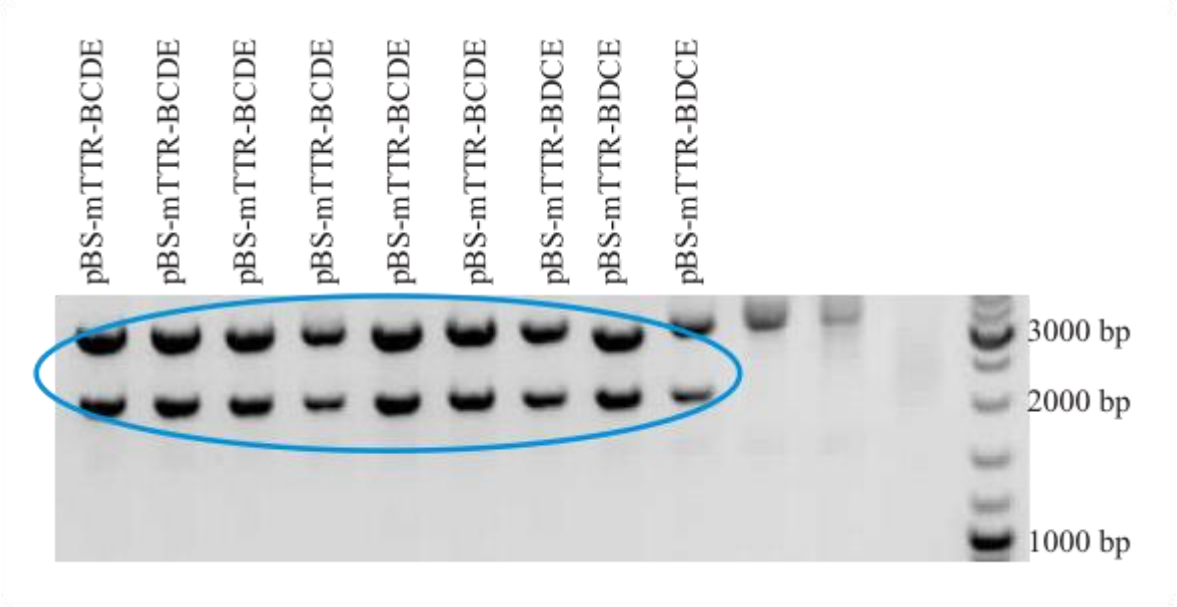

Figure 5.9: *Bgl* II restriction analysis of polycistronic cassette within the packaging plasmid.

Fragments encoding the polycistronic cassettes were digested out of pCI-pri-miRNA-BCDE and pCI-pri-miRNA-BDCE and ligated into both pBS-CMV-pA and pBS-mTTR-pA. Positive clones for all four vectors were initially screened with *Bgl* II restriction digest the expected banding pattern of 3 215 bp and 2 036 bp for pBS-mTTR- BCDE-pA and pBS-mTTR- BDCE-pA. Positive clones were also identified for pBS-CMV-BCDE-pA and pBS-CMV-BDCE-pA. Lane 1-6: pBS-mTTR-BCDE-pA, 7-9: pBS-mTTR-BDCE-pA, 13: O'GeneRuler™ DNA ladder mix (Thermo Fischer Scientific, MA, USA).

5.1.7 Assessment of silencing activity of anti-HCV pri-miR mimics.

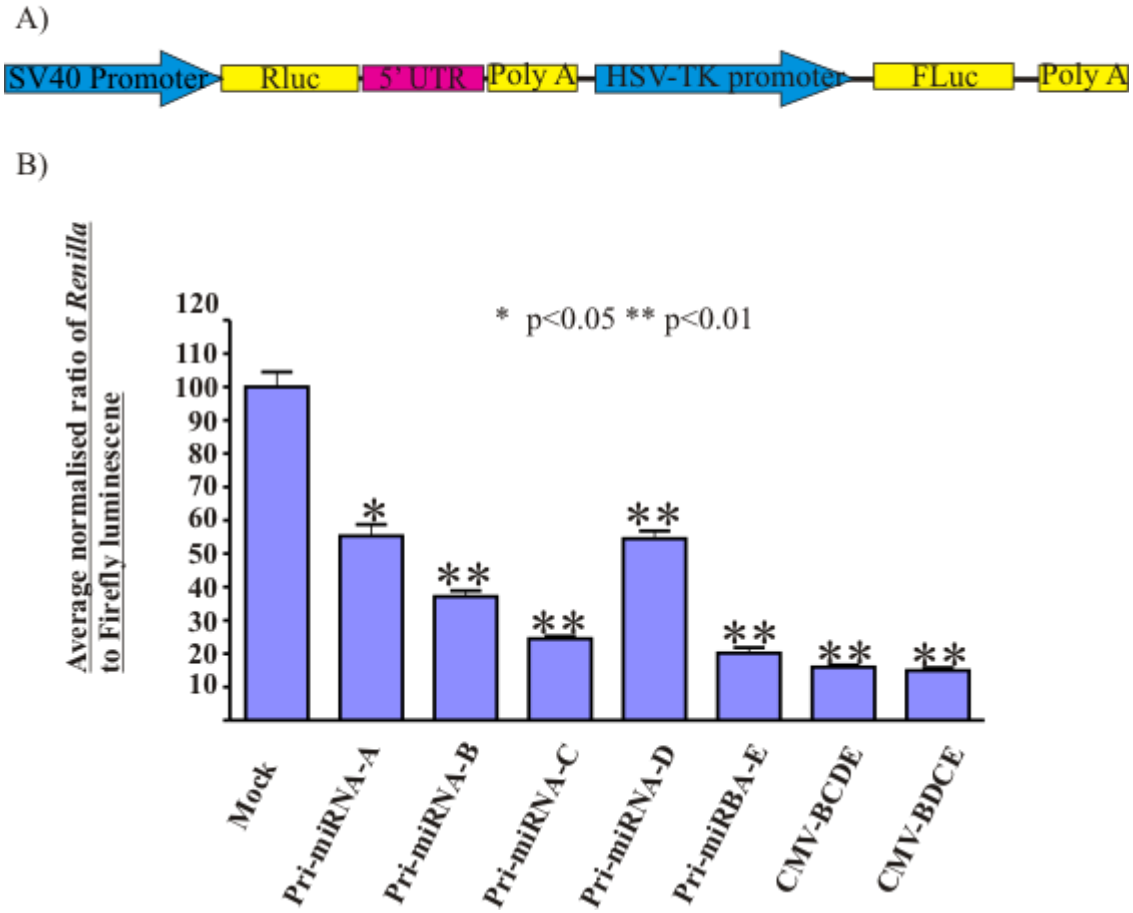

Figure 5.10: Silencing activitity of anti-HCV monomeric pri-miR mimics.

To determine the efficacy of each construct dual luciferase assay was performed. a) The 5' UTR region of HCV was cloned downstream of Renilla luciferase (Rluc) and is expressed as part of the mRNA. Firefly luciferase (Fluc) is expressed independently of Renilla luciferase as it has its own HSV-TK promoter. b) Luciferase assays were performed on lysates from cells transfected in triplicate with the indicated constructs and data plotted as the average ratio of Renilla/Firefly luciferase activity. Data was normalized to mock (pCI-neo).

5.1.8 Hirt extraction of rAAV vector genomic DNA.

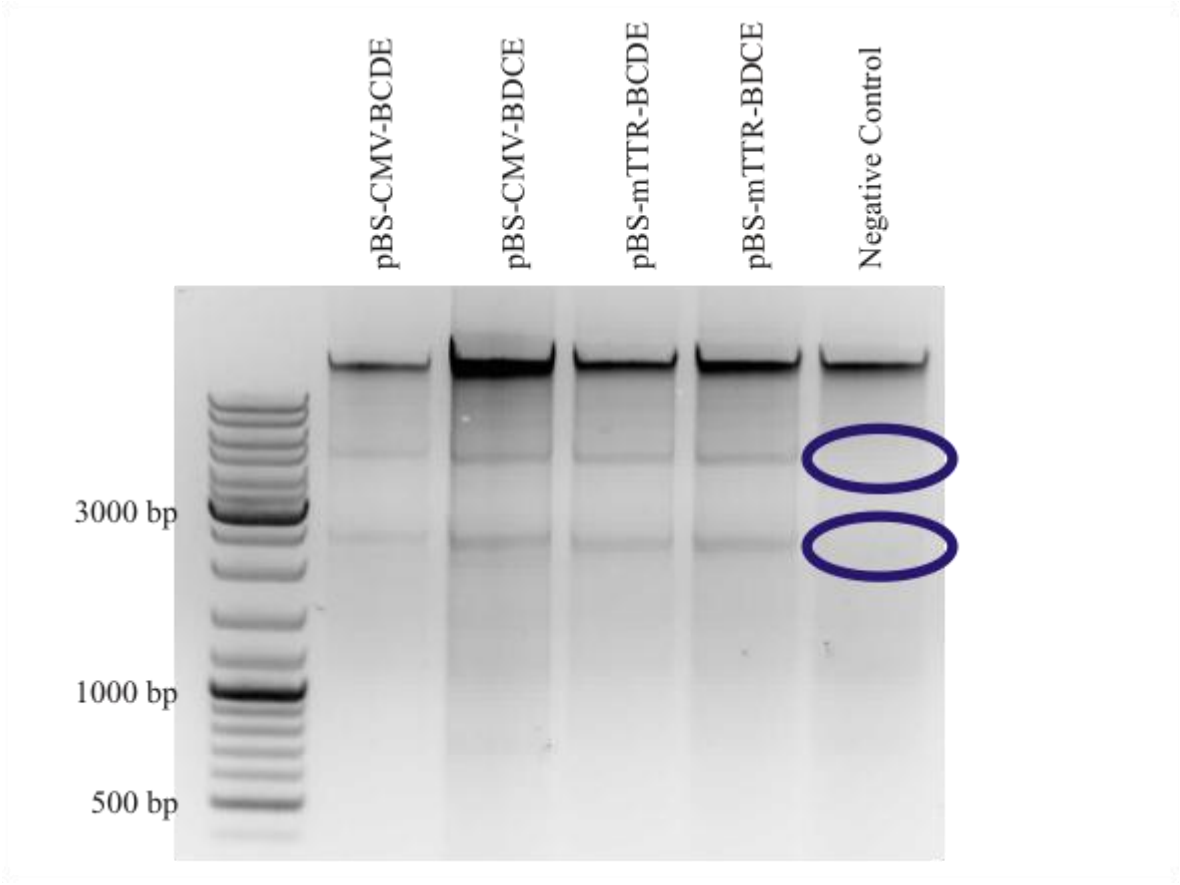

Figure 5.11: Hirt extractions of rAAV vector genomic DNA.

Hirt extractions were performed to allow for the detection of single and double stranded rAAV genomes, expected banding pattern would occur at 5 500 bp and 2 500 bp. These bands are not present in the negative control as indicated by the circles (No helper plasmid was included in this sample thus no viral propagation occurred).

5.2 Laboratory Techniques

5.2.1 Gel extraction.

5.2.1.1 MinElute Gel Extraction Kit (Qiagen, CA, USA)

Reagents

MinElute Gel Extraction Kit (Qiagen, CA, USA)

Protocol

DNA fragments were excised from an agarose gel using a clean, sharp scalpel blade and each fragment placed in a microcentrifuge tube. Fragments were then weighed and three volumes of buffer QG to one volume of the gel was added to the microcentrifuge tube. The samples were subsequently incubated at 50°C for ten minutes with mixing every two to three minutes during the incubation to dissolve the gel. One gel volume of isopropanol was added to the samples and mixed by inverting the tubes. The samples were placed into a MinElute® column and centrifuged for one minute at 16 100× g. The flow-through was discarded, the MinElute® column was placed back into the same collection tube and 500 µl of Buffer QG was added to the column. The columns were centrifuged for an additional minute at 16 100 ×g. The flow-through was discarded and the MinElute® column was washed with 750 µl of buffer PE. The flow-through was discarded and the MinElute® column was centrifuged for an additional minute at 16 100× g. The MinElute® column was placed into a clean 1.5 ml microcentrifuge tube and 10 µl of distilled water was placed on the MinElute® membrane and left to stand for a minute. The samples were centrifuged for one minute at 16 100× g to elute the DNA.

5.2.1.2 Phenol: chloroform extraction method.

Reagents

Salt saturated Phenol

Five hundred grams of phenol crystals were dissolved in 100 ml of 2 M Tris-HCl (pH 7.4) and 130 ml of water at 37 °C with stirring. The upper aqueous phase was removed and 100 ml of 2 M Tris-HCl (pH 7.4), 25 ml of m-creosol, 1 ml of beta-mercaptoethanol and 500 mg of 8-hydroxyquinoline added. The solution was mixed and the phases allowed to separate. The yellow salt-saturated phenol forms the top layer. The solution was stored in a dark container at room temperature.

Phenol:chloroform solution

Twenty-five millitres of salt-saturated phenol was added to 25 ml of chloroform to create a 50 ml solution.

3M Sodium Acetate

In 80 ml of distilled water, 40.8 g of sodium acetate was dissolved and adjusted to pH of 7. The volume was adjusted to 100 ml and the solution sterilised by autoclaving.

Protocol

Fragments of interest were excised form the agarose gel and placed in a microcentrifuge tube containing a filter. Excised samples were centrifuged for 30 minutes at 16 100× g to allow DNA to flow through the filter into a clean microcentrifuge tube. An equal volume of phenol:chloroform was added to the flow-through and mixed by vortexing. The microcentrifuge tube was centrifuged for one minute at 16 100× g. The aqueous phase was removed and placed in a new 1.5 ml microcentrifuge tube and the phenol:chloroform extraction was repeated. The extraction was repeated twice with chloroform only. Sodium

acetate (3 M) and 100% ethanol, equivalent 0.1× and 2.5× the volume of the removed aqueous phase was added. The sample was incubated at -20°C overnight. Following overnight incubation samples were centrifuged at 4°C for 30 minutes at 16 100× g to pellet the DNA. The 100% ethanol was removed and the pellet washed in 70% ethanol followed by centrifugation at 4°C for 5 minutes. The pellet was allowed to air dry and the DNA was re-suspended in 20 µl of distilled water.

5.2.1.3 EconoSpin™ pre-packed silica Membrane spin columns (Epoch Life Science, Inc., TX, USA) Extraction.

Protocol

Fragments from the gel were excised, and three volumes of GEX buffer (5.5 M guanidine thiocyanate; 20 mM Tris-HCl; pH 6.6) was added to the sample. Samples were then incubated at 55°C for ten minutes. The sample was transferred to an EconoSpin™ column and centrifuged for one minute at 16 100× g. The flow-through was discarded and 500 µl of PB buffer (5 M guanidine hydrochloride; 20 mM Tris-HCl (pH 6.6); 38% ethanol) was added to the column. The column was centrifuged at 16 100× g for one minute. Five hundred microlitres of WS buffer (10 mM Tris-HCl (pH 7.5); 80% ethanol) was added to the column and the samples were centrifuged at 16 100× g for one minute. Samples were centrifuged twice more followed by removing the column and placing it in a 1.5 ml microcentrifuge tube. Twenty microlitres of EB buffer (10 mM Tris-HCl; pH 8.5) was added to the column and allowed to stand for two minutes. The column was centrifuged for one minute at 16 100× g to elute the DNA.

5.2.2 Total DNA extraction using QIAamp DNA mini kit (Qiagen, CA, USA).

Protocol

Twenty microlitres of Proteinase K was added to 200 µl of virus sample, followed by the addition of 200 µl of AL Buffer. Samples were pulse-vortexed for 15 seconds and incubated at 56°C for 10 minutes. The samples were briefly centrifuged and 200 µl of 100% ethanol was added to each sample followed by 15 seconds of vortexing. The samples were again briefly centrifuged and 700 µl of the solution was then added to the QIAamp Mini spin column. The columns were placed in collection tubes and centrifuged for 1 minute at 6 000× g. The mini spin columns were removed and placed in new collection tubes and the old ones were discarded. Five hundred microlitres of Buffer AW1 was applied to the mini spin columns and the columns were centrifuged for 1 minute at 6 000× g. The old collection tubes were discarded and the mini spin columns placed in fresh collection tubes. Five hundred microliters of buffer AW2 was then added to the columns and the columns were centrifuged at 20 000× g for 3 minutes. The mini spin columns were placed in microcentrfuge tubes and centrifuged for an additional minute at 20 000× g. Mini spin columns were placed in fresh microcentrifuge tubes and distilled water was applied to the columns and left to incubate at room temperature for 5 minutes. Finally the columns were centrifuged at 6 000× g for 1 minute the filtrate was stored while the mini column was discarded and the filtrate was stored.

5.2.3 Preparing Luria-Bertani plate containing Ampicillin, IPTG, X-gal.

Reagents

1000× Ampicillin (10ml)

One gram of Ampicillin (Roche, Germany) was added to five millilitres of deionised water and five millilitres of ethanol.

Luria-Bertani agar plates containing ampicillin.

Ten grams of Bacto-tryptone (Oxoid, England), 5 g of Yeast extract (Oxoid, England), 10 g of Bacteriological agar (Oxoid, England), and 5 g of NaCl were dissolved in one litre of deionised water and autoclaved for 30 minutes at 121°C and 1 kg/cm^2. Ampicillin (1000×) was added to a final concentration of 100 µg/ml. Luria Bertani agar was then poured into perti dishes and allowed to solidify at room temperature.

5-bromo-4-chloro-3-indolyl-β-D-hiogalactopyranoside (X-gal) stock solution.

Twenty milligrams of X-gal (Sigma, MO, USA) was dissolved in one millilitre of dimethyl formamide, the microcentrifuge tube was then wrapped in aluminium foil as the solution is light sensitive and stored at -20°C.

Isopropyl-β -D-thiogalactopyranosid (IPTG) stock solution

One hundred milligrams of IPTG (Roche, Germany) was added to one millilitre of sterile water. The resulting solution was then filter sterilised.

Protocol

Eight microlitres of IPTG and forty microliters of X-gal stock solutions was added to the LB agar plates and spread evenly across the surface. The plates are then allowed to dry in the incubator for 30 minutes at 37°C.

5.2.4 Preparation and transformation of competent *E.coli*.

Reagents

Luria-Bertani media

Ten grams Bacto-tryptone (Oxoid, England), 5 g Yeast extract (Oxoid, England), and 5 g NaCl were dissolved in deionised water and autoclaved for 30 minutes at 121°C and 1 kg/cm^2.

Transformation Buffer (100ml)

Transformation buffer was prepared as follows: 1.47 g $CaCl_2.2H_2O$ (100 mM), 0.302 g PIPES (10 mM), 15 ml glycerol (15%) were dissolved in 80 ml of deionised water, the pH of the solution was adjusted to pH 7.0 with NaOH. The solution was then made up to 100 ml using deionised water. The buffer was then autoclaved at 121°C and 1 kg/cm^2 and stored at -20°C.

Protocol

Preparation of competent *E.coli*

A starter culture of *E. coli* (DH5α, Life Technologies, CA, USA) was prepared by inoculating 10 ml of LB broth with a glycerol stock of the bacteria and incubating overnight at 37°C with shaking. Fifty millilitres of Luria-Bertani broth was inoculated with one millilitre of *E. coli* culture incubated at 37°C until the absorbance read 0.4 at 600 nm. The cells were collected by centrifugation at 2 790× g for ten minutes. The pellet was then re-suspended in 20 ml of Transformation buffer and incubated on ice for 30 minutes. Following centrifugation for 15 minutes at 1090× g the cells were re-suspended in one millilitre of Transformation buffer. Aliquots of 100 µl were placed in sterile microcentrifuge tube and the competent *E. coli* was stored at -80°C.

Transformation of competent *E. coli*.

Five to ten microlitres of ligation mix was added to 100 µl of competent *E. coli* and incubated on ice for 30 minutes. Competent *E. coli* were then heat shocked at 42°C for 90 seconds followed by incubation on ice for five minutes. The transformed *E. coli* were then plated on agar containing selective antibiotics.

5.2.5 Blue/white screening of transformed *E. coli.*

Protocol

Transformed *E. coli* were plated onto the LB agar plates that contain X-gal and IPTG. The *E. coli* are then incubated at 37°C overnight. IPTG induces the expression of β-galactosidase, which will digest X-gal producing a blue colour. Inserts that have been successfully cloned into the plasmid will disrupt the *LacZ* gene and thus produce a mutant β-galactosidase resulting in a bacterial colony that has a white colour. Unsuccessful cloning results in the production of fully functional β-galactosidase and therefore these colonies have a blue appearance.

5.2.6 Plasmid preparation.

5.2.6.1 High Pure plasmid isolation kit (Roche Applied Science, Germany)

Reagents

Luria-Bertani media (Appendix 5.2.4)

High pure plasmid isolation kit (Roche Applied Science, Germany)

Protocol.

Ten millilitres of LB media was inoculated with a single bacterial colony and incubated overnight at 37°C with shaking. Cells were pelleted through centrifugation at 2 790× g for

10 minutes at 4°C. The pellets were re-suspended in 250 µl Suspension buffer (50 mM Tris-HCl; 10 mM EDTA; pH 8.0) containing RNAse A (10 mg/ml). Two hundred and fifty microlitres of Lysis buffer (0.2 M NaOH and 1% SDS) was subsequently added to the samples and allowed to incubate at room temperature for five minutes. Three hundred and fifty microlitres of chilled Binding buffer (4 M Guanidine hydrochloride; 0.5 M potassium acetate (pH 4.2)) was then added to each sample and incubated on ice for five minutes. The samples were then centrifuged for 10 minutes at 16 100× g. The supernatant was subsequently transferred to a High Pure filter column and centrifuged at 16 100× g for one minute. The flow-through was discarded and 700 µl of Wash buffer II (20 mM NaCl; 2 mM Tris-HCl (pH 7.5)) was added to the High pure filter column. Samples were centrifuged at 16 100× g for one minute the flow-through discarded and the samples were centrifuged for another minute at 16 100× g. The High Pure filter column was placed in a 1.5 ml microcentrifuge tube, 100 µl of the Elution buffer was added and the samples were centrifuged for 1 minute at 16 100× g.

5.2.6.2 Alkaline lysis method.

Reagents

Luria-Bertani media (Appendix 5.2.4)

Resuspension Buffer (Glucose buffer).

25 mM Tris pH 8, 50 mM Glucose and 10 mM EDTA.

Lysis Buffer (1L)

Eight grams of NaOH was added to 100 ml of 10% SDS. The solution was made up to one litre with distilled water.

Neutralising buffer (500 ml)

One hundred and forty seven grams of potassium acetate was added to 400 ml of distilled water, following this 57.5 ml glacial acetic acid was added to the solution. The solution was made up to 500 ml with distilled water.

Phenol:Chloroform solution (Appendix 5.2.1.2)

3M Sodium Acetate solution (pH 7) (Appendix 5.2.1.2)

Ethanol

Protocol.

Ten millilitres of LB media was inoculated with a single bacterial colony and incubated at 37°C overnight with shaking. The LB culture was then centrifuged at 6 000× g for 10 minutes at room temperature. The supernatant was discarded and the pellet was re-suspended in 150 µl Glucose buffer (25 mM Tris pH 8, 50 mM Glucose and 10 mM EDTA) and mixed by vortexing for 5 seconds. The sample was placed into a clean microcentrifuge tube and 300 µl of the Lysis buffer was added and mixed. Three hundred microlitres of the Neutralising buffer was added and the sample was centrifuged at 15 700× g for 15 minutes at room temperature. The supernatant was placed into a clean microcentrifuge tube. An equal volume of phenol:chloroform solution was added and the sample was centrifuged at 15 700× g for 10 min. The supernatant was placed in a fresh microcentrifuge tube and an equal volume of chloroform was added and the sample was centrifuged at 15 700× g for 10 minutes. The supernatant was placed into a fresh microcentrifuge tube and one tenth of this volume of a 3 M sodium acetate solution (pH 7) was added. Two and a half times the supernatant volume of ice cold 100% ethanol was added to the solution and mixed well. The solution was incubated at -20°C for 30 minutes. The sample was centrifuged at 15 700× g for 15 minutes, the supernatant discarded and the

pellet washed with 500 µl of 70% ethanol. The pellet was centrifuged at 15 700× g for 10 minutes and supernatant discarded. The pellet was allowed to air dry and was re-suspended in 50 µl of distilled water.

5.2.6.3 Plasmid Maxi Kit (Qiagen, CA, USA).

Reagents

Luria-Bertani media (Appendix 5.2.4)

Plasmid Maxi Kit (Qiagen, CA, USA).

Protocol

One hundred millilitres of LB media was inoculated with a single colony and incubated at 37°C overnight with shaking. The LB culture was then centrifuged at 6 000× g for 15 minutes at 4°C. Ten millilitres of Re-suspension buffer (Buffer P1: 50 mM Tris-HCl, 10 mM EDTA; pH 8.0) was then used to re-suspend the pellet. Ten millilitres of Lysis buffer (Buffer P2: 200 mM NaOH; 1% SDS) was subsequently added and the samples mixed by inverting the tube. The solution was incubated at room temperature for five minutes. Ten millilitres of chilled Binding buffer (Buffer P3: 3 M KAc, pH 5.5) was added to the samples and was mixed thoroughly by inverting the tube. This solution was then subjected to centrifugation at 6 000× g for 30 minutes at 4°C. The solution was centrifuged for 15 minutes at 20 000× g at 4°C. During centrifugation the QIAGEN-tip 500 was equilibrated by applying ten millilitres of Equilibration buffer (Buffer QBT: 750 mM NaCl; 50 mM MOPS, 15% isopropanol; 0.15% Triton® X-100; pH 7.0). The buffer was allowed to drain through the QIAGEN-tip 500 by gravity flow. The supernatant from the sample was applied to the QIAGEN-tip 500 and allowed to enter the column by gravity flow. The QIAGEN-tip 500 was subsequently washed using 60 ml of Wash buffer (Buffer QC: 1 M

NaCl; 50 mM MOPS; 15% isopropanol; pH 7.0). The QIAGEN-tip 500 was then placed into a sterile 50 ml tube and DNA was eluted by applying 15 ml of Elution buffer (Buffer QF: 1.25 M NaCl; 50 mM Tris-Cl; 15% isopropanol; pH 8.5). DNA was subsequently precipitated by adding 10.5 ml of room temperature isopropanol and incubated overnight at -20°C. The samples were centrifuged at 15 000× g for 30 minutes at 4°C. The isopropanol was removed and the DNA was washed using room temperature 70% ethanol. The samples were centrifuged at 15 000× g for 10 minutes and the 70% ethanol was removed. The pellet was allowed to air dry and was re-suspended in 200 µl of distilled water.

5.2.7 Tissue culture:

Reagents:

1 000× Pen/Strep

One gram of streptomycin and 0.62 g of penicillin were dissolved in 10 ml of distilled water and subsequently filter sterilised.

Geneticin (2 000 µg/ml)

One millilitre of distilled water was used to dissolve 1.39 g of Geneticin. Solution was mixed by vortexing and filter sterilised before use.

TrypLE Express (Life Technologies, CA, USA).

FCS (delta bioproducts, South Africa)

Opti-MEM (Life Technologies, CA, USA).)

Saline with 0.01% EDTA

DMEM medium (Life Technologies, CA, USA).

Protocol

Maintaining cells

Huh 7, Huh 7-11-7, Huh-luc-ET and HEK293T cells were incubated in a humidified incubator with 5% CO_2 at 37°C. Huh 7 and HEK293T cells were maintained in DMEM (Life Technologies, CA, USA) supplemented with FCS and antibiotics, penicillin (100 000 U/ml) and streptomycin (100 µg/ml) (Life Technologies, CA, USA). Huh-11-7 and Huh-luc-ET were grown in DMEM (Life Technologies, CA, USA) supplemented with 2 mM L-glutamine, 2.5% FCS, penicillin (100 000 U/ml), streptomycin (100 µg/ml) (Life Technologies, CA, USA) and Geneticin (1000 ng/ml) (Life Technologies, CA, USA). When cells reached a confluency of 90% or greater, cells were passaged into a new culture dish. Cells were first washed with five millilitres of saline containing 0.01% EDTA, followed by incubating the cells in fresh saline and EDTA for 10 minutes. Following this incubation, saline and EDTA was removed and 500 µl if TrypLE Express was added to the cells. The cells were dislodged from the culture dish by aspiration. HEK293T do not require treatment with EDTA or TrypLE Express to be dislodged. Cells were added to a new 10 cm tissue culture dish at a confluency of 25%. Ten millilitres of DMEM was added to the 10 cm dish and cells were incubated at 37°C with 5% CO_2. Growth medium was replaced every 24 hours until the cells were ready to be passaged.

5.2.8 RNA extraction.

Reagents

TRI Reagent® (Sigma-Aldrich, MO, USA).

Chloroform

Isopropanol

75% ethanol

Protocol 24-well plate (values in parentheses indicate volumes used for 6-well plates and 10 cm dishes, respectively)

Media was removed from the cells and 250 µl (500 µl; 1 000 µl) TRI Reagent® was added to each well. The cells were re-suspended and transferred to a microcentrifuge tube and incubated for 5 minutes at room temperature. Fifty microlitres (100 µl; 200 µl) of chloroform was added and samples were shaken vigorously for 15 seconds. The samples were incubated at room temperature for a further 2 minutes and centrifuged for 20 minutes at 4°C at 12 000× g. The aqueous layer was removed, placed in a clean microcentrifuge tube, an equal volume of isopropanol was added and the samples were stored overnight at -70°C. Following the overnight incubation the samples were centrifuged at 12 000× g for 20 minutes at 4°C. The supernatant was discarded, the pellet was washed with 250 µl (500 µl; 1000 µl) of 75% ethanol and subsequently centrifuged at 7 500× g for 5 minutes at 4°C. The supernatant was discarded and pellets were allowed for air dry for 5 minutes before being re-suspened in 50 µl (100 µl; 200 µl) of distilled water.

5.2.9 Protein quantification using Pierce™ BCA Protein assay kit (Thermo Fischer Scientific, MA, USA)

Reagents:

BCA Reagent A:

Sodium carbonate, Sodium bicarbonate, bicinchoninic acid, and sodium tartate in 0.1 M sodium hydroxide.

BCA Reagent B:

25 ml solution containing 4% cupric sulphate.

Bovine serum albumin (BSA) at 2 mg/ml in 0.9% Saline with 0.05% Sodium azide.

Protocol:

Dilutions of the BSA standards were made as indicated in Table 5.1. A working solution was made by mixing 50 parts of BCA reagent A with one part of BCA reagent B. One hundred microlitres of each standard and sample was transferred into a 2 ml microcentrifuge tube and 2 000 µl of working solution added. Samples were then incubated at room temperature for 2 hours or at 37°C for 30 minutes. The absorbance of each sample was then measured at 562 nm.

Table 5.1: Dilution series of BSA standard.

Vial	Volume of BSA	Volume of Diluent	Final Concentration
A	300 µl of stock.	0	2 000 µg/ml
B	375 µl of stock.	125 µl	1 500 µg/ml
C	325 µl of stock.	325 µl	1 000 µg/ml
D	175 µl if Vial B	175 µl	750 µg/ml
E	325 µl of vial C	325 µl	500 µg/ml
F	325 µl of vial E	325 µl	250 µg/ml
G	325 µl of vial F	325 µl	125 µg/ml
H	100 µl of vial G	400 µl	25 µg/ml
I	0	400 µl	0 µg/ml

5.2.10 Northern blot analysis.

Reagents:

TE Buffer:

10 mM Tris-HCl (pH8), 1 mM EDTA.

Sephadex

Five grams of sephadex G-25 was added to 50 ml TE buffer. This solution was left to incubate at room temperature overnight with rotation. The solution was centrifuged at 2 790× g for 2 minutes and the supernatant discarded. Fifty millilitres of fresh TE buffer was added and the solution centrifuged again at 2 790× g for 2 minutes. This was repeated 2-3 times.

10× TBE Buffer:

Four hundred millilitres of distilled water was used to dissolve 27.7 g boric acid powder and 50 g of Tris. Twenty millilitres of 0.5 M EDTA (pH 8) solution the pH adjusted to 8 and the solution was made up to 500 ml with distilled water. The solution was subsequently autoclaved.

20× SSC (3 M sodium chloride and 0.3 M sodium citrate).

One hundred and seventy five grams per litre of NaCl and 88 g/L Trisodium citrate dihydrate were dissolved in 900 ml of distilled water the pH was adjusted to 7.

10% SDS

Ten grams of SDS was dissolved in 100 ml of water.

15% Denaturing polyacrylamide gel.

Six millilitres of the 10× TBE buffer was mixed 28.8 g Urea (pH 8), 8.55 g acrylamide and 0.45 g bis-acrylamide and the solution was made up to 60 ml with distilled water. The solution was warmed to ensure all reagents dissolved and 300 µl of 10% ammonium persulphate and 30 µl of TEMED were then added to the solution. The gel was then poured into the casting apparatus and allowed to set at room temperature.

CHAPTER 6

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