EXPRESSION OF ANTI-HBV PRIMARY MICRO-RNA SHUTTLES USING AN INDUCIBLE PROMOTER SYSTEM

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

of

Master of Science in Medicine

Johannesburg, 2013

DECLARATION

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

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....27th...day of.....September....., 2013

To my biggest inspirations,

my father, Muzi and my mother and guardian angel, Catherine

CONFERENCE PRESENTATIONS

1. Mlambo T, Mowa B, Arbuthnot, PB. Expression of anti-HBV primary micro-RNA shuttles using an inducible promoter system. Molecular Biosciences Research Thrust (MBRT) Research Day; 2011 Dec 7th; Braamfontein, South Africa.

2. Mlambo T, Mowa B, Arbuthnot, PB. Expression of anti-HBV primary micro-RNA shuttles using an inducible promoter system. The South African Society for Biochemistry and Molecular Biology (SASBMB) Congress; 2012 Jan 29th-Feb 1st; The Drakensburg, South Africa.

3. Mlambo T, Mowa B, Arbuthnot, PB. Expression of anti-HBV primary micro-RNA shuttles using an inducible promoter system. The Faculty of Health Sciences Research Day; 2012 Sep 19th; Parktown, South Africa.

4. Mlambo T, Mowa B, Arbuthnot, PB. Inducible expression of primary micro-RNA mimics for the inhibition of HBV replication. The University of the Witwatersrand Cross Faculty Post-Graduate Symposium; 2012 Oct 19th & 22nd; Braamfontein, South Africa.

5. Mlambo T, Mowa B, Arbuthnot, PB. An inducible and liver-specific RNAi-activating expression system for the inhibition of HBV replication. Molecular Biosciences Research Thrust (MBRT) Research Day; 2012 Dec 5th; Braamfontein, South Africa.

ABSTRACT

Hepatitis B virus (HBV) infection is an important global health concern and chronic carriers of the virus are at high risk of developing hepatocellular carcinoma (HCC) and cirrhosis. Current therapies are only partially effective, which emphasises the need for improved treatment strategies. Harnessing the RNA interference (RNAi) pathway as a treatment strategy against HBV has shown great promise. However, there are obstacles that need to be overcome before RNAi-based treatment of HBV infection is realised. These include problems of liver tissue targeting and dose regulation. This study investigated the use of a liver specific and mifepristone-inducible RNA polymerase (Pol) II promoter system for the specific and precise regulation of anti-HBV sequence expression. The inducible system used consists of two expression cassettes; one containing the regulator/transactivator protein and another containing the transgene. Natural primary microRNA (pri-miR) mimics, pri-miR-31/5 and pri-miR-31/5/8/9, were used as anti-HBV sequences. *Firefly luciferase* gene expression was used to test modulation by the inducible system and to determine optimal induction conditions. The pri-miR-31/5, pri-miR-31/5/8/9 and luciferase encoding fragments were incorporated into the plasmid vector pRS17 that bears the inducible promoter, creating pRS-31/5, pRS-31/5/8/9 and pRS-Luc respectively. Firefly luciferase expression with this system was shown to be inducible and mifepristone dose-dependent. Effective knockdown of HBV gene expression was achieved with both pRS-31/5 and pRS-31/5/8/9 *in vitro* and *in vivo*. However, with high vector amounts, similar efficiency in silencing of HBV gene expression was observed in the presence and absence of the inducer mifepristone suggesting leaky expression of the pri-miRs. To

confirm this, knockdown studies were carried out with the pri-miR-31/5/8/9-expressing cassette separated from the transactivator cassette. HBV gene expression knockdown was observed with the pri-miR-31/5/8/9 cassette alone confirming leaky expression from the inducible system. Leakiness appears to be as a result of the *E1B* promoter driving the expression of the pri-miRs in the absence of mifepristone. However, reducing the vector amounts decreased basal expression and improved the inducibility of the system in cell culture studies. Successful propagation of an inducible and liver-specific RNAi-activating expression system will address the difficulty of achieving dose control of RNAi effectors and contribute to advancing the use of RNAi for HBV treatment.

ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to my supervisors Professor Patrick Arbuthnot and Dr Betty Mowa without whose guidance and support I could not have completed this dissertation.

I wish to thank Dr Abdullah Ely for his patience and assistance with various aspects of my project.

I am grateful to Dr Gloria Gonzalez–Aseguinolaza and Dr Ruben Hernandez-Alcoceba for their help with the pRS17 vector.

I would also like to thank my colleagues at the Antiviral Gene Therapy Research Unit for their assistance and helpful discussions.

I am sincerely thankful for the funding I have received from the University of the Witwatersrand Postgraduate Merit Award and Merit Scholarship, the Poliomyelitis Research Foundation and the National Research Foundation.

Special thanks also go to my family and friends for their constant support and encouragement.

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aa	-	amino acid
AAT	-	α_1-antitrypsin
AAV	-	adeno-associated virus
ATP	-	adenosine triphoshate
bp	-	base pair
BGHpA	-	bovine growth hormone polyadenylation signal
CAT	-	chloramphenicol acetyltransferase
cccDNA	-	covalently closed circular DNA
CMV	-	cytomegalovirus
COS-1	-	African green monkey kidney fibroblast-like cell line
Cox-2	-	cyclooxygenase-2
DBD	-	DNA binding domain
DGCR8	-	di George Critical Region 8
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	deoxyribonucleic acid
dNTP	-	deoxyribonucleotide triphosphate
DsRed2	-	*Discosoma* sp. Red fluorescent protein variant 2
dsRNA	-	double-stranded RNA

E.coli	-	*Escherichia coli*
EcR	-	ecdysone receptor
EDTA	-	ethylenediaminetetraacetic acid
EGFP	-	enhanced green fluorescent protein
ELISA	-	enzyme linked immunosorbent assay
Epo	-	erythropoietin
FCS	-	foetal calf serum
FRAP	-	FKBP12-rapamycin-associated protein
g	-	gravitational acceleration
HBeAg	-	hepatitis B virus e antigen
HBsAg	-	hepatitis B virus surface antigen
HBV	-	hepatitis B virus
HBx	-	hepatitis B virus X protein
HCC	-	hepatocellular carcinoma
HCV	-	hepatitis C virus
HEK293	-	human embryonic kidney cell line
HepaRG	-	human hepatoma cell line
HepG 2.2.15	-	human hepatoma cell line
HFN1	-	hepatocyte nuclear factor 1
hGH	-	human growth hormone
HIV	-	human immmunodeficiency virus

HSV	-	herpes simplex virus
Huh7	-	human hepatoma cell line
hVEGF	-	human vascular endothelial growth factor
IL-12	-	interleukin-12
i.p	-	intraperitoneal
IPTG	-	isopropyl-beta-D-thiogalactopyranoside
IRF	-	interferon regulatory factor
KRAB	-	Krüppel-associated box
LA	-	Luria Bertani agar
LB	-	Luria Bertani medium
lhRNA	-	long hairpin RNA
LTR	-	long terminal repeat
mfp	-	mifepristone
MHI	-	murine hydrodynamic injection
miR	-	microRNA
mRNA	-	messenger RNA
NF-κB	-	nuclear factor-kappaB
nt	-	nucleotide
NVV	-	non-viral vector
OD	-	optical density
ORF	-	open reading frame

PCR	-	Polymerase Chain Reaction
PEG	-	polyethylene glycol
PKR	-	protein kinase receptor
Pol	-	RNA polymerase
pre-miR	-	precursor miR
pre-mRNA	-	precursor mRNA
pre-rRNA	-	precursor rRNA
pri-miR	-	primary miR
PRLBD	-	progesterone ligand binding domain
qRT-PCR	-	quantitative reverse transcription PCR
rcDNA	-	relaxed circular DNA
RISC	-	RNA-induced silencing complex
RNA	-	ribonucleic acid
RNAi	-	RNA interference
RNase	-	ribonuclease
rpm	-	revolutions per minute
rRNA	-	ribosomal RNA
RXR	-	retinoid X receptor
SEAP	-	secreted human placental alkaline phosphatase
shRNA	-	short hairpin RNA
siRNA	-	short interfering RNA

snRNA	-	small nuclear RNA
SNALP	-	stable nucleic acid lipid particle
snoRNA	-	small nucleolar RNA
SNP	-	single nucleotide polymorphism
SV40pA	-	simian virus 40 polyadenylation signal
TAE buffer	-	tris-acetate-EDTA buffer
tet	-	tetracycline
*tet*R	-	tetracycline repressor
*tet*O	-	tetracycline operator
TERT	-	telomerase reverse transcriptase
tk	-	thymine kinase
TLR	-	toll-like receptor
TRBP	-	TAR-RNA-binding protein
TTR	-	transthyretin
tRNA	-	transfer RNA
UAS	-	upstream activation sequence
USP	-	ultraspiracle
UTR	-	untranslated region
UV	-	ultraviolet
VP16	-	virion protein 16
VPEs	-	viral particle equivalents

WHV	-	woodchuck hepatitis virus
X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZFHD1	-	zinc finger homeodomain 1

CHAPTER 1

1 INTRODUCTION

1.1 HBV infection and current treatment strategies

Hepatitis B virus (HBV) is a major global health burden and it is estimated that more than 2 billion people worldwide have been infected with HBV. Of these, between 350 and 400 million are chronically infected with the virus (1). Chronic carriers of HBV are at high risk of developing complications such as hepatocellular carcinoma (HCC) and cirrhosis (1-3). HCC is responsible for 85-90% of primary liver cancers (4) which represent the fifth most common cancer worldwide and the second most common cause of cancer-related mortality in men and the seventh most commonly diagnosed cancer and the sixth most common cause of cancer death in women (5). The virus is transmitted percutaneously, sexually and perinatally and is highly endemic in many regions of Africa (6, 7). An effective vaccine for HBV has been available since 1981, however, the vaccine must preferably be administered soon after birth before exposure to infection can occur (1, 8). As of 2008, there are seven licensed treatments for HBV: the nucleoside/nucleotide analogues adefovir, lamivudine, telbivudine, tenofovir and entecavir as well as the injected interferons, interferon α and pegylated interferon α (9, 10). One of the major limitations associated with the use of nucleoside/nucleotide analogues (particularly lamivudine) for the treatment of HBV is the emergence of viral resistance and this has led to a combinatorial approach to treatment (11, 12). The use of interferons has been restricted as a result of the high cost as well as the side effects and risk of hepatic injury associated with treatment (6, 13). These limitations necessitate novel treatment strategies for HBV which will allow for the sustained management of the virus with limited side effects.

1.2 RNA interference

The need for alternative HBV infection treatment strategies directed interest to the RNA interference (RNAi) pathway and the possibility of harnessing the pathway to advance effective antiviral therapeutics against HBV. RNAi was demonstrated in *Caenorhabditis elegans* by Fire, Mello and colleagues in 1998 (14). The RNAi pathway has since been identified in most eukaryotic cells and is characterised by the silencing of homologous genes by short, duplex RNA sequences usually between 21 and 23 nt in length (15, 16). The first step to produce these regulatory RNAi effectors in humans occurs in the nucleus and involves the transcription of microRNA (miR)-encoding genes by RNA polymerase (Pol) II to produce primary microRNA (pri-miR) sequences (Figure 1.1) (17). These sequences are then processed by the microprocessor complex comprising Drosha and DGCR8 proteins (di George Critical Region 8) to form precursor miR (pre-miR) hairpins, typically between 60-80 nt in length (18, 19). The nuclear karyopherin exportin 5 facilitates the export of these sequences from the nucleus to the cytoplasm. Here, they are further processed by Dicer and TRBP (TAR-RNA-binding protein) to form miR duplexes of between 21-23 nt in length containing 2-nt 3' hydroxyl overhangs (20-25). These duplexes are then presented to the RNA-induced silencing complex (RISC) which retains one strand, the "guide strand", while the other strand, the "passenger strand", is released from the complex (26-30). The guide strand, which is associated with Argonaute 2 (a component of RISC), subsequently guides the complex to a specific mRNA typically by binding to the 3' untranslated region (UTR) of the target mRNA. If the guide shares complete complementarity with the target site Argonaute 2 cleaves the target mRNA ultimately leading to degradation of the mRNA. (31-33). If the guide is partially complementary to its target translation will be suppressed (17). Mature miRs are rarely

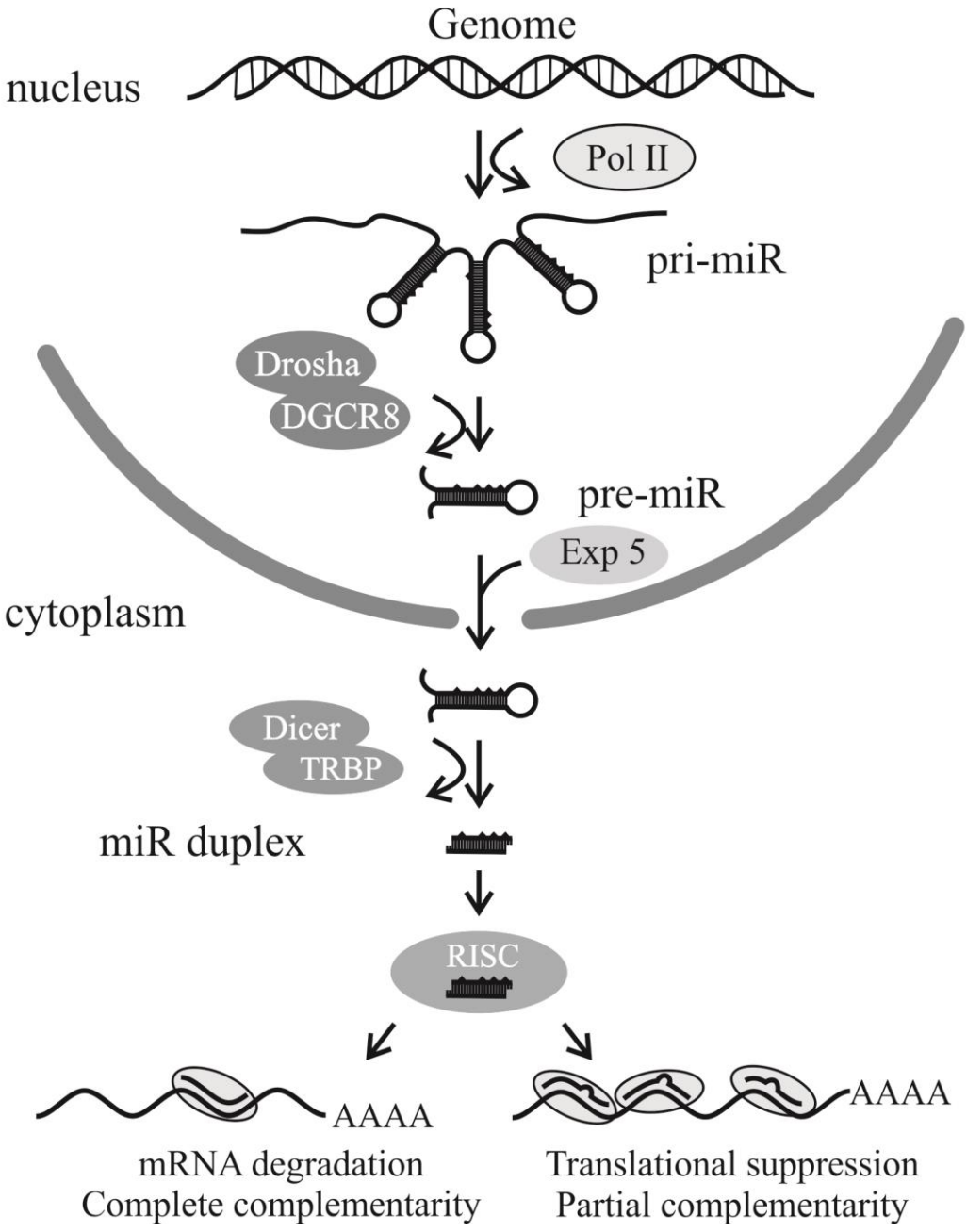


Figure 1.1: The RNAi pathway.

Transcription of miR-encoding genes by Pol II occurs in the nucleus to produce pri-miR sequences. These sequences are processed by Drosha and DGCR8 proteins to form pre-miR hairpins. The pre-miRs are exported to the cytoplasm by exportin 5 where they are processed by Dicer and TRBP to form miR duplexes of between 21-23 nt in length. These duplexes are incorporated into RISC which subsequently targets homologous mRNA. Adapted from Arbuthnot and Thompson (17).

entirely complementary to their targets and hybridisation between the target and nucleotides 2-7 in the 5' end of the guide strand, termed the "seed region", is sufficient to induce translational suppression (17, 34).

1.2.1 HBV as an RNAi target

HBV is a noncytopathic virus that belongs to the *Hepadnaviridae* family of hepatotropic DNA viruses. Its genome exists as partially double-stranded, relaxed circular DNA (rcDNA) of 3.2 kb. The HBV genome replicates via an RNA intermediate and rcDNA is converted to covalently closed circular DNA (cccDNA) within infected hepatocytes (13, 17, 35-37). The viral genome is compact and encodes four overlapping open reading frames (ORFs): *precore*/*core*, *polymerase*, *surface* and *X* (Figure 1.2). The compact genome and limited sequence plasticity makes HBV a good candidate for treatment involving nucleic acid hybridisation. In addition, the overlapping reading frames limit the possibility of viral escape (17, 38). Harnessing the RNAi pathway as a treatment strategy against HBV involves the use of exogenous RNAi effectors. These have proven to be valuable in antiviral therapeutics as they can be targeted to specific sites in the viral genome. These effectors can either be expressed or synthetic and activate RNAi at different stages of the pathway. However, a major limitation associated with RNAi-based treatment of HBV is the inability to eliminate the stable pool of cccDNA which is maintained within hepatocytes during infection with the virus (39).

The availability of valuable HBV infection models has facilitated the development of RNAi against HBV. Cell culture models of HBV infection typically make use of

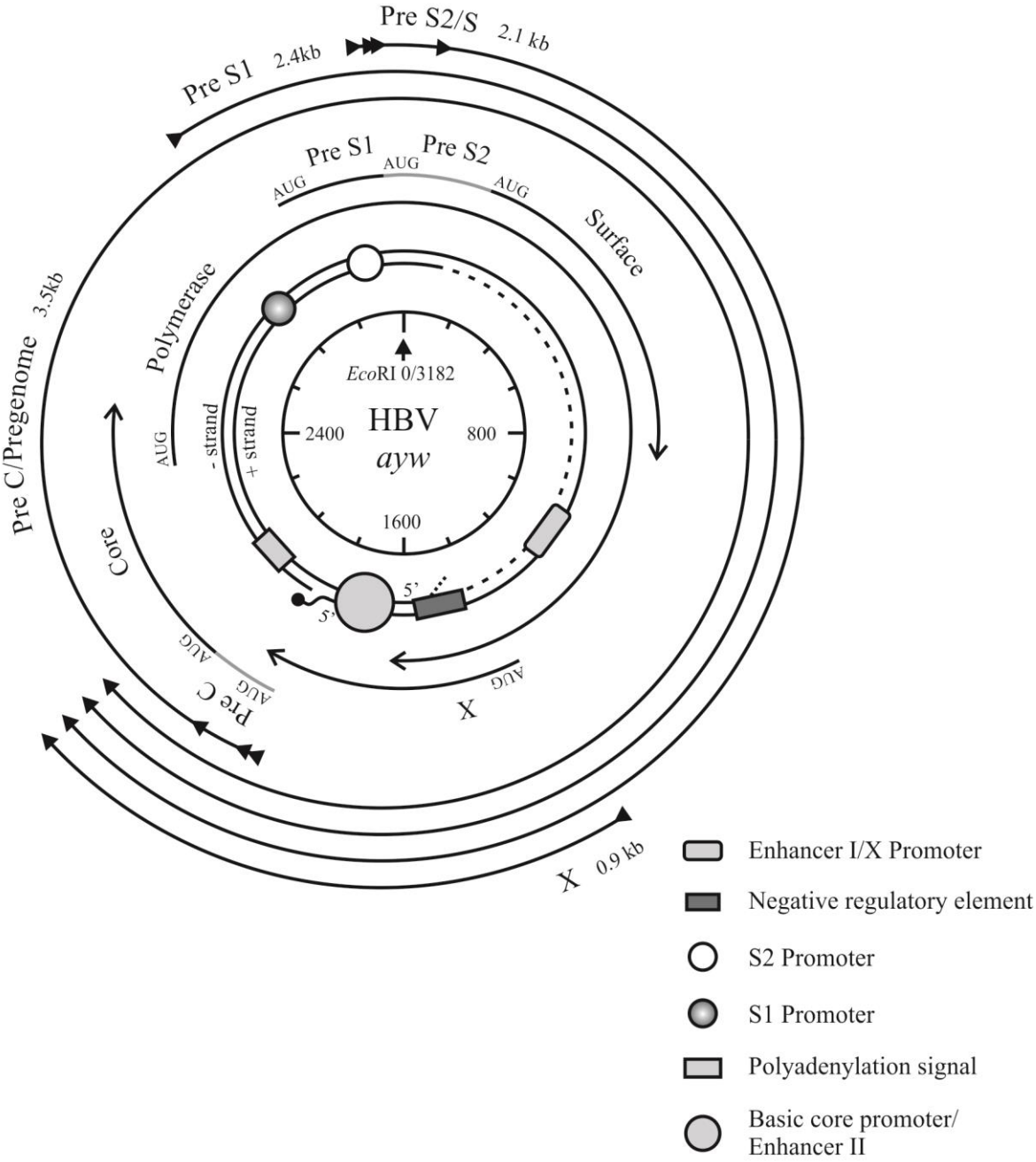


Figure 1.2: The organisation of the HBV genome.

The genome exists as partially double-stranded, relaxed circular DNA of 3.2 kb and encodes four overlapping ORFs: *precore*/*core*, *polymerase*, *surface* and *X*. Arrows immediately surrounding the genome indicate the four ORFs. The four outer arrows represent viral transcripts. Shaded rectangles and circles indicate regulatory elements. Coordinates are shown relative to the *Eco*RI restriction site. Adapted from Passman, Weinberg, Kew and Arbuthnot (40).

transiently transfected cells or cell lines which constitutively produce HBV particles (e.g. HepG 2.2.15 cells) (38). The development of the HepaRG cell line, which is the only cell line known to be susceptible to HBV infection, provided a more accurate model of HBV replication in cell culture. HepaRG cells also exhibit hepatocyte-like morphology, show specific hepatic functions and may contribute to elucidating the entry mechanism of HBV (41). Methods used to assess the inhibition of HBV replication *in vitro* include measuring hepatitis B surface antigen (HBsAg) secretion and reduction of viral RNAs and viral particle equivalents (VPEs) (38, 42, 43). Reporter assays (e.g. luciferase) have also been widely used to assess HBV gene expression knockdown *in vitro*. psiCHECK-*HBx* (44), which contains the *HBx* sequence downstream of the Renilla *luciferase* ORF, is an example of a plasmid that is used as a reporter target vector for the luciferase assay.

The murine hydrodynamic injection (MHI) provided the first *in vivo* model of HBV replication. This technique involves the high pressure injection of a HBV replication-competent plasmid through the tail vein of mice. Although the procedure itself is hepatotoxic, it results in the efficient uptake of the plasmid by the hepatocytes (45). Transgenic mice have also been used to simulate HBV viral replication. These animals have a replication-competent HBV sequence integrated into their genomes, but infection of hepatocytes does not occur as mice lack the required HBV entry receptor (46, 47). Nonetheless, this model mimics chronic infection in humans as HBV replication continues for the duration of the animal's life (48). The model commonly used for HBV infection involves chimaeric immunodeficient mice which have been engrafted with human hepatocytes. Induction of liver damage and subsequent loss of murine hepatocytes allows for the repopulation of the liver with human hepatocytes (49-52). This model is particularly

valuable because cccDNA is formed during viral replication in the human hepatocytes in contrast to the murine hydrodynamic injection and transgenic mouse models (53). Larger animal models for HBV replication have been identified including woodchucks (54) and chimpanzees (55); however, their use is restricted by the requirement for specialised housing, cost and ethical considerations (47, 56). Inhibition of HBV *in vivo* can be assessed by measuring markers of replication such as HBsAg and hepatitis B virus e antigen (HBeAg) and circulating viral particles in the serum (38, 43). Firefly luciferase expression can also be measured using bioluminescence imaging of live animals as a means of determining HBV inhibition. Typically, the RNAi target sequence is placed downstream of the *Firefly luciferase* gene such that silencing of the target results in diminished bioluminescence (57).

1.2.2 Advances in the use of RNAi against HBV

The exploitation of the RNAi pathway has mainly been achieved using synthetic short interfering RNAs (siRNAs) which are mimics of the dsRNA formed after processing by Dicer and TRBP, precursor microRNA mimics (pre-miRs) or short hairpin RNAs (shRNAs) that enter the pathway after Drosha processing and primary microRNA mimics (pri-miRs) (Figure 1.1) (58). Mimics of pre- and pri-miRs are commonly expressed from Pol III (e.g. U6 and H1) and Pol II promoters respectively (17, 59). In the case of HBV, there has been considerable progress using all three strategies over the past decade.

Initial studies by several groups demonstrated the efficacy of siRNAs against HBV expression and replication *in vitro*. Shlomai and Shaul showed that siRNAs targeted to the

HBx and *core* ORFs efficiently reduced the levels of the corresponding viral transcripts and proteins (60). The antiviral effect was sequence-specific and did not require active viral replication. Several regions of the HBV genome have since been targeted using RNAi. The first successful targeting of siRNAs to the liver was achieved by McCaffrey and colleagues (61). The hydrodynamic injection method was used to deliver a target construct containing the NS5B region of hepatitis C virus fused to luciferase RNA and an siRNA against NS5B. RNAi-mediated silencing was observed and demonstrated the efficacy of siRNAs in mice. Shortly after, a mouse model for HBV infection was developed using the hydrodynamic injection method which enabled the application of RNAi against actively replicating virus *in vivo* (62-64). In addition, McCaffrey and colleagues (64) as well as Giladi and colleagues (63) demonstrated the *in vivo* efficacy of U6 promoter cassettes encoding anti-HBV shRNAs and anti-HBV siRNAs respectively. While synthetic siRNAs have rapid and dose controlled effects (63), they are rapidly cleared from the liver and have a short half-life in serum. Therefore, repeated delivery would be necessary to achieve more sustained expression (58). In contrast, shRNAs result in more sustained expression (65).

Artificial pri-miRs have been developed in which the guide and complementary sequences of natural miRs are replaced with those of shRNAs (66). This approach takes advantage of the natural transcription of cellular miRs from Pol II promoters (59). In addition, expression from Pol II promoters allows for tissue-specific and regulated expression; the latter reducing the risk associated with oversaturation of the endogenous RNAi machinery (67). Zeng *et al*. first demonstrated that by substituting the stem sequence of miR-30 with unrelated base-paired sequences, novel miRs could be generated (68). This revealed the

potential of artificial miRs as therapeutic RNAi sequences. The pri-miR-30 backbone has since been widely utilised and characterised (66, 69, 70). Ely and colleagues exploited the liver-specificity and natural processing of miR-122, the most abundant miR in the human liver (71), by incorporating anti-HBV sequences into the pri-miR-122 backbone (57). Many miRs are encoded in genomic clusters which are transcribed as polycistronic miRs; this allows for the production of multiple miRs from a single transcription unit (72-74). This is particularly useful in limiting viral escape; and in the case of HBV provides the potential for targeting a range of HBV genotypes (70). The miR-106b and miR-17-92 polycistronic clusters have been used to generate multiplexed RNAi activators against HIV (75, 76). Chung and colleagues designed miR-155-based vectors which they demonstrated could be used to express up to 8 tandem copies of a synthetic miR from a single polycistronic unit to increase the inhibition of a single target mRNA (77). Progress has also been made in the use of expressed polycistronic cassettes against HBV. Ely *et al*. generated trimeric anti-HBV Pol II expression cassettes encoding mimics of miR-31 (78), thus harnessing the efficient processing of miR-31 by Drosha (79). The polycistronic pri-miR-31/5/8/9 (78) and monocistronic pri-miR-31/5 (57) anti-HBV expression cassettes designed by Ely and colleagues were used in this study. Snyder and colleagues went on to design a polycistronic Pol II-driven expression cassette against HBV which incorporated a liver-specific promoter thus affording the opportunity for tissue-specific expression (70). Artificial miRs have the added advantage of improved safety compared to shRNAs *in vitro* and *in vivo*. Boudreau *et al*. demonstrated that artificial miRs do not interfere with cellular processes such as miR biogenesis and are better tolerated than shRNAs (80). In addition, it has been proposed that the superior silencing observed with miRs can be attributed to their processing by Drosha/DGCR8 which simulates natural miR processing. The Drosha step is bypassed by shRNAs and may result in less efficient entry into the RNAi pathway (76).

1.2.3 Challenges associated with RNAi-based therapeutics against HBV

The discovery of RNAi evoked great interest and attracted focus to its potential in different applications. Although significant progress has been made in the development of antiviral strategies using RNAi, there are obstacles that must be overcome before it can become a clinically feasible treatment strategy. Ideal RNAi effectors against HBV must be potent, stable, effectively delivered at the specified doses and have limited toxicity as well as limited off-target effects (56). The main challenges, some of which are addressed in this study, are discussed below.

1.2.3.1 Off-target and immunostimulatory effects of RNAi activators

The presence of dsRNA in a cell can be indicative of an invading virus and will therefore trigger an immune response characterised by the release of inflammatory cytokines as well as the activation of the interferon response (81, 82). These off-target effects can be induced by dsRNA through the activation of dsRNA-dependent protein kinase receptor (PKR) and the interaction of dsRNA with toll-like receptors (TLRs) (81). The resulting cascade leads to the activation of transcription factors such as NF-κB, IRF3 and IRF7 and the increased expression of genes including inflammatory cytokines and interferons (17, 83, 84). This leads to the inhibition of protein synthesis and programmed cell death (82). It has also been shown that the type of RNAi effector and its specific properties may have an effect on immunostimulation (85). For example, synthetic siRNAs that are longer than 30 bp (86) and lack 2-nt 3'overhangs (87), among other characteristics, have been found to stimulate the immune response. In addition, several 'danger' motifs such as GU rich sequences have been found to be immunostimulatory (88). Hybridisation of RNAi effectors to unintended mRNA may also result in off-target effects. This non-specific silencing occurs as a result

of partial identity to the unintended target. It has been shown that sequences bearing as few as 11 contiguous complementary sequences can be directly silenced (89).

Off target effects have been attenuated in RNAi activators by various chemical modifications to siRNAs. The addition of a 5'-O-methyl group on the terminal ribose of the sense strand ensures that the antisense strand is preferentially incorporated into RISC thus limiting non-specific gene silencing (90). A 2'-O-methyl modification at position 2 of the guide strand has been shown to reduce the silencing of off-target sequences with partial complementarity to the seed region of the siRNA (91). Algorithms and guidelines have been developed to aid in the efficient design of RNAi effectors by predicting silencing efficiency thus preventing unintended silencing of mRNAs (92-94). In addition, microarray analysis of cellular transcripts has been proposed as a means of predicting off-target effects and ensuring the safety of RNA-based therapeutics (17). Importantly, targeted gene expression through the use of tissue-specific promoters and transcriptional control elements ensures that silencing is confined to specific tissues; thus limiting off-target effects brought about by non-specific binding or immune stimulation (95-97).

1.2.3.2 Optimising delivery vectors

The effective delivery of RNAi effectors to human patients in a clinical setting remains a major obstacle to advancement of RNAi-based therapy against HBV. Studies are ongoing in the development of safe and efficient delivery mechanisms that will facilitate targeted delivery to organs of choice. Both viral and non viral vectors have been investigated for their potential as RNAi delivery vehicles. The hydrodynamic injection method, while useful in animal models of HBV, is not a feasible method of delivery in the clinic. Proven

methods for siRNA delivery involve injection directly into the tissue thus reducing off-target effects, degradation and clearance of the siRNAs. However, many organs are not easily accessible for local delivery and such a method would be considerably invasive (98).

Morissey and colleagues demonstrated the benefit of chemical modifications for increasing the stability of siRNAs delivered intravenously in a mouse model (99). Various strategies have since been used to generate non-viral vectors (NVVs) to deliver siRNAs to the liver such as cationic lipid-containing nucleic acid complexes (lipoplexes) (100) and SNALPs (stable nucleic acid lipid particles) (101). The conjugation of siRNAs to lipids or steroids increases their hydrophobicity allowing transport across the cell membrane and protecting the siRNAs from nuclease activity (98). Nanoparticles containing siRNAs have also been generated using the 'stealth' polymer polyethylene glycol (PEG) to improve stability and passive hepatotropism (102). Components that target the liver and improve specificity such as apolipoprotein A-1 have also been incorporated into the design of siRNA NVVs (103). NVVs have the benefits of compatibility with siRNAs and the ability to be chemically modified and produced on a large scale thus making them useful tools for gene therapy applications (47).

Significant progress has also been made in developing a viral delivery strategy for anti-HBV RNAi effectors. Carmona *et al.* (104) and Uprichard *et al.* (105) demonstrated that hepatotropic recombinant adenoviral vectors expressing shRNAs from Pol III promoters suppressed pre-existing HBV gene expression and replication in transgenic mice. Adeno-associated viruses (AAVs) have also been shown to be promising delivery vectors for anti-HBV therapeutics (67). Both adenoviruses and AAVs are capable of efficiently

transducing hepatocytes *in vivo* (46) and infecting both dividing and non-dividing cells (106). However, the use of adenoviruses has been limited by their toxicity as a result of triggering the innate and adaptive response therefore limiting repeated administration (107, 108). The newer generation helper-dependent or 'gutless' adenoviruses which lack all viral coding sequences have reduced immunostimulatory effects and have been shown to deliver shRNAs (109) and pri-miRs (110) efficiently to the liver (109). While more difficult to propagate, these vectors are capable of long-term gene transfer (>2 years) *in vivo* (111). A study by Crowther *et al*. showed that the immunogenic effects of adenoviruses can be attenuated by PEG modification of the virus (42). In contrast, AAVs are nonpathogenic and do not stimulate the immune response. Many AAV serotypes have been described with AAV2 being the most well characterised and AAV8 being of particular interest for HBV therapy because of its natural hepatotropism (46). Chen *et al*. demonstrated that AAV2 could be combined with the capsid of AAV8 to deliver anti-HBV shRNAs efficiently to the liver without significant side effects (112). Lentiviral vectors based on the lentivirus genus of retroviruses (which includes HIV) have also been developed. These vectors have the benefit of infecting non-dividing and terminally undifferentiated cells (113). In addition, stable proviral DNA integration can enable long-term expression of RNAi effectors and thus sustained silencing of HBV (47, 114).

1.2.3.3 Viral escape

An important consideration in the development of antiviral therapeutics is the emergence of viral escape mutants which has led to a combinatorial approach to treatment. The same consideration must be applied to RNAi-based therapeutics. It has been demonstrated that single nucleotide substitutions within target mRNA, especially within the seed region, are

sufficient to provide protection against silencing by RNAi (115). RNAi resistance in HIV has been widely studied and shown to occur at various levels including: base substitutions or deletions within the target, substitution mutations upstream of the target resulting in altered viral mRNA secondary structures thus preventing access of RISC to its cognate target and mutations in the non-targeted long terminal repeat (LTR) promoter sequences which lead to upregulation of viral gene transcription (116)]. However, RNAi selection mutants have also been reported *in vitro* for poliovirus (115), HCV (117) and HBV (118). Combinatorial RNAi has proven to be a promising strategy against the selection of escape mutants. Multi-target gene silencing of conserved regions in viral genomes has been of particular interest (116).

Pol III-driven shRNA expression cassettes can be placed adjacent to each other within a single vector resulting in the expression of multiple shRNAs. However, viral escape can still occur, albeit at a delayed rate compared to the single shRNAs (116). Another strategy involves the use of long hairpin RNAs (lhRNAs) which can then be processed by Dicer into multiple siRNAs. Weinberg and colleagues designed lhRNAs targeting the *HBx* ORF of HBV as a means of limiting viral escape. Although having the advantage of targeting several sites of the genome simultaneously, processing across the lhRNA duplex was not equally efficient (44). In addition, there are concerns associated with the possible immunostimulatory effects of dsRNAs that are longer than 30 bp (86). However, in agreement with other studies (85), Weinberg and colleagues also demonstrated that expressed dsRNAs, including lhRNAs, do not induce the interferon response (44). Polycistronic miR mimics have also been widely used for multiple gene silencing and provide a means for limiting viral escape. Ely and colleagues were able to demonstrate effective HBV knockdown using expressed polycistronic miR cassettes targeting three

different viral genome sites. In addition, there was no evidence of unintended off-target effects (78). There has also been evidence of the potential of combining RNAi effectors with established licensed drugs for HBV. One study demonstrated the antiviral synergy between shRNAs and an existing HBV therapeutic-lamivudine; this may further reduce viral escape (119).

1.2.3.4 Dose regulation

Grimm *et al*. showed that U6-promoter-driven shRNAs expressed from recombinant AAVs resulted in dose-dependent liver injury and fatality in mice (67). Toxicity was demonstrated to be as a result of oversaturation of the miR processing machinery, specifically the karyopherin exportin 5, highlighting the importance of achieving dose control of expressed RNAi effectors. Pol III promoter-driven shRNAs have been found to result in significant HBV knockdown both *in vitro* and *in vivo* (104, 105); however, they are constitutively active and as such may result in overexpression of shRNAs and toxicity. Pol II promoters are tissue-specific and can be more easily regulated and have therefore proven to be valuable tools in the development of safe and efficient RNAi-based therapeutics (120). The mechanisms used to achieve dose regulation are discussed in detail in the following sections.

1.3 Mammalian promoters for the expression of RNAi effectors

Nuclear DNA transcription in eukaryotes is carried out by three RNA polymerases: Pol I, Pol II and Pol III. Each polymerase is highly specialised and catalyses the transcription of a specific group of genes. Pol I catalyses the transcription of precursor ribosomal RNA (pre-rRNA), which is processed into 28S, 5.8S and 18S rRNAs. Pol II is involved in the transcription of a wide range of genes including all protein-coding mRNAs, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and miRs. Pol III, similarly to Pol I, catalyses the transcription of a small subset of genes, the most abundant being the tRNAs (transfer RNAs) and the 5S rRNA (121-124). For transcription to take place, genes must contain specific promoters which are typically made up of two elements, the basal promoter elements and the modulator promoter elements. The basal elements drive low levels of transcription while the modulator elements enhance or reduce the basal levels of transcription. The polymerases cannot identify their target promoters directly, therefore basal promoter elements are first recognised by specific transcription elements which can then recruit the correct polymerase (125).

Pol II and Pol III promoters have been widely used for the expression of RNAi effectors. Based on the promoter structures and the requirements for transcription, genes transcribed by Pol III can be categorised in three ways: type 1 include the 5S rRNA genes, type 2 include the tRNA genes and type 3 include the snRNA U6 and H1 genes (125, 126). The two most commonly used promoters to drive the expression of shRNAs are the U6 and H1 promoters. The U6 snRNA is essential for pre-mRNA splicing (124) while H1 is the RNA component of the nuclear RNase P which cleaves tRNA precursors to produce the mature

5'-termini (127). The U6 and H1 promoters are favoured because they have a well-defined transcription start and stop site, simple structure and they naturally drive the expression of small RNAs (114, 127, 128). However, U6-driven expression of shRNAs has been shown to saturate the endogenous RNAi pathway and result in toxicity (67). In addition, it has been demonstrated that shRNA expression from the U6 promoter is more likely to induce the interferon response than expression from the H1 promoter (129). The weaker H1 and tRNA promoters may therefore be preferred for some applications (47). Modified tRNA promoters have been demonstrated to drive shRNA expression effectively and effect comparable inhibition of HIV replication to levels observed with U6 and H1 promoters (130, 131). However, tRNA promoters give rise to longer dsRNAs than H1 and U6 promoters thus increasing the risk associated with inducing the interferon response (114). The discovery that Pol III promoters can also transcribe miR genes (132) and that tissue specific expression can be achieved with tRNA promoters (133) may provide exciting new possibilities in the use of Pol III promoters.

With the exception of U6 snRNA, the major vertebrate U snRNAs (U1-U5) are transcribed by Pol II (134). Pol II promoters can be divided into two large classes: the mRNA and snRNA promoters; in addition Pol II has been found to naturally transcribe miR genes (59, 125). Tissue specific and inducible Pol II promoters can be used to address the dose regulation and tissue targeting problems associated with RNAi therapy. The cytomegalovirus (CMV) Pol II promoter has been widely used and demonstrated to drive high levels of transgene expression in the liver following the hydrodynamic injection (135). However, the use of Pol II promoters to express hairpin sequences has been limited by the variable silencing of conventional shRNAs; as observed with CMV-driven shRNAs

which were several orders of magnitude less efficient in knocking down the target compared to U6-driven constructs (92). These observations necessitate optimised design to achieve potent silencing. Nonetheless, it is evident that promoter choice is an important consideration for RNAi-based therapeutics.

1.4 Gene expression regulation and tissue targeting in gene therapy

Dose regulation and tissue targeting remain important obstacles preventing the advancement of gene therapy into the clinic. As a result, several studies have been conducted and considerable progress has been made in the development of expression systems that allow for the temporal and spatial control of therapeutic sequence expression. The ability to control gene expression will be particularly useful where potentially toxic transgenes or therapies are delivered. An important example relevant to the treatment of HBV is interferon therapy which is often not well-tolerated by patients and is associated with many side effects (6, 13). Therefore a system which will allow for the delivery of specified doses of interferon to the liver will be a major step towards developing safe and effective treatment against HBV (136).

1.4.1 Liver specific gene expression

The tissue-specific transcription of genes is an important feature of eukaryotic cells; however, for a long time the molecular mechanisms behind tissue-specific transcriptional control were unknown. Tissue specific transcription factors, the majority of which bind to promoter or enhancer regions, have since been identified as the major contributors to tissue-restricted transcription (137-139). The ability to achieve tissue specific gene

expression has been harnessed in the development of tissue specific gene delivery vectors targeting various tissues, organs and cells including the eye, liver, heart, pancreas, muscle, endothelium, neurons, central nervous system, T cells, dendritic cells, epithelial cells and haematopoietic system [reviewed in (140)]. Promoters classified as tumour-specific such as the promoters of the cyclooxygenase-2 (Cox-2) and telomerase reverse transcriptase (TERT) genes have also attracted interest as they enhance the expression of certain genes in numerous tumour types but not in normal tissues [reviewed in (141)].

The tissue-specific transcription of genes in the liver has been widely studied and has resulted in the identification of DNA binding transcription factors which interact with hepatocyte-specific promoters and enhancers. These transcription factors are not exclusively found in the liver but in other cell types as well. However, as illustrated with the transcription factor hepatocyte nuclear factor 1 (HNF1), binding sites for liver-specific transcription factors are more prevalent in liver-specific genes than in genes expressed in other tissues (138). Interestingly, HNF1 was found to bind to the promoter of the surface antigen of HBV possibly contributing to its hepatotropism (142). In addition, the natural tropism of HBV has been exploited to generate gene transfer vectors based on the HBV surface antigen L particle for liver-specific delivery (143). HNF1 has also been demonstrated to interact with the essential promoter regions of many liver-specific genes including albumin, α-fetoprotein and transthyretin (142).

Several promoters and enhancers of hepatic genes have since been used to achieve targeting to the liver, including those from albumin, α_1-antitrypsin (AAT), transthyretin and haemopexin genes. Albumin is an abundant protein which is mainly expressed in

hepatocytes (144, 145). AAT is a proteinase inhibitor that is also mainly synthesised in the liver (137, 145). Haemopexin is a glycoprotein whose synthesis is increased in response to acute infections in the liver (146) while the synthesis of transthyretin (prealbumin) is decreased in response to acute inflammation (147). A study comparing different liver specific promoters demonstrated that high-levels of gene expression were achieved and specificity was increased when the promoters were combined with albumin and hepatitis B-derived enhancers. (145). It has also been demonstrated that liver-specific promoters can be incorporated into viral vectors as a potential approach for targeting gene therapy agents to hepatic cells (148). This provides evidence of the great potential of liver-specific promoters in the development of anti-HBV therapeutics.

1.4.2 Inducible gene expression systems

The ability to control gene expression *in vitro* and *in vivo* is a valuable tool for many applications particularly in the development of clinical gene therapeutics. Inducible systems initially comprised promoters that were responsive to stimuli such as heavy metals, heat-shock and hormones. However, these are unsuitable for clinical applications owing to the pleiotropic effects of the inducing agents caused by interference with normal cellular physiology (149, 150). The ideal regulatory system should be active in the presence of an exogenous, orally bioavailable and physiologically inert small molecule and promote high levels of expression. However, induction should occur over a wide dose range of the inducer to allow for dose-responsive control of gene expression. In addition, it should show low basal activity in the absence of the inducer and be able to switch between the ‘on’ and ‘off’ state. Importantly, the constituent components should not interfere with endogenous cellular processes and should ideally be of human origin to reduce

immunogenic effects. Tissue-specific expression is also an important requirement to minimise off-target effects. Compatibility with conventional viral and non-viral vectors would also be an added advantage (151-154). Several strategies have been developed to enable gene expression under the control of a small exogenous molecule. The majority are based on the tetracycline repressor or the ecdysone, rapamycin and progesterone receptors.

The tetracycline (tet) inducible system developed by Gossen and Bujard is the most well characterised and most widely used and has been used for various applications *in vitro* and *in vivo*. It is based on the regulatory elements of the transposon Tn10 (Tn*10*)-specified tetracycline-resistance operon in *E.coli*. The main component is the tetracycline repressor (*tet*R) which negatively regulates the transcription of tet resistance-mediating genes (155). The first derivative of the tet inducible system, tet-OFF, was developed by combining *tet*R with the C-terminal domain of virion protein 16 (VP16) from the herpes simplex virus (HSV) which is essential for the transcription of immediate early viral genes. The resultant hybrid transactivator (tTA) stimulates minimal promoters fused to tetracycline operator (*tet*O) sequences (155). In the presence of tetracycline, the drug forms a complex with tTA therefore the binding of tTA to *tet*O sequences is inhibited and transcription does not occur (155). However, the continuous presence of tetracycline to switch off gene expression is not ideal. In addition, tetracycline deposits in the bone and is consequently cleared slowly *in vivo* preventing rapid induction of gene expression (156, 157). Therefore the Tet-OFF has been replaced in many applications by the Tet-ON system which shows the opposite mechanism. The limitations associated with both the tet-ON and tet-OFF systems include the toxicity of the tetracyclines themselves (158), toxicity of the tTA protein (157) and the variable basal expression observed in different cell types and tissues (150, 157, 159).

The ecdysone system harnesses insect hormone responsiveness and transfers it to mammalian cells giving rise to a means of achieving highly effective gene regulation (160). Steroid receptors belong to a family of transcription factors and their activity is tightly regulated by the binding of their cognate steroid ligand (161). During metamorphosis in *Drosophila melanogaster*, morphological changes are triggered by the steroid molting hormone 20-OH ecdysone through the ecdysone receptor. This response is mediated by the functional ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle (USP) gene (162). Hormone responsiveness similar to the natural mechanism in insects can be achieved in cultured mammalian cells by co-transfection with EcR, USP, an ecdysone responsive reporter and treatment with ecdysone or the synthetic analogue muristerone A (160). No and colleagues increased the sensitivity of the system by fusing a truncated ecdysone receptor to the activation domain of VP16 and replacing USP with its mammalian homologue, the retinoid X receptor (RXR). The benefits of ecdysteroids include their lipophilicity allowing penetration into all tissues and their short half-lives allowing for precise and potent inductions (160). However, limitations include possible immunogenic effects resulting from the expression of insect-derived proteins and slower clearance and metabolism of the lipid soluble steroid hormones (159).

The rapamycin system was developed by taking advantage of the ability of small immunosuppressive molecules to bind to members of the immunophilin protein family (150). Rapamycin is a natural heterodimeriser which complexes with the immunophilin FKBP12 and FKBP12-rapamycin-associated protein (FRAP) (157). To create the inducible system, a human chimaeric DNA binding domain ZFHD1 was joined to FKBP and the p65

activator domain derived from NF-κB was fused to FRAP. The addition of rapamycin results in dimerisation of the two fusion proteins and allows transcription of genes downstream of ZFHD1 binding sites (152, 163). Rapamycin is an ideal inducer as it is capable of entering most tissues and has a short half live *in vivo*. However, a major limitation is the growth inhibitory and immunosuppressive effects of rapamycin (157). This limitation has led to the use of rapamycin analogues (rapalogs) which do not bind efficiently to the wild-type FRAP kinase (164).

1.4.2.1 The mifepristone inducible system

Used in this study is the mifepristone inducible system, which has been well characterised. It is a valuable tool for gene therapy particularly for applications requiring efficient spatiotemporal gene expression with minimal toxic effects.

Mifepristone is a synthetic steroid known to have antiprogestin and antiglucocorticoid activity (165). It has been approved for human use for the termination of pregnancy and has been shown to be effective at doses ranging from 200-600 mg (166). Mifepristone has been well characterised and its metabolism, pharmacokinetics and pharmacodynamics in humans have been elucidated (167),((165). It has also been identified as having potential in the treatment of steroid-dependent tumours (168) and Cushing's syndrome (169). In addition, the side effects associated with mifepristone are moderate and it has been used safely long-term at doses up to 20 mg/kg in humans (170, 171).

The mifepristone inducible system was designed following the observation that a 42 amino

acid deletion in the C-terminal of the ligand-binding domain (LBD) of the human progesterone receptor rendered it incapable of binding to natural progestins. However, this mutant (hPRB891) retained its ability to bind and respond to synthetic antiprogestins such as mifepristone (RU486), not as antagonists but as agonists (172). Activation of gene expression by mifepristone is ideal compared to progestin agonists as it does not activate endogenous progesterone receptors which could result in pleiotropic effects in cells. The hPRB891 mutant was further modified to prevent activation of progesterone receptor-mediated genes in the presence of mifepristone thus giving rise to the mifepristone inducible system (173).

The main component of the first version of this system was the chimaeric regulator (GLVP) (Figure 1.3a) comprising the modified C-terminal ligand-binding domain of hPRB891 fused to the yeast transcriptional activator GAL4 and the activation domain from the HSV protein VP16 (174). The GAL4 region used serves a DNA-binding function (175), a GLVP dimerisation function (176) and contains a nuclear localisation signal (177). Replacing the DNA-binding domain of the progesterone receptor with that of GAL4 eliminated the possibility of activating endogenous progesterone-responsive genes. This modification also ensured that only genes with GAL4 DNA-binding sites would be activated in the presence of mifepristone. In addition, GAL4-activated genes have as yet not been identified in mammalian cells further ensuring that only the target gene would be activated (174).

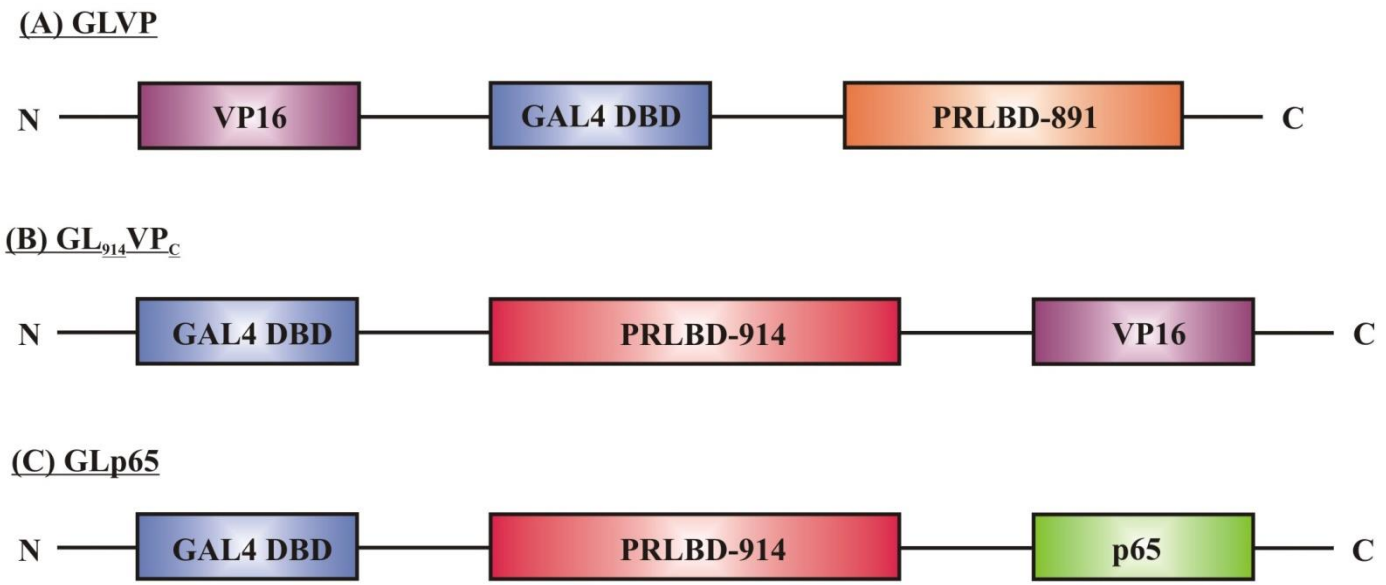


Figure 1.3: The structures of the mifepristone transactivator (TA) proteins.

(a) The first-generation transactivator, GLVP, comprises the activation domain VP16 derived from the HSV, the GAL4 DNA binding domain (GAL4 DBD) which is derived from yeast and the mutated progesterone ligand binding domain with a truncation of 42 C-terminal amino acids (PRLBD-891). (b) The structure of the modified GLVP transactivator. In $GL_{914}VP_C$ the VP16 domain is located in the C-terminus of the molecule and the length of the mutated progesterone ligand-binding domain is increased to 914 aa. (c) The structure of the second-generation transactivator, GLp65. It consists of the GAL4 DNA binding domain, the mutated progesterone ligand binding domain, PRLBD-914, with a truncation of 19 C-terminal amino acids and the activation domain of the p65 protein derived from NF-κB.

Following the observation that the GLVP regulator could effectively activate genes under the control of the thymine kinase gene (*tk*) promoter but was weaker under the control of a minimal promoter such as the TATA box, modifications were made to improve the transactivation potential of the regulator. Firstly, the VP16 domain was moved from the N terminus of the molecule to the C terminus. Secondly, the length of the mutated progesterone ligand-binding domain was increased by adding 23 amino acids, increasing its affinity for mifepristone. The new $GL_{914}VP_c$ (Figure 1.3b) construct exhibited enhanced transactivation potential and responded to concentrations of mifepristone 10-fold lower than the original construct without increasing the basal activity in the absence of the inducer (178).

The newest generation of the mifepristone inducible system consists of the GLp65 transactivator and is similar in function to the first generation system but differs structurally in two ways (Figure 1.3c). Firstly, to reduce immunogenic effects the virally derived VP16 activation domain was replaced with the human p65 transactivation domain, a component of the NF-κB complex (179). Secondly, it consists of the previously mentioned PRLBD-914 which is the truncated form of the progesterone ligand-binding domain with a deletion of 19 C-terminal amino acids instead of the original 42. This modified system shows lower basal activity in the absence of mifepristone and enhanced transgene activation when the inducer is administered. Tissue specific transgene expression was also achieved with GLp65 by coupling the regulator to the liver specific TTR promoter (180). The safety concerns associated with the constitutive expression of the GLp65 transactivator protein have also been addressed. The activation domain of GLp65 has been shown to interact with various transcription regulation and signaling factors. In

addition, the ligand-binding domain can bind heat-shock proteins and transcriptional co-activators. However, genome-wide microarray analysis performed in mice that had expressed the protein for a month revealed that there were no morphological or biochemical changes in the livers of the mice suggesting that the protein is well-tolerated (181). The absence of toxicity further demonstrates the safety of the mifepristone inducible system for use *in vivo*.

Despite the modifications, both generations of the mifepristone regulatory system function similarly (Figure 1.4). Using the GLp65 system as an example: in the absence of mifepristone, the GLp65 protein is constitutively expressed in tissues but remains inactive. Binding of mifepristone to the PRLBD-914 domain results in dimerisation of GLp65 and translocation to the nucleus. In the nucleus, the GAL4 DNA-binding domain interacts with the GAL4 upstream activation sequences (UAS) in the promoter of the target gene. This results in the p65 transactivation domain being in close enough proximity to activate target gene expression (173).

The system typically consists of two expression cassettes; one containing the regulator/transactivator protein and another containing the gene of interest. The transactivator can be under the control of any promoter and the target can be any gene placed under the control of a minimal promoter e.g. TATA box and four high-affinity GAL4 DNA-binding sites (173) (Figure 1.4).

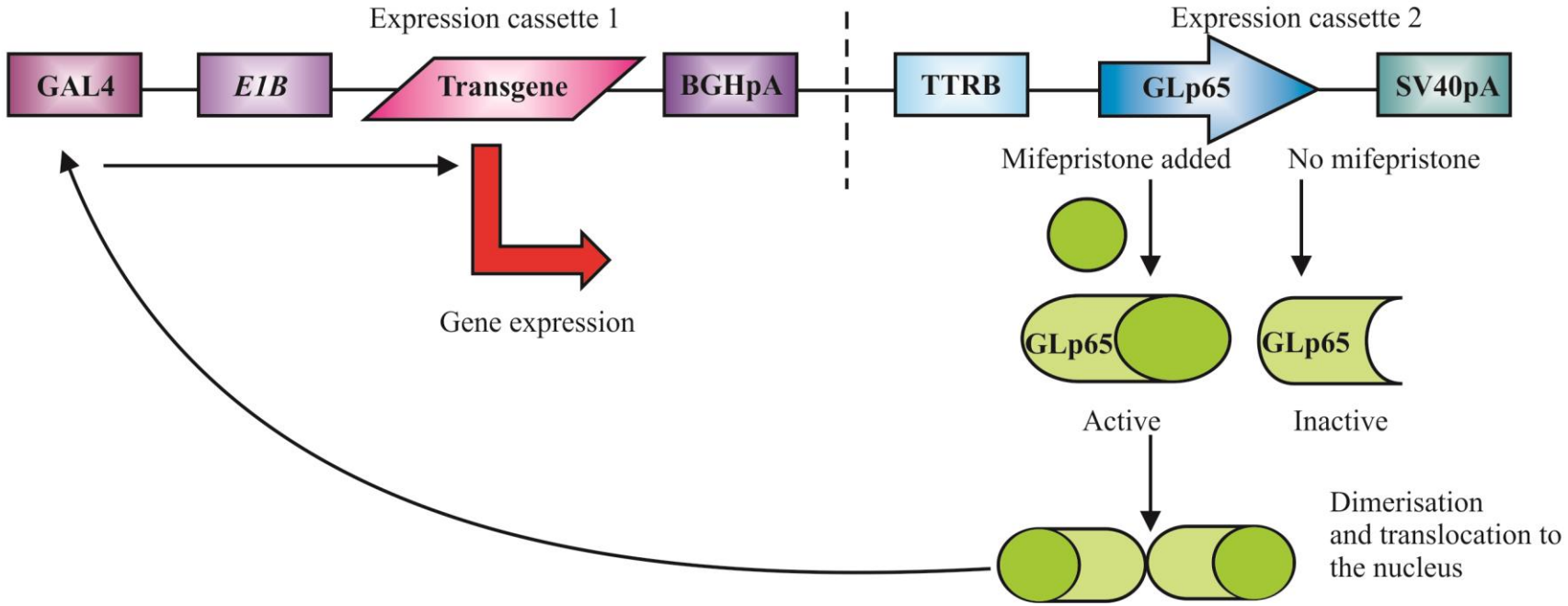


Figure 1.4: Mechanism and structure of the mifepristone inducible system used in this study.

The first expression cassette consists of a GAL4 binding site, the *ElB* TATA promoter upstream of the transgene and the BGHpA transcription stop signal. The second expression cassette consists of the TTRB fragment comprising a liver-specific promoter and enhancer from which the GLp65 transactivator is expressed and the SV40pA transcription stop signal. The GLp65 transactivator consists of the GAL4 DNA binding domain, the mutated progesterone ligand-binding domain PRLBD-914 and the transactivation domain p65. Binding of mifepristone to the PRLBD-914 domain results in dimerisation of GLp65 and translocation to the nucleus. In the nucleus, the GAL4 DBD domain interacts with the GAL4 upstream activation sequences (UAS) in the *E1B* promoter. This results in the activation of target gene expression by p65.

Activation of gene expression *in vitro* using this strategy was observed with concentrations of mifepristone as low as 0.1 nM and maximal levels of expression were achieved with 1 nM of the inducer (174). Wang and colleagues demonstrated that gene expression could be activated at mifepristone concentrations about 1000-fold lower than those required for termination of pregnancy (600 mg or about 10 mg/kg) (174). *In vivo* studies were carried out in transgenic mice where GLVP was expressed from the liver specific transthyretin (TTR) promoter. The authors demonstrated 1500-fold and 3500-fold induction after the oral administration of 250 µg/kg and 500 µg/kg of mifepristone respectively. Gene expression was confined to the liver and minimal expression was observed in the absence of mifepristone. In addition, a daily dose of 100 µg/kg of mifepristone from day 1 of mating did not cause pregnant mice to abort and did not result in developmental defects in the offspring (182). This indicates that the concentrations of mifepristone required for gene expression will not likely lead to detrimental effects.

The mifepristone inducible system has been shown to be versatile and amenable to use in different applications. Wang and colleagues demonstrated its function as an inducible repressor of gene expression by replacing the VP16 activation domain with the Krüppel-associated box (KRAB) repression domain. The inducible repressor inhibited chloramphenicol acetyltransferase (CAT) activity two to six-fold in the presence of mifepristone (178). In another variation, the promoter for the regulator protein is replaced with an autoinducible promoter that consists of GAL4 sites linked to a minimal *tk* promoter. When mifepristone is added, the regulator protein, initially present at low levels, becomes activated and binds to its own GAL4 sites as well as those of the transgene. This results in the activation of the transgene as well as the regulator itself in a progressive self-

amplifying process. This autoinducible system has been demonstrated to exhibit tighter regulation of gene expression compared to the original system (183). To avoid the possible toxicity associated with high levels of GAL4 expression, the GAL4 DBD has been substituted with the DNA-binding domain of the bacterial LexA repressor. This modification allowed inducible and specific gene expression in transgenic zebrafish (184).

Compatibility with viral vectors is an added advantage and delivery has been achieved *in vivo* using adenoviral (185), helper-dependent adenoviral (180), lentiviral (186) and HSV vectors (187). In addition, delivery has been demonstrated using non viral methods- direct injection of plasmid DNA into the hind-limb muscles of mice followed by electroporation. The authors demonstrated that multiple cycles of transgene regulation could be achieved by repeated induction with mifepristone (188). This system has also previously been used for the long-term, regulated and tissue specific expression of human interleukin-12 (hIL-12) as a potential therapy for liver cancer (189). Crettaz and colleagues went on to express murine interleukin-12 (mIL-12) successfully in a mifepristone-dependent manner using a helper-dependent adenoviral vector in the woodchuck model of HBV infection. Woodchucks treated with mIL-12 showed a marked and sustained reduction of viraemia as well as a reduction in woodchuck hepatitis virus (WHV) DNA, a loss of e and surface antigen and improved liver histology. In addition, the authors demonstrated that IL-12 gene transfer to the liver is capable of breaking the immunotolerance to viral antigens that is observed following neonatal infection with the virus (190).

1.4.2.2 Other inducible systems

The inducible systems discussed all have the added benefit of compatibility with viral vectors thus facilitating efficient delivery [reviewed in (153)]. Apart from systems induced by small molecules, several others have been designed to respond to other stimuli such as pH (191), heat shock (192), light (193), radiation (194), glucose (195) and hypoxia (196) although not all will be useful in clinical applications. In addition, gene regulation has been achieved with two- or three- component small-molecule activated gene switches [reviewed in (194)]. For example, the heat-shock induction of hsp70 has recently been combined with a rapamycin-dependent gene switch to further improve the spatiotemporal control of gene expression (197). The latest generation of inducible systems involves technologies that allow for the coordinated expression of several transgenes as well as the independent regulation of different sets of transgenes, In addition, epigenetic expression switches and gene networks with intrinsic expression memory have also been investigated [reviewed in (198)]. Another potentially powerful tool in gene regulation is the ability to control the removal of a gene when desired as has been achieved with the Cre-Lox system. The Cre-Lox system is comprised of a gene flanked by LoxP sites thus allowing the gene to be deleted when the Cre recombinase enzyme is expressed in the cell (199). This strategy has previously been used for the conditional, Cre-Lox regulated control of RNAi (200). In addition, coupling of this system with a regulatory system such as the mifepristone inducible system enables the inducible knockout of specific genes and thus contributes to generating valuable gene ablation models (201). More advances in the development of regulatable systems of gene expression are anticipated and will contribute to the feasibility of gene therapy strategies in the clinic.

1.5 Problem statement

The mifepristone inducible system is evidently a powerful tool with potential use in many applications and could contribute to the development of a safe and efficient treatment strategy against HBV and the overall advancement of RNAi to the clinic.

This study used this system to address some of the major limitations preventing the development of RNAi-based therapeutics against HBV. Current treatment strategies are only partially effective highlighting the urgent need for alternative treatments. The current limitations regarding the use of RNAi in the treatment of HBV infection mainly involve problems of delivery, targeting, dose regulation and safety. Therefore this study made use of the mifepristone inducible system for the tissue-specific and regulatable expression of anti-HBV pri-miR mimics. The Pol II CMV promoter driven mono- and polycistronic mimics of naturally occurring pri-miR-31 were designed in our lab, whereby anti-HBV sequences were used to substitute the guide and the complementary sequences (pri-miR-31/5 and pri-miR-31/5/8/9). These CMV-expressed mimics of pri-miR-31 have previously been demonstrated to effect efficient knockdown of HBV replication (57, 78). In this study the pri- miR-31/5 and pri-miR-31/5/8/9 sequences were incorporated into the mifepristone inducible system to enable their dose regulation and liver specific expression. The regulator used is under the control of a liver-specific promoter thus ensuring gene expression is confined to hepatocytes, the primary site of HBV infection. In addition, the regulator is designed to only activate the expression of the pri- miRs in the presence of the inducer mifepristone. The ability to fine-tune transgene expression with mifepristone will address the dose-regulation and toxicity concerns associated with expressed RNAi activators.

1.6 Aims and objectives

The main aim of this study was to express the anti-HBV pri-miR-31/5 and pri-miR-31/5/8/9 sequences under the control of a liver-specific and inducible promoter *in vitro* and *in vivo*.

Objectives:

1. To construct mifepristone inducible promoter-driven pri-miR-31/5, pri-miR-31/5/8/9 and the *Firefly luciferase* gene-containing plasmid.
2. To use the luciferase-expressing construct to test modulation by the inducible promoter in liver-derived Huh7 cells and in mice by measuring *Firefly luciferase* gene expression at different concentrations of the inducer mifepristone.
3. To assess HBV knockdown in Huh7 cells transfected with pri-miR-expressing and HBV target constructs using ELISA and the Dual Luciferase assay.
4. To measure HBV knockdown in mice injected with anti-HBV and target plasmids via tail vein hydrodynamic injection using bioluminescence imaging.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Bacterial methods

2.1.1 Culturing of *Escherichia coli* in Luria Bertani medium

The XL1-Blue strain of *E.coli* was grown on Luria Bertani agar (LA) or in Luria Bertani medium (LB) (Appendix 6.1) overnight at 37°C. Liquid cultures were shaken at 200-250 rpm. *E.coli* strains carrying ampicillin resistance plasmid were cultured in medium containing ampicillin antibiotic (Appendix 6.1) at a concentration of 100 µg/ml.

2.1.2 Preparation of chemically competent *E.coli* cells

E.coli cells from a freezer stock or a single colony were inoculated into LB and the culture incubated overnight at 37°C with shaking at 200-250 rpm. The pre-culture was diluted 100-fold and incubated at 37°C with shaking at 200-250 rpm until the absorbance reading at 600 nm was between 0.4-0.6. Following this, the cells were centrifuged at 3200 *g* for 15 minutes at 4°C. The pellet was resuspended in 20 ml of transformation buffer (Appendix 6.1) for every 100 ml of culture and incubated on ice for 20 minutes. The cells were centrifuged at 800 *g* for 15 minutes at 4°C and the pellet resuspended in 1 ml of transformation buffer. Aliquots of 100 µl were transferred to sterile microcentrifuge tubes and stored at -70°C.

2.1.3 Transformation of chemically competent *E.coli* cells

Competent *E.coli* cells were added to 1 ng of DNA and incubated for 10 minutes on ice. For the transformation of ligation reactions (Section 2.3.5), the whole ligation mixture was used. After incubation, the cells were placed in a water bath at 42°C for 90 seconds. Immediately after heat-shock 500 µl of LB pre-warmed at 37°C was added to the cells and incubation carried out at 37°C for 1 hour. After 1 hour the cells were plated onto LA plates pre-warmed at 37°C and were incubated overnight at 37°C.

For blue-white screening, 40 µl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Appendix 6.1) and 8 µl of IPTG (isopropyl-beta-D-thiogalactopyranoside) (Appendix 6.1) were added to each LA plate and evenly spread on the surface. The plates were dried at 37 °C for 10 minutes. The transformed cells were plated and incubated overnight at 37°C. White colonies were picked for screening.

2.2 Plasmid DNA isolation and purification

2.2.1 Small scale plasmid preparation (‘mini prep’)

A single colony containing the plasmid of interest was inoculated into 1 ml of LB containing ampicillin at a concentration of 100 µg/ml and incubated overnight at 37°C with shaking at 200-250 rpm. The culture was transferred to a microcentrifuge tube and centrifuged at 12 000 *g* for 30 seconds at room temperature. The pellet was resuspended in 100 µl of Buffer P1 (Appendix 6.1) then the cells lysed by adding 200 µl of Buffer P2 (Appendix 6.1). The solution was mixed thoroughly and incubated at room temperature for

10 minutes. Following incubation, 150 µl of Buffer P3 (Appendix 6.1) was added. The solution was mixed thoroughly and incubated on ice for 5 minutes. The bacterial lysate was centrifuged at 16 100 *g* for 10 minutes at 4°C. The supernatant was transferred to a clean microcentrifuge tube and left at room temperature for 5 minutes. DNA precipitation was carried out by adding an equal volume of room-temperature isopropanol and incubating at -20°C for 5 minutes. After incubation, the DNA was centrifuged at 16 100 *g* for 15 minutes at 4°C and the supernatant decanted. The DNA pellet was washed with 200 µl of 70% ethanol chilled to 4°C then centrifuged at 16 100 *g* for 1 minute at 4°C. The supernatant was decanted and the pellet air-dried at room temperature for 20 minutes. The DNA was redissolved by adding 50 µl of deionised water and incubating at room temperature for 30 minutes.

Small scale preparations of DNA for sequencing were purified using the Roche High Pure Plasmid Isolation kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions.

2.2.2 Large scale plasmid preparation ('bulk prep')

A single colony containing the plasmid of interest was inoculated into 400 ml of LB containing ampicillin at a concentration of 100 µg/ml and incubated overnight at 37°C with shaking at 200-250 rpm. The culture was centrifuged at 6800 *g* for 5 minutes at room temperature. The pellet was resuspended in 5 ml of Buffer P1 (Appendix 6.1) then the cells lysed by adding 10 ml of Buffer P2 (Appendix 6.1). The solution was mixed thoroughly and incubated at room temperature for 10 minutes. Following incubation, 7.5 ml of Buffer

P3 (Appendix 6.1) was added. The solution was mixed thoroughly and incubated on ice for 5 minutes. The bacterial lysate was centrifuged at 3200 *g* for 30 minutes at 4°C then passed through cheese cloth. DNA precipitation was carried out by adding an equal volume of room-temperature isopropanol and incubating at -20°C for 30 minutes. After incubation, the DNA was centrifuged at 3200 *g* for 30 minutes at 4°C and the supernatant decanted. The DNA pellet was washed with 5 ml of 70% ethanol chilled to 4°C then centrifuged at 3200 *g* for 5 minutes at 4°C. The supernatant was decanted and the pellet air-dried at room temperature for 20 minutes. The DNA was redissolved by adding 200 µl of deionised water and incubating at 37°C for 30 minutes. The concentration of the DNA was measured on a Nanodrop® Spectrophotometer.

DNA used *in vivo* was isolated using either the Endofree® Plasmid Maxi Kit (Qiagen, MD, USA) or the Nucleobond Xtra Maxi EF kit (Macherey Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions.

2.2.3 Phenol-chloroform extraction

One third of the volume of phenol and one third of the volume of chloroform were added to the DNA. The mixture was centrifuged at 12 000 *g* for 5 minutes at room temperature. The top layer was removed and transferred to a clean microcentrifuge tube. One third of the volume of chloroform was again added and the mixture was centrifuged at 12 000 *g* for 1 minute at room temperature. The top layer was removed and transferred to a clean microcentrifuge tube. An equal volume of room-temperature isopropanol was added. The tube was inverted to ensure thorough mixing then centrifuged at 16 100 *g* for 20 minutes at 4°C. The supernatant was carefully poured off and 150 µl of 70% ethanol chilled to 4°C

was added to wash the pellet. The pellet was centrifuged at 16 100 *g* for 5 minutes at 4°C. The ethanol was poured off and the pellet air-dried at room temperature for 20 minutes. The pellet was resuspended in 200 µl of deionised water and incubated at 37°C for 30 minutes.

2.2.4 DNA purification from an agarose gel

Agarose gel electrophoresis

To make a 1% gel, 0.5 g of agarose was added to 50 ml of 1× TAE (tris-acetate-EDTA) buffer (Appendix 6.1) and heated in a microwave for 2 minutes at maximum power. Deionised water was used to make the volume up to 50 ml after heating. The agarose was left to cool then 5 µl of 2 mg/ml ethidium bromide was added to enable visualisation of DNA. The agarose was poured into a gel tray and allowed to set at room temperature. Once the gel had solidified it was placed into a gel tank containing 1× TAE buffer. Orange DNA Loading Dye (1×) (Thermo Fisher Scientific, MA, USA) was added to the DNA and the samples loaded into the gel wells. O' GeneRuler DNA Ladder Mix (1 µg) (Thermo Fisher Scientific, MA, USA) was loaded in one well alongside the samples. Electrophoresis was carried out for 1 hour at 100V.

DNA purification

The agarose gel was placed under UV light and a sharp clean scalpel used to excise the desired DNA fragment. Nylon filter wool was placed in a 500 µl microcentrifuge tube and a small hole pierced in the bottom of the tube using a sterile heated needle. The gel fragment was placed in the 500 µl microcentrifuge tube which was then placed inside a 2

ml microcentrifuge tube. The gel fragment was centrifuged at 16 100 *g* at 4°C until all the liquid had passed into the 2 ml microcentrifuge tube. The DNA was purified by phenol chloroform extraction (Section 2.2.3)

PCR products were subjected to electrophoresis on a 1% agarose gel and purified using the MinElute® Gel Extraction Kit (Qiagen, MD, USA) according to the manufacturer's instructions.

2.3 Enzymatic manipulations of DNA

2.3.1 Restriction digestion

Restriction digestion was used for the preparation of cloning vectors and inserts as well as for the screening of transformants. Each digestion mixture contained up to 2 µg of DNA. Digestions were carried out with restriction enzyme in the manufacturer's recommended restriction buffer and at the recommended temperature overnight. Restriction enzymes were purchased from Thermo Fisher Scientific (MA, USA) or New England Biolabs (MA, USA). The amounts of enzyme and buffer were scaled up accordingly for the digestion of greater amounts of DNA. The digested DNA fragments were separated by electrophoresis on a 1% agarose gel (Section 2.2.4).

2.3.2 Vector desphosphorylation

Up to 5 µg of DNA was digested and the restriction enzyme inactivated at 65 °C for 20 minutes for both *Swa*I and *Cla*I. To remove 5' phosphate groups from the ends of the

vector and prevent re-ligation, one tenth of the volume of 10× Antarctic Phosphatase Reaction Buffer and 1 µl of Antarctic Phosphatase (New England Biolabs, MA, USA) was added to the DNA. The mixture was incubated at 37 °C for 60 minutes for 5' extensions or blunt ends and 120 minutes for 3' extensions. The reaction was heat inactivated at 65°C for 5 minutes and the DNA purified using gel extraction (Section 2.2.4).

2.3.3 Klenow reaction

To fill in 5' overhangs following restriction digestion the Klenow reaction was carried out. Up to 4 µg of linear DNA was added to each reaction mixture. To each reaction, 2 µl of 10× reaction buffer and 0.5 µl dNTP mix (2 mM each) was added followed by 1-5 U of the Klenow fragment (Thermo Fisher Scientific, MA, USA). The mixture was made up to 20 µl with nuclease-free water then mixed thoroughly and incubated at 37°C for 10 minutes.

2.3.4 Polymerase Chain Reaction

The amplification of DNA was carried out using PCR. Each reaction contained 1-10 ng of template DNA, 1× DreamTaq buffer, 0.2 mM dNTP mix, 0.2 µM forward and reverse primer, DreamTaq DNA Polymerase (5 U/µl) (Thermo Fisher Scientific, MA, USA) and nuclease-free water up to 50 µl according to the manufacturer's instructions. For each PCR, the cycling conditions were as follows: Initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C. A suitable extension time was calculated for each reaction based on the size of the DNA sequence to be amplified. A final extension step was carried out at 72°C for 5 minutes. Colony PCR was used for the screening of transformants. One

colony was added to 10 µl of deionised water and 1 µl per reaction used as the template. For the amplification of DNA to be used for cloning, High Fidelity enzyme (Thermo Fisher Scientific, MA, USA) was used.

2.3.5 DNA ligation

Ligations were carried out using the Fast-Link DNA Ligation kit (Epicentre Biotechnologies, WI, USA). For ligations of insert DNA with cohesive ends, each reaction contained 1.5 µl of 10× Fast-Link Ligation buffer, 1.5 µl of 10 mM ATP, 1 µl of Fast-Link DNA Ligase (2 U/µl) and different ratios of vector and insert. Each reaction was made up to 15 µl with deionised water. For ligations of insert DNA with blunt ends, each reaction contained 1.5 µl of 10× Fast-Link Ligation buffer, 0.75 µl of 10 mM ATP, 1 µl of Fast-Link DNA Ligase (2 U/µl) and different ratios of vector and insert. Each reaction was made up to 15 µl with deionised water. The ligations were incubated at 4°C overnight. Following incubation the reactions were placed in a heating block at 70°C for 15 minutes to inactivate the Fast-Link DNA ligase. The reactions were centrifuged briefly and the entire ligation mixture transformed into *E.coli* cells.

For cloning of PCR products, the InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific, MA, USA) was used. Each reaction mixture contained 1 µl of the vector pTZ57R/T (55 ng/µl), 6 µl of 5× ligation buffer, PCR product to a 1:3 ratio vector: insert, 1 µl of T4 DNA ligase (5 U/µl) and nuclease-free water up to 30 µl. The ligations were incubated at 4°C overnight. The entire ligation mixture was transformed into *E.coli* cells.

2.4 Plasmids

Table 2.1: Plasmids used and constructed in this study

Plasmid	Description	Source/reference
pCI-neo	Mammalian expression vector	Promega, WI, USA
pCI-pri-miR-31/5	pCI-neo carrying the monocistronic anti-HBV pri-miR-31/5 sequence	Ely *et al*., 2008 (57)
pCI-pri-miR-31/5/8/9	pCI-neo carrying the polycistronic anti-HBV pri-miR-31/5/8/9 sequence	Ely *et al*., 2009 (78)
pCI-FLuc	pCI-neo carrying the *Firefly luciferase* gene	Ely and Arbuthnot, 2010 (40)
pTZ57R/T	TA cloning vector	Thermo Fisher Scientific, MA, USA
pTZ-31/5	pTZ57R/T carrying the pri-miR-31/5 sequence with engineered NarI sites	This work
pTZ-31/5/8/9	pTZ57R/T carrying the pri-miR-31/5/8/9 sequence with engineered SwaI and NarI sites	This work
pTZ-Luc	pTZ57R/T carrying the *Firefly luciferase* gene with engineered ClaI sites	This work
pRS17	Plasmid carrying the mifepristone inducible system	Dr Gonzalez-Aseguinolaza (CIMA, Spain)
pRS-31/5	pRS17 carrying the pri-miR-31/5 sequence	This work
pRS-31/5/8/9	pRS17 carrying the pri-miR-31/5/8/9 sequence	This work
pRS-Luc	pRS17 carrying the *Firefly luciferase* gene	This work
phRL-CMV	Mammalian expression and reporter vector which constitutively expresses *Renilla* luciferase from a CMV promoter. Firefly luciferase activity is relativised to *Renilla* luciferase.	Promega, WI, USA
pCH-9/3091	Target vector which contains a greater than genome length HBV sequence	Nassal, 1992 (39)
pCH Firefly Luc	Target vector in which the *preS2/S* ORF of pCH-9/3091 is substituted with the *Firefly luciferase* gene	Ely *et al*., 2008 (57)
psiCHECK-*HBx*	Reporter target vector which contains the *HBx* sequence downstream of the Renilla *luciferase* ORF	Weinberg *et al*., 2006 (44)
pCI-neo eGFP	Plasmid vector which constitutively expresses enhanced green fluorescent protein (EGFP)	Passman *et al*., 2000 (40)

2.5 Construction of plasmids expressing anti-HBV sequences and the *Firefly luciferase* gene from the mifepristone inducible system

2.5.1 Construction of pRS-31/5

A large scale preparation of pRS17 (Table 2.1) was digested with *Cla*I and vector dephosphorylation carried out. The pri-miR-31/5 sequence was amplified from pCI-pri-miR-31/5 (Table 2.1) using oligonucleotide primers engineered to incorporate *Nar*I restriction sites (Table 2.2) at both ends of the sequence. Digestion with *Nar*I produces ends compatible with the *Cla*I restriction site, therefore incorporating *Nar*I sites facilitated cloning into the pRS17 vector digested with *Cla*I. The pri-miR-31/5 amplicon was ligated into the pTZ57R/T vector (Table 2.1) using the InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific, MA, USA) giving pTZ-31/5. To check for inadvertent errors which may have been introduced by PCR, one positive clone was sequenced (Inqaba Biotec, South Africa). The pri-miR-31/5 fragment was excised from pTZ-31/5 using *Nar*I and ligated into the *Cla*I site of pRS17 using the Fast-Link DNA Ligation Kit (Epicentre Biotechnologies, WI, USA) protocol for cohesive ends to create pRS-31/5 (Figure 2.1).

2.5.2 Construction of pRS-31/5/8/9

A large scale preparation of pRS17 was digested with *Cla*I followed by digestion with *Swa*I and vector dephosphorylation carried out. The pri-miR-31/5/8/9 sequence was amplified from pCI-pri-miR-31/5/8/9 (Table 2.1) using oligonucleotide primers engineered to incorporate *Swa*I and *Nar*I restriction sites at either end of the sequence (Table 2.2). Engineering *Swa*I and *Nar*I restriction sites facilitated cloning into the pRS17 vector digested with *Swa*I and *Cla*I. The pri-miR-31/5/8/9 amplicon was ligated into the

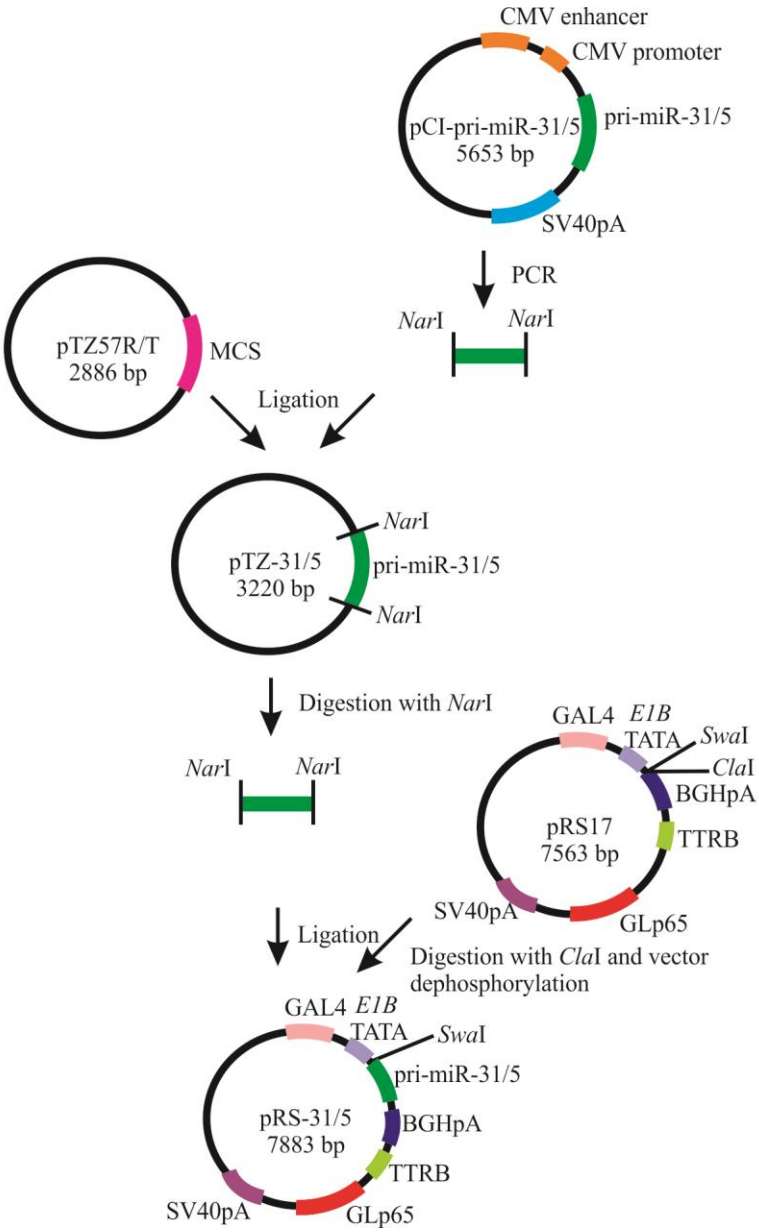


Figure 2. 1: Construction of pRS-31/5.

The empty vector pRS17 was digested with *Cla*I and vector dephosphorylation carried out. The pri-miR-31/5 sequence was amplified from pCI-pri-miR-31/5 using oligonucleotide primers engineered to incorporate *Nar*I restriction sites at both ends of the sequence. The pri-miR-31/5 amplicon was ligated into the pTZ57R/T vector giving pTZ-31/5. The pri-miR-31/5 fragment was excised from pTZ-31/5 using *Nar*I and ligated into the *Cla*I site of pRS17 to create pRS-31/5. pRS-31/5/8/9 and pRS-Luc were constructed similarly with *Swa*I/*Nar*I or *Cla*I sites incorporated at both ends of the sequence respectively.

pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit giving pTZ-31/5/8/9. To check for inadvertent errors which may have been introduced by PCR, one positive clone was sequenced (Inqaba Biotec, South Africa). The pri-miR-31/5/8/9 fragment was excised from pTZ-31/5/8/9 using *Swa*I and *Nar*I and ligated into the *Swa*I and *Cla*I sites of pRS17 using the Fast-Link DNA Ligation Kit protocol for blunt ends to create pRS-31/5/8/9.

2.5.3 Construction of pRS-Luc

A large scale preparation of pRS17 was digested with *Cla*1 and vector dephosphorylation carried out. The *Firefly luciferase* gene was amplified from pCI-FLuc (Table 2.1) using oligonucleotide primers engineered to incorporate *Cla*I restriction sites (Table 2.2) at both ends of the sequence. Engineering *Cla*I restriction sites onto the *luciferase* fragment facilitated cloning into the pRS17 vector digested with *Cla*I. The *luciferase* amplicon was ligated into the pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit giving pTZ-Luc. To check for inadvertent errors which may have been introduced by PCR, one positive clone was sequenced (Inqaba Biotec, South Africa). The *luciferase* fragment was excised from pTZ-Luc using *Cla*I and ligated into the *Cla*I site of pRS17 using the Fast-Link DNA Ligation Kit protocol for cohesive ends to create pRS-Luc.

2.5.4 Separating pRS-31/5/8/9 into the transgene and transactivator expression cassettes

To obtain a plasmid containing the GLp65 transactivator only, the cassette containing the pri-miR-31/5/8/9 sequence was deleted from pRS-31/5/8/9 by digestion with *Asc*I followed by the Klenow reaction and digestion with *Pme*I, creating the GLp65 plasmid.

The transgene expression cassette containing the pri-miR-31/5/8/9 sequence was obtained by amplification from pRS-31/5/8/9 using primers Cassette1F (5'-AGGCACCCCAGGCTTTAC-3) and Cassette1R (5'-GGGGATCCTCTAGAGCTACCTG-3'). The pri-miR-31/5/8/9-containing amplicon was ligated into the pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit giving pTZ-pri-miR. To check for inadvertent errors which may have been introduced by PCR, one positive clone was sequenced (Inqaba Biotec, South Africa).

Table 2.2: Oligonucleotides used for the amplification of the pri-miR-31/5 and pri-miR-31/5/8/9 sequences and the *Firefly luciferase* gene

	Pri-miR-31/5
*Nar*IF	5′-GATC**GGCGCC**CAGGTGTCCACTCCCAGTTC-3′
*Nar*IR	5′-GATC**GGCGCC**CCTCACTAAAGGGAAGCGGC-3′
	Pri-miR-31/5/8/9
*Swa*IF	5′-GATC**ATTTAAAT**CAGGTGTCCACTCCCAGTTC-3′
*Nar*IR	5′-GATC**GGCGCC**CCTCACTAAAGGGAAGCGGC-3′
	Firefly luciferase
*Cla*IF	5′-GATC**ATCGAT**CAGGTGTCCACTCCCAGTTC-3′
*Cla*IR	5′-GATC**ATCGAT**CCTCACTAAAGGGAAGCGGC-3′

Restriction sites are indicated in bold

2.6 Tissue culture methods

2.6.1 Cell culture growth conditions

The human hepatoma cell line, Huh7, was maintained in a humidified incubator at 37°C and 5% CO_2 in DMEM growth medium (Appendix 6.2) supplemented with 10% (w/v) foetal calf serum (FCS) (Thermo Fisher Scientific, MA, USA) and Penicillin (100 000 U/ml) and Streptomycin (100 µg/ml) (Sigma-Aldrich, MO, USA).

2.6.2 Passaging of Huh7 cells

The cells were cultured in 75 cm^2 flasks and were passaged when they reached a density covering 90% of the culture flask surface. The culture medium was aspirated and discarded. Following this, the cells were washed once with 10 ml of saline containing 0.01% EDTA and incubated in 5ml of saline-EDTA for 5 minutes in a humidified incubator at 37°C and 5% CO_2. Following incubation, the saline-EDTA was discarded and 1 ml of 0.5× trypsin-EDTA (Appendix 6.2) added to the cells. After incubation for 1 minute in a humidified incubator at 37°C and 5% CO_2, the cells were detached from the surface of the culture flask by gently tapping the flask then aspirated and transferred to a clean 50 ml tube. The volume of cells required for the desired cell density was left in the tube and the rest of the cells were discarded. To inactivate the trypsin, 20 ml of DMEM growth medium containing 10% (w/v) FCS and Penicillin/Streptomycin warmed to 37°C was added to the cells and the cells vortexed briefly. Following this, the cells were transferred to a clean culture flask and incubated in a humidified incubator at 37°C and 5% CO_2.

For seeding in 24 well plates, after the cells were detached from the culture flask antibiotic-free DMEM growth medium containing FCS was added. The cells were seeded at a density of 3×10^5 cells per well and grown overnight in a humidified incubator at 37°C and 5% CO_2.

2.6.3 Transfection of eukaryotic cells

Transfection of Huh7 cells was carried out using Lipofectamine 2000™ (Life Technologies, CA, USA) according to the manufacturer's instructions. Twenty-four hours prior to transfection cells were transferred to antibiotic-free medium and seeded at a density of 3×10^5 cells per well in a 24-well plate. Each transfection mixture contained up to 800 ng of effector DNA, 100 ng of target plasmid and 100 ng of pCI-neo eGFP (Table 2.1). pCI-neo eGFP was included in each co-transfection as a means of verifying equivalent transfection efficiency using fluorescence microscopy. To each microgram of DNA to be transfected, 50µl of Opti-MEM (Life Technologies, CA, USA) was added and the mixture incubated at room temperature for 5 minutes. For each microgram of DNA, 1 µl of Lipofectamine 2000™ was mixed with 49 µl of Opti-MEM and the mixture incubated at room temperature for 5 minutes. After incubation, the DNA and Lipofectamine mixtures were combined and incubated at room temperature for 20 minutes for complex formation. To each well, 100 µl of the transfection mixture was added dropwise. Five hours after transfection, the medium was carefully removed from each well and replaced with 500 µl of antibiotic-free medium.

2.6.4 Measurement of Firefly luciferase activity in lysates

Huh7 cells in a 24 well plate were co-transfected with up to 500 ng of pRS-Luc, 100 ng of phRL-CMV (Table 2.1) to normalise Firefly luciferase expression and 100 ng of pCI-neo eGFP. Five hours after transfection the medium was carefully removed from each well and replaced with 500 µl of antibiotic-free medium containing different concentrations of mifepristone (10^{-10}-10^{-5} M) for induced cells or ethanol for uninduced cells. Twenty-four hours after induction the medium was removed from the cells and the cell lysate used for the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) according to the manufacturer's instructions.

2.6.5 Assessment of the *in vitro* efficacy of pri-miRs incorporated into the mifepristone inducible system

2.6.5.1 Assessing the efficacy of pri-miRs by ELISA

Huh7 cells in a 24 well plate were co-transfected with up to 500 ng of pRS-31/5 or pRS-31/5/8/9, 100 ng of target plasmid (pCH-9/3091) (Table 2.1) and 100 ng of pCI-neo eGFP. Five hours after transfection the medium was carefully removed from each well and replaced with 500 µl of antibiotic-free medium containing 10^{-9} M, 10^{-8} M or 10^{-7} M mifepristone (Appendix 6.2) for induced cells or ethanol for uninduced cells. Twenty-four hours after induction the supernatant was collected from the co-transfected cells. Knockdown of HBV replication was assessed by measuring the secretion of HBV surface antigen (HBsAg), a marker of HBV gene expression, in the culture supernatants by ELISA using the Monolisa™ HBsAg Ultra Kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions.

2.6.5.2 Assessing the efficacy of pri-miRs using the Dual Luciferase Assay

Huh7 cells in a 24 well plate were co-transfected with up to 100 ng of pRS-31/5 or pRS-31/5/8/9, 100 ng of target plasmid (psiCHECK-*HBx*) (Table 2.1) and 100 ng of pCI-neo eGFP. Five hours after transfection the medium was carefully removed from each well and replaced with 500 µl of antibiotic-free medium containing 10^{-10} M, 10^{-8} M or 10^{-5} M mifepristone for induced cells or ethanol for uninduced cells. Twenty-four hours after induction the medium was removed from the cells and the cell lysate used for the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) according to the manufacturer's instructions.

2.7 Mouse studies

Female NMRI mice were used for all experiments (South African Vaccine Producers, South Africa). All procedures were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. Animal ethics clearance number: 2010/10/04.

2.7.1 Measurement of Firefly luciferase activity *in vivo*

To assess gene expression under the control of the inducible system, mice were injected with the pRS-Luc construct and induced with 250 µg/kg or 500 µg/kg mifepristone or oil only (180, 189, 202). Mice received an intraperitoneal injection of mifepristone (Appendix 6.3) in a total volume of 100 µl prior to plasmid delivery and for subsequent inductions. Up to 20 µg of DNA was administered per mouse (78). The DNA was made up to a volume of 2.5 ml using saline and delivered to the mice using the hydrodynamic injection.

The luciferase substrate D-Luciferin, potassium salt (Gold Biotechnology, MO, USA), was dissolved in phosphate buffered saline to a final concentration of 15 mg/ml, filter sterilised and administered to the mice (150 mg/kg) via intraperitoneal injection 5-10 minutes prior to imaging. The mice were anaesthetised with isofluorane and imaging carried out using the Xenogen IVIS® imaging system at specified time points (PerkinElmer, MA, USA). Bioluminescence was quantified using the Living Image® software (PerkinElmer, MA, USA).

2.7.2 Assessment of the *in vivo* efficacy of pri-miRs incorporated into the mifepristone inducible system

To assess the HBV knockdown *in vivo*, mice were injected with pRS-31/5/8/9 and the target plasmid pCH Firefly Luc (Table 2.1) and induced with 250 µg/kg, 500 µg/kg or 1 mg/kg mifepristone or oil only (180, 188, 189, 202). Mice received an intraperitoneal injection of mifepristone in a total volume of 100 µl prior to plasmid delivery. Subsequently, induction was carried out daily. Up to 10 µg of DNA was administered per mouse. The required amount of DNA was made up to a volume of 2.5 ml using saline and delivered to the mice using the hydrodynamic injection. D-Luciferin was administered to the mice via intraperitoneal injection 5-10 minutes prior to imaging. The mice were anaesthetised with isofluorane and imaging carried out using the Xenogen IVIS® imaging system at 5 hours, day 3 and day 6. Bioluminescence was quantified using the Living Image® software.

2.8 Statistical analysis

Each assay was conducted a minimum of two times. Data are expressed as the normalised mean ± the normalised standard deviation. Statistical difference was considered significant when $p<0.05$ and was determined according to the Student's paired two-tailed t-test. Calculations were done with the GraphPad software package (GraphPad Software Inc., CA, USA).

CHAPTER 3

3 RESULTS

3.1 Expression of the *Firefly luciferase* gene with the mifepristone inducible system *in vitro*

3. 1. 1 Induction of *Firefly luciferase* gene expression with mifepristone

The toxicity of RNAi effectors in hepatocytes remains an obstacle to the development of RNAi-based HBV treatment (67). The use of inducible promoters, whereby transgene expression is fine-tuned, could abolish these toxic effects. The mifepristone inducible system has been used to achieve inducible and tissue specific gene expression and therefore could contribute to developing a novel treatment strategy for HBV [reviewed in (173, 203)]. The inducible system used in this study consists of two expression cassettes; one containing the regulator/transactivator protein and another containing the transgene. The regulator of the mifepristone inducible system is under the control of a liver-specific promoter thus ensuring gene expression is confined to hepatocytes, the primary site of HBV infection. In addition, the regulator is designed to only activate gene expression in the presence of the inducer mifepristone.

Reporter assays such as the luciferase assay provide an easy method of assessing the levels of gene expression. Therefore a plasmid expressing Firefly luciferase under the control of the mifepristone inducible system was constructed. Briefly, the *Firefly luciferase* gene sequence was amplified using PCR and cloned into the *Cla*I site of the pRS17 vector bearing the mifepristone inducible system creating pRS-Luc.

pRS-Luc was then used to assess the regulation of gene expression and determine the optimal induction conditions in our hands. Huh7 cells were transfected with different amounts of the pRS-Luc construct and 100 ng of the phRL-CMV plasmid, which constitutively expresses *Renilla* luciferase. Firefly luciferase activity is therefore relativised to the *Renilla* luciferase background and gene expression is calculated as a ratio of the two. As a negative control, cells were transfected with 500 ng of the empty vector pRS17, corresponding to the highest amount of pRS-Luc transfected. Cells were transfected with 100 ng of pCI-Fluc in which the *Firefly luciferase* gene is incorporated into pCI-neo as a positive control, corresponding to the lowest amount of pRS-Luc transfected, or 100 ng of pCI-neo to normalise luciferase expression from pCI-Fluc. pCI-neo eGFP (100 ng) was included in each co-transfection and in subsequent experiments as a means of verifying equivalent transfection efficiency using fluorescence microscopy. Induction of gene expression has previously been achieved with 10^{-8} M mifepristone and has been shown to be dose responsive with higher expression observed at higher concentrations of mifepristone (10^{-7} M) (202). Therefore 10^{-8} M and 10^{-6} M mifepristone were the initial induction concentrations investigated. Firefly luciferase activity was measured using the Dual Luciferase Assay. Fold induction is expressed as the ratio of the average expression value obtained with each concentration of mifepristone and the average value obtained with the uninduced cells.

With all vector amounts of the pRS-Luc construct, significant gene expression was observed with both concentrations of mifepristone relative to the uninduced cells ($0.0004>p<0.007$). Overall, the induction of *Firefly luciferase* gene expression observed was 10-30-fold (Figure 3.1). These findings suggest that gene expression under the control of the mifepristone inducible system is inducible. However, there appears to be higher

basal activity in the absence of mifepristone with increasing amounts of pRS-Luc as shown by the decreased fold induction with higher amounts of pRS-Luc (Figure 3.1)

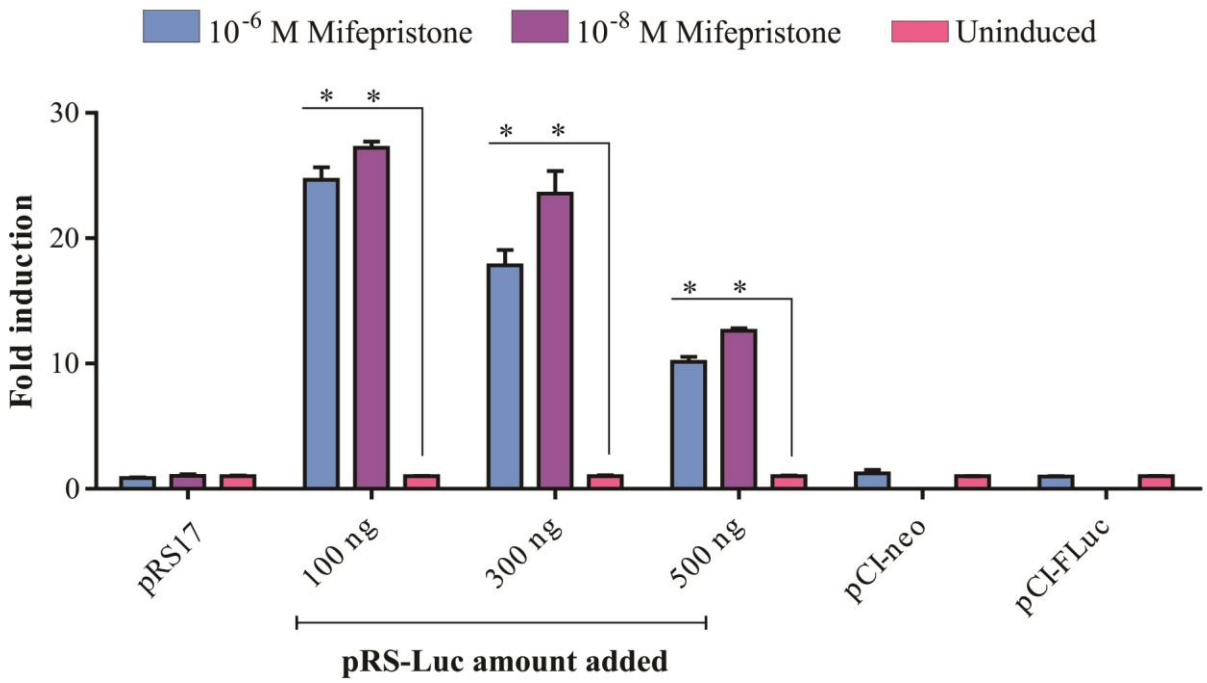


Figure 3.1: *Firefly luciferase* gene expression with the inducible system *in vitro*.

Huh7 cells were transfected with 100 ng of phRL-CMV and different amounts of pRS-Luc or 500 ng of the empty vector pRS17 as a negative control. Cells were transfected with 100 ng of pCI-FLuc as a positive control or 100 ng of pCI-neo to normalise Firefly luciferase expression from pCI-Fluc. Five hours after transfection induction was carried out with 10^{-8} M mifepristone or 10^{-6} M mifepristone for induced cells or ethanol for uninduced cells. The Dual Luciferase Assay was carried out 24 hours after induction. Error bars indicate the normalised standard deviation (n=3). *$p < 0.01$, t test, relative to the uninduced cells.

3. 1. 2 Expression of the *Firefly luciferase* gene with the mifepristone inducible system is dose-dependent

Gene expression under the control of the mifepristone inducible system has been shown to be dependent on the concentration of mifepristone both *in vitro* and *in vivo* (180, 188, 189, 202). To test for dose dependency, expression of Firefly luciferase from pRS-Luc was assessed in Huh7 cells induced with a wider range of mifepristone concentrations. As a result of minimal basal expression that was observed (Figure 3.1), 300 ng of the pRS-Luc construct was used. Huh7 cells were transfected with 100 ng of phRL-CMV and 300 ng of the pRS-Luc construct or 300 ng of pRS17, pCI-FLuc or pCI-neo. Transfected cells were induced with different concentrations of mifepristone or left uninduced. The Dual Luciferase Assay was carried out 24 hours after induction.

A significant increase in induction of *Firefly luciferase* gene expression was observed at all concentrations of mifepristone compared to the uninduced cells ($0.0001>p<0.03$), with the greatest induction achieved with 10^{-9} M mifepristone (Figure 3.2). Interestingly, reduced induction was observed with the highest mifepristone concentrations investigated (10^{-5} M and 10^{-6} M). However, there was no significant difference between the induction achieved with 10^{-9} M mifepristone, 10^{-8} M mifepristone or 10^{-7} M mifepristone ($p>0.05$). The difference between the induction achieved with 10^{-6} M mifepristone or 10^{-5} M mifepristone was also not significant ($p=0.98$). Based on these observations, induction of gene expression under the control of the mifepristone inducible system appears to be dependent on the concentration of mifepristone.

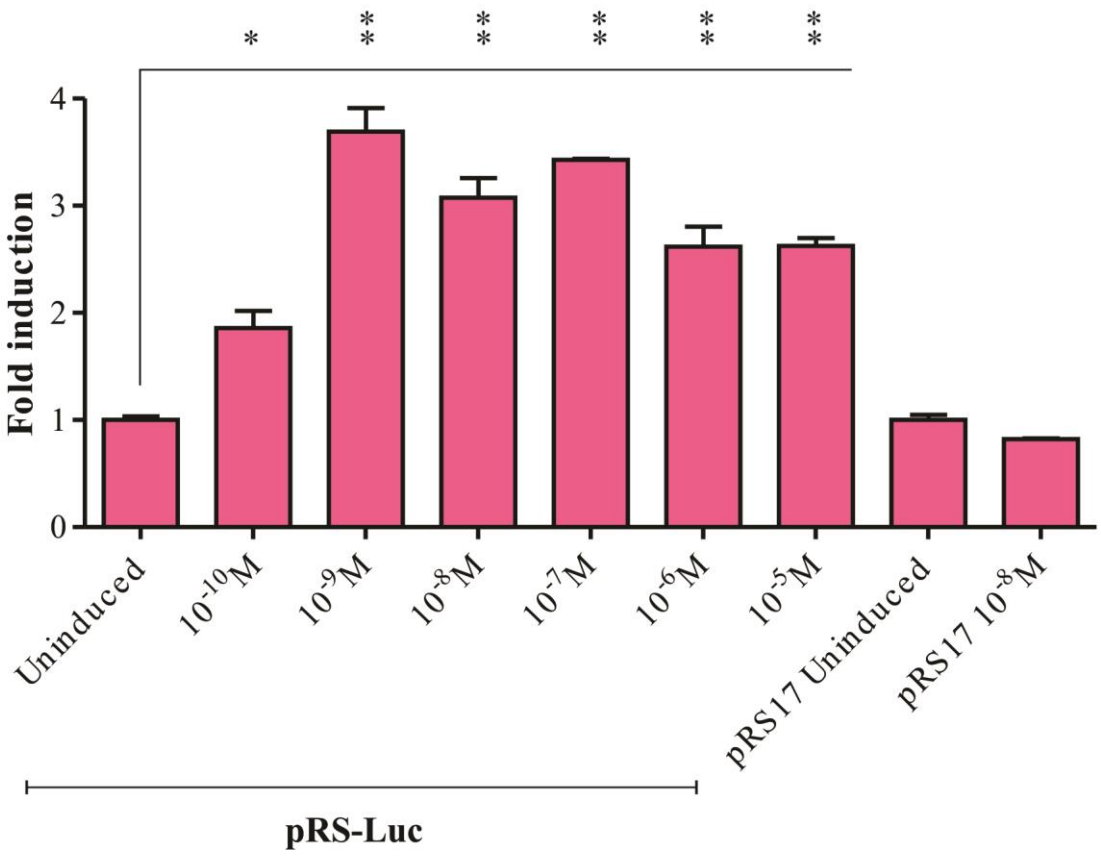


Figure 3.2: Fold induction of *Firefly luciferase* gene expression *in vitro* at different concentrations of mifepristone.

Huh7 cells were transfected with 100 ng of phRL-CMV and 300 ng of pRS-Luc or 300 ng of the empty vector pRS17 as a negative control. Cells were transfected with 300 ng of pCI-FLuc as a positive control or 300 ng of pCI-neo to normalise Firefly luciferase expression from pCI-FLuc. Five hours after transfection, induction was carried out with different concentrations of mifepristone or ethanol for uninduced cells. The Dual Luciferase Assay was carried out 24 hours after induction. Error bars indicate the normalised standard deviation (n=3). *p< 0.05, **p<0.01, t test, relative to the uninduced cells.

3.2 Knockdown of HBV gene expression with pri-miR mimics expressed under the control of the mifepristone inducible system *in vitro*

3.2.1 pri-miR-31/5/8/9 expressed under the control of the mifepristone inducible system reduces HBsAg secretion

Pri-miR mimics have been found to result in significant HBV gene expression knockdown *in vitro* (57, 78). Pol II and Pol III promoters have been widely used for the expression of RNAi effectors. However, Pol III promoters are constitutively active and have been shown to result in toxicity as a consequence of saturating the endogenous RNAi pathway (67). In addition, induction of the interferon response has been observed with Pol III promoters (129). In contrast, tissue specific and inducible expression can be achieved with Pol II promoters thus addressing the dose regulation and targeting problems associated with RNAi therapy. This study made use of an inducible system in which pri-miR expression is regulated by a transactivator expressed from a tissue-specific promoter. The ability to achieve inducible expression of RNAi effectors is an important consideration in the development of RNAi-based therapeutics.

HBsAg secretion is a marker of HBV gene expression and was used to investigate the antiviral efficacy of pri-miR-31/5/8/9 expressed from the mifepristone inducible system. Briefly, the pri-miR-31/5/8/9 sequence was amplified using PCR and cloned into the *Swa*I and *ClaI* sites of the pRS17 vector bearing the mifepristone inducible system creating pRS-31-5/8/9.

Huh7 cells were transfected with 100 ng of the target plasmid, pCH-9/3091, which contains a greater than genome length HBV sequence, and different amounts of pRS-31/5/8/9. As a negative control, cells were transfected with 500 ng of the empty vector pRS17 corresponding to the highest amount of pRS-31/5/8/9 transfected. Cells were transfected with either 500 ng of pCI-pri-miR-31/5/8/9 as a positive control or 500 ng of pCI-neo to normalise pCI-pri-miR-31/5/8/9. As a result of similar induction observed with 10^{-9} M, 10^{-8} M and 10^{-7} M mifepristone (Figure 3.2), the lowest concentration (10^{-9} M mifepristone) was used in this experiment; which may minimise the side effects associated with the antiprogestin and antiglucocorticoid activity of mifepristone (204). Therefore, cells were induced with 10^{-9} M mifepristone or left uninduced. After 24 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion.

A significant reduction in HBsAg was observed with all vector amounts of pRS-31/5/8/9 after induction compared to pRS17 ($0.0006>p<0.02$) (Figure 3.3). However, a significant reduction in HBsAg was also observed with 300 ng and 500 ng of pRS-31/5/8/9 without induction compared to pRS17 ($p=0.0090$ and $p=0.0069$ respectively). There was no significant difference observed when comparing induced and uninduced cells with all vector amounts of pRS-31/5/8/9 ($p>0.05$) suggesting that there was no induction of gene expression. In addition, the reduction in HBsAg secretion observed with uninduced cells suggests that there is leaky expression from pRS-31/5/8/9. We have also observed that there is high basal expression of pri-miR-31/5/8/9 *in vitro* with higher vector amounts (800 ng, data not shown) which could mask induction; therefore even lower amounts of pRS-31/5/8/9 were investigated.

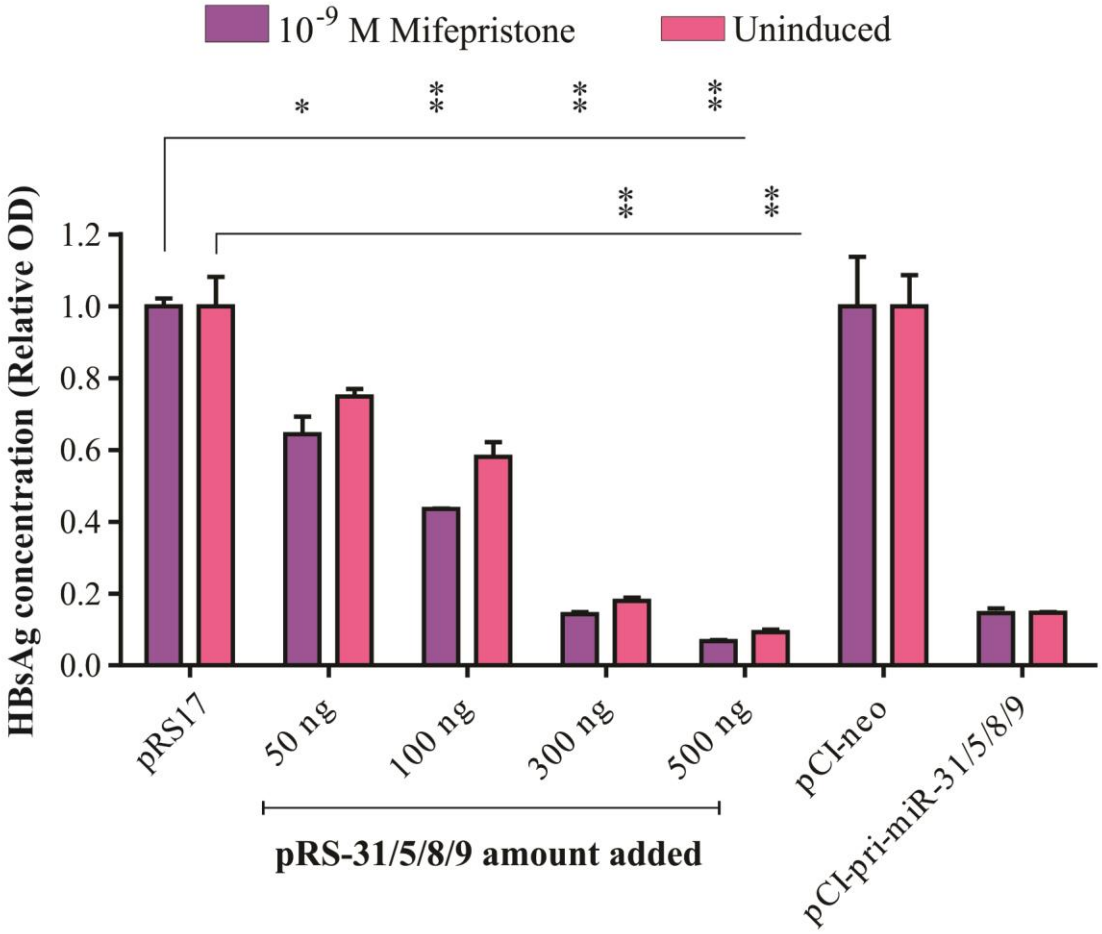


Figure 3.3: Assessment of HBsAg secretion from Huh7 cells transfected with the pri-miR-31/5/8/9 construct using ELISA.

Huh7 cells were transfected with 100 ng of pCH-9/3091 and different amounts of pRS-31/5/8/9 or 500 ng of the empty vector pRS17 as a negative control. Cells were transfected with 500 ng of pCI-pri-miR-31/5/8/9 as a positive control or 500 ng of pCI-neo to normalise pCI-pri-miR-31/5/8/9. Five hours after transfection induction was carried out with 10^{-9} M mifepristone or ethanol for uninduced cells. After 24 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion. Error bars indicate the normalised standard deviation (n=3). *$p< 0.05$, **$p<0.01$, t test, relative to the pRS17 induced and uninduced cells.

3.2.2 Determining the optimum vector amount of pRS-31/5/8/9 required to reduce basal expression *in vitro*

Variations in the levels of gene expression and induction have been observed with the mifepristone inducible system (180, 188, 189). This variation seems to be dependent on factors such as the particular gene being expressed, the cell lines being used, whether expression is *in vitro* or *in vivo* and the delivery mechanism in the case of *in vivo* studies. Therefore it is important to optimise the system for each specific application.

High vector amounts of the pri-miR-31/5/8/9-expressing construct result in high basal levels of expression which could mask the induction that is achieved with the mifepristone inducible system. To reduce basal expression, the amount of pRS-31/5/8/9 would need to be optimised such that induction of gene expression can be achieved with minimal basal expression. Therefore low amounts of pRS-31/5/8/9 were investigated with and without induction.

The measurement of HBsAg secretion was again used to assess the expression of pri-miR-31/5/8/9 from the mifepristone inducible system. Cells were transfected with 100 ng of the target plasmid, pCH-9/3091, and different amounts of pRS-31/5/8/9. As a negative control, cells were transfected with 50 ng of the empty vector pRS17 corresponding to the highest amount of pRS-31/5/8/9 transfected. Cells were transfected with either 800 ng of pCI-pri-miR-31/5/8/9 as a positive control or 800 ng of pCI-neo to normalise pCI-pri-miR-31/5/8/9. At first, no induction was carried out to determine the vector amount of pRS-31/5/8/9 at which there is no or negligible basal expression in the absence of mifepristone.

After 48 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion.

Without induction, there was no significant reduction in HBsAg observed with all amounts of pRS-31/5/8/9 compared to pRS17 ($p>0.05$) (Figure 3.4A). This suggests that at the vector amounts investigated there was no basal expression of pri-miR-31/5/8/9.

In the subsequent experiment, induction was carried out to determine the lowest amount of pRS-31/5/8/9 which can be used to achieve induction with the mifepristone inducible system. Only the three lowest vector amounts (10 ng, 20 ng and 30 ng) of pRS-31/5/8/9 were investigated as they seemed to result in the least basal expression in the absence of mifepristone (Figure 3.4A). Cells were transfected with 100 ng of the target plasmid, pCH-9/3091, and different amounts of pRS-31/5/8/9. As a negative control, cells were transfected with 30 ng of pRS17 corresponding to the highest amount of pRS-31/5/8/9 transfected. As a result of the high induction observed with 10^{-9} M, 10^{-8} M and 10^{-7} M mifepristone (Figure 3.2), all three concentrations were investigated. Therefore 5 hours after transfection cells were induced with 10^{-9} M, 10^{-8} M or 10^{-7} M mifepristone or left uninduced. After 24 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion.

Upon addition of mifepristone there was no significant induction (expressed as fold knockdown) observed with any vector amounts of pRS-31/5/8/9 compared to the uninduced cells ($p>0.05$) (Figure 3.4B). These observations suggest that for the expression of pri-miRs using the mifepristone inducible system, a balance between basal expression

and induction must be achieved. Both must be at acceptable levels such that tight regulation of gene expression can still be achieved. Therefore, in subsequent experiments a much wider range of vector amounts was investigated but attempts were made to keep the amounts added as low as possible.

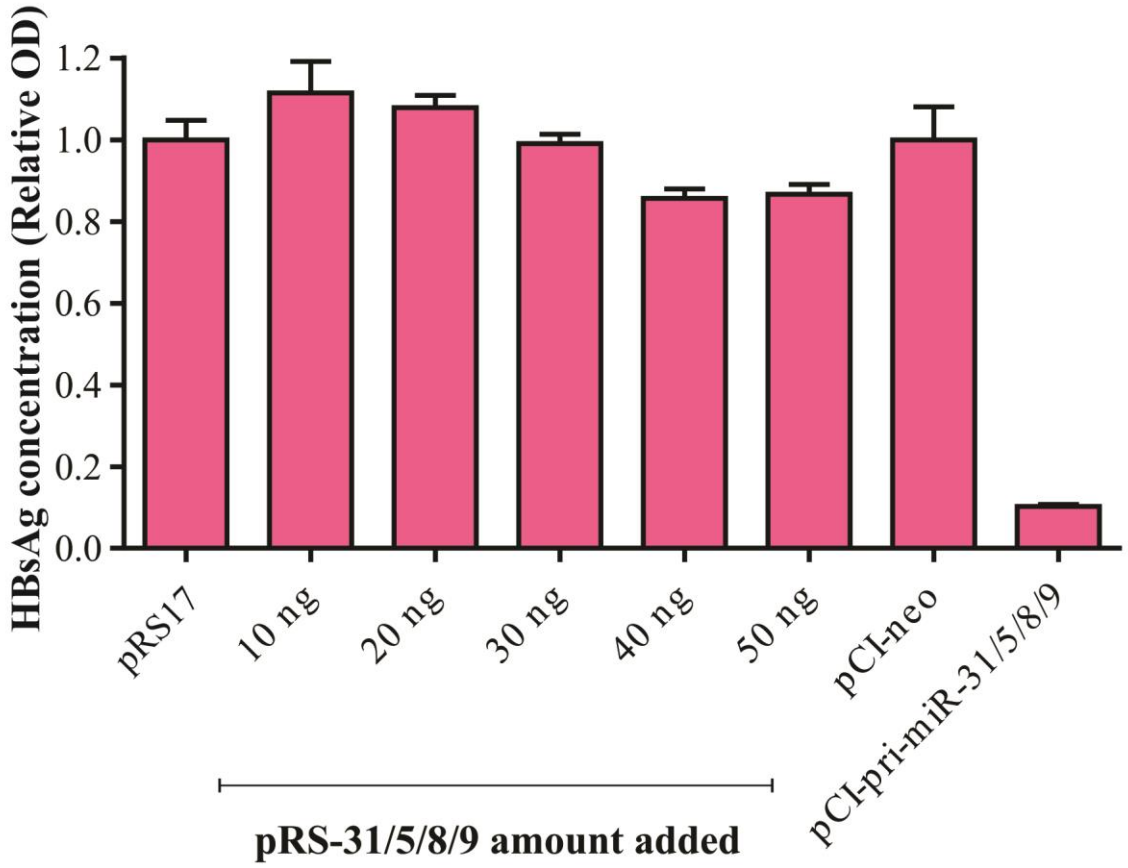


Figure 3.4A: Determining the basal expression from pRS-31/5/8/9 *in vitro*.

Huh7 cells were transfected with 100 ng of pCH-9/3091 and different amounts of pRS-31/5/8/9 or 50 ng of the empty vector pRS17 as a negative control. Cells were transfected with 800 ng of pCI-pri-miR-31/5/8/9 as a positive control or 800 ng of pCI-neo to normalise pCI-pri-miR-31/5/8/9. No induction was carried out after transfection. After 48 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion. Error bars indicate the normalised standard deviation (n=3).

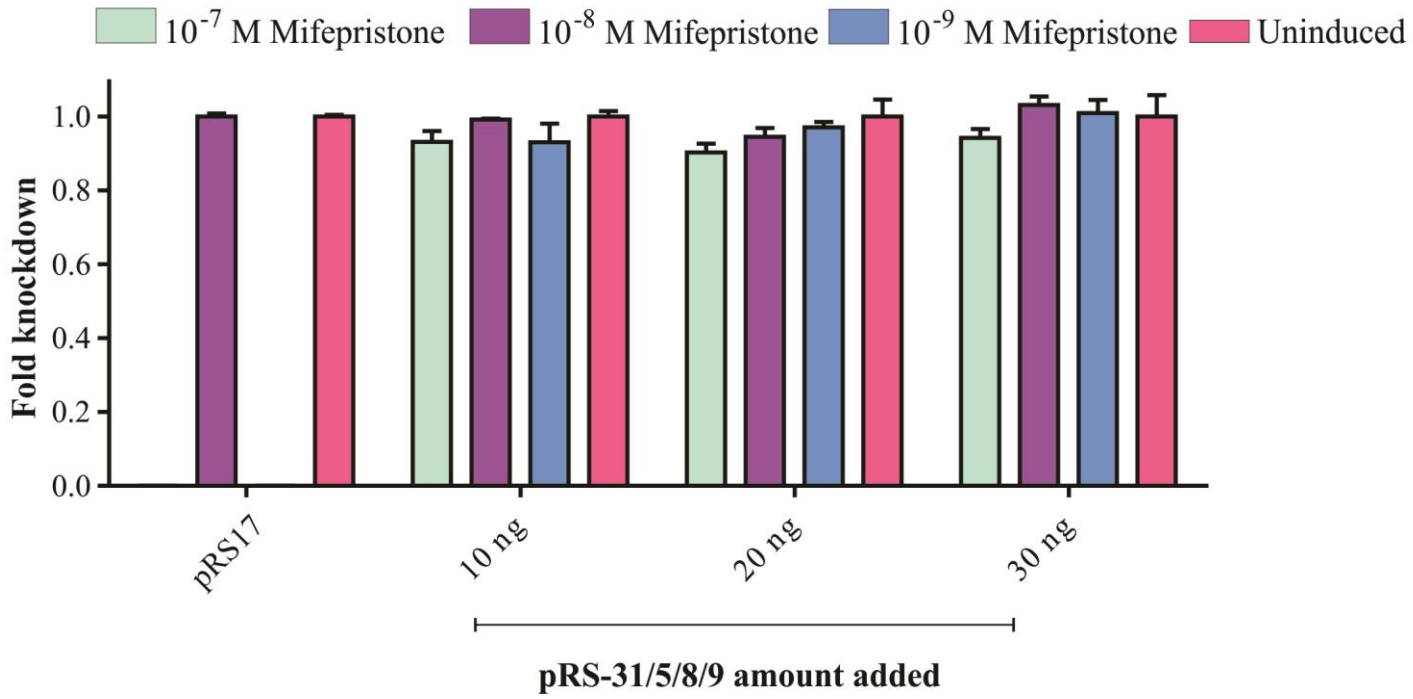


Figure 3.4B: Optimising induction conditions for the expression of pri-miR-31/5/8/9 *in vitro*.

Huh7 cells were transfected with 100 ng of pCH-9/3091, and different amounts of pRS-31/5/8/9 or 30 ng of the empty vector pRS17 as a negative control. Five hours after transfection cells were induced with 10^{-9} M, 10^{-8} M or 10^{-7} M mifepristone or ethanol for uninduced cells. After 24 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion. Error bars indicate the normalised standard deviation (n=3).

3.2.3 pri-miR-31/5 expressed under the control of the mifepristone inducible promoter reduces HBsAg secretion

To further investigate the possibility of using the mifepristone inducible system to regulate anti-HBV sequence expression, the plasmid expressing pri-miR-31/5 under the control of the inducible system was constructed. Briefly, the pri-miR-31/5 sequence was amplified using PCR and cloned into the *Cla*I site of the pRS17 vector bearing the mifepristone inducible system creating pRS-31/5.

Cells were transfected with 100 ng of the target plasmid, pCH-9/3091, and different amounts of pRS-31/5. As a negative control, cells were transfected with 500 ng of pRS17 corresponding to the highest amount of pRS-31/5 transfected. Cells were transfected with either 500 ng of pCI-pri-miR-31/5 as a positive control or 500 ng of pCI-neo to normalise pCI-pri-miR-31/5. Five hours after transfection cells were induced with 10^{-9} M mifepristone or left uninduced. After 24 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion.

A significant difference in HBsAg was observed with 50 ng, 300 ng and 500 ng of pRS-31/5 after induction compared to pRS17 ($0.01>p<0.05$) (Figure 3.5). However, with 50 ng and 300 ng of pRS-31/5 uninduced cells also resulted in a significant reduction in HBsAg compared to pRS17 ($p=0.020$ and $p=0.012$ respectively). Nonetheless, there was a significant difference in HBsAg secretion when comparing induced and uninduced cells observed with 50 and 500 ng of pRS-31/5 ($p= 0.0049$ and 0.013 respectively) indicating that there was some induction achieved with the pRS-31/5 construct. However, the reduction in HBsAg secretion observed with uninduced cells suggests that there is leaky

expression from pRS-31/5. These observations confirm the results obtained with the pRS-31/5/8/9 construct (Figure 3.3). Therefore, pri-miR expression from the mifepristone inducible system appears to be leaky *in vitro*. Interestingly, the pRS-31/5/8/9 construct appears to result in reduction of HBsAg to a greater extent than the pRS-31/5 construct when compared to the pRS17 ($p<0.01$ compared to $p<0.05$) (Figure 3.3 & Figure 3.5).

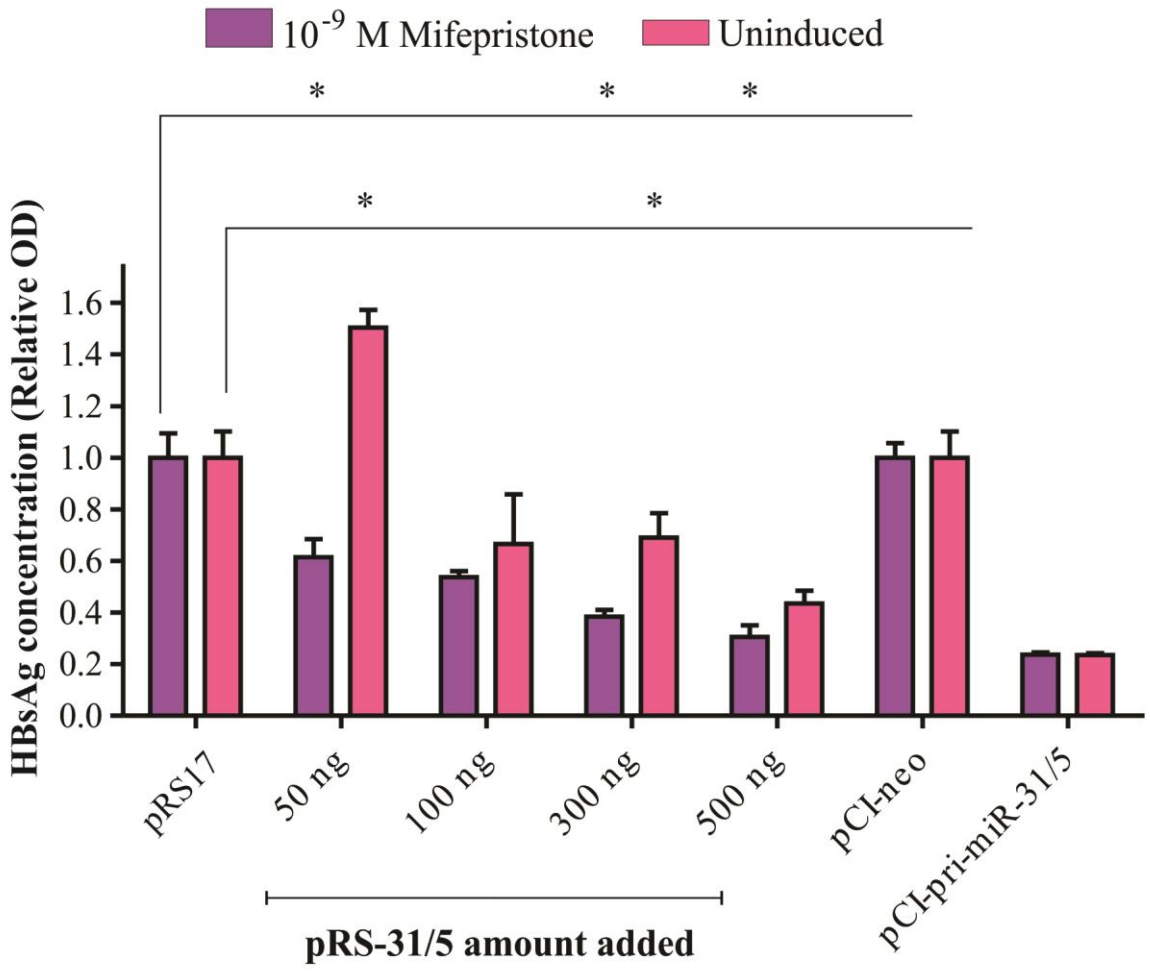


Figure 3.5: Assessment of HBsAg secretion from Huh7 cells transfected with the pri-miR-31/5 construct using ELISA.

Huh7 cells were transfected with 100 ng of pCH-9/3091 and different amounts of pRS-31/5 or 500 ng of the empty vector pRS17 as a negative control. Cells were transfected with 500 ng of pCI-pri-miR-31/5 as a positive control or 500 ng of pCI-neo to normalise for pCI-pri-miR-31/5. Five hours after transfection cells were induced with 10^{-9} M mifepristone or ethanol for uninduced cells. After 24 hours the supernatant was collected and ELISA carried out to measure HBsAg secretion. Error bars indicate the normalised standard deviation (n=3). *$p < 0.05$, t test, relative to the pRS17 induced and uninduced cells.

3.3 Assessment of knockdown of HBV gene expression and mifepristone dose dependence following induction of pri-miR expression using the Dual Luciferase Assay

To confirm the results observed with ELISA, the Dual Luciferase Assay was used. The target plasmid used for the Dual Luciferase Assay, psiCHECK-*HBx*, contains the *HBx* sequence downstream of the Renilla *luciferase* ORF. Firefly luciferase is expressed constitutively, whereas *Renilla* luciferase is susceptible to silencing by anti-*HBx* sequences. Therefore a ratio of *Renilla* to Firefly luciferase activity is used as an indicator of target knockdown.

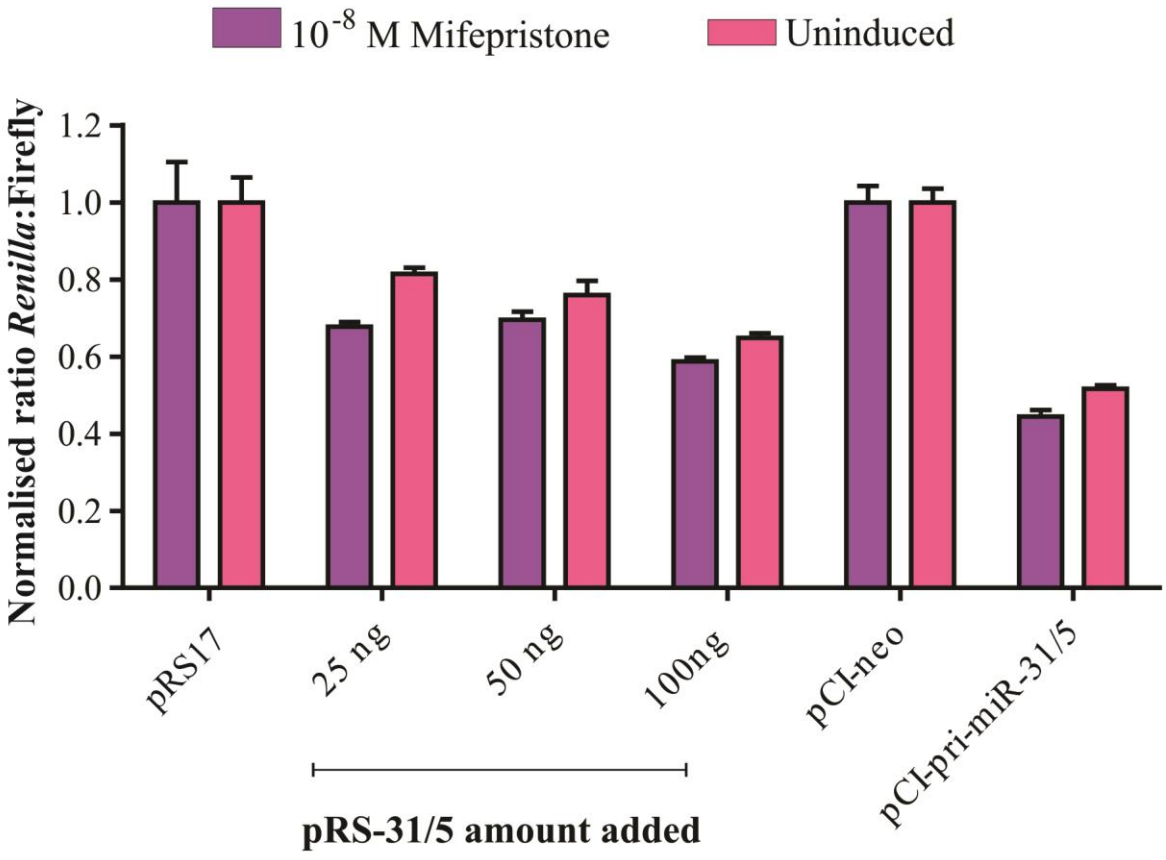
Cells were transfected with 100 ng of the target plasmid, psiCHECK-*HBx*, and different amounts of pRS-31/5 or pRS-31/5/8/9. As a negative control, cells were transfected with 100 ng of the empty vector pRS17 corresponding to the highest amount of pRS-31/5 and pRS-31/5/8/9 transfected. To minimise the basal expression, 100 ng was the highest amount of pRS-31/5 and pRS-31/5/8/9 investigated. Cells were transfected with either 100 ng of pCI-pri-miR-31/5 or pCI-pri-miR-31/5/8/9 as positive controls or 100 ng of pCI-neo to normalise pCI-pri-miR-31/5 and pCI-pri-miR-31/5/8/9. Five hours after transfection cells were induced with 10^{-10} M, 10^{-8} M and 10^{-5} M mifepristone or left uninduced. These concentrations of mifepristone were chosen because they resulted in significantly different induction of gene expression with the luciferase-expressing construct (Figure 3.2). After 24 hours the lysate was collected and used for the Dual Luciferase Assay.

Overall, with pRS-31/5, there was no significant HBV gene expression knockdown with both induced and uninduced cells compared to pRS17 under the conditions investigated

(p= 0.063-0.14) (Figure 3.6A). Interestingly, mifepristone dose-dependent knockdown was observed when a wide range of mifepristone concentrations was used (Figure 3.6B). A significant difference in knockdown was observed when comparing induced to uninduced cells with all vector amounts of pRS-31/5 with 10^{-5} M and with 25 ng and 100 ng with 10^{-8} M mifepristone. Unlike with pRS-Luc (Figure 3.2), 10^{-5} M mifepristone resulted in the greatest knockdown fold change. No significant difference was observed with any amount of pRS-31/5 with 10^{-10} M mifepristone ($p>0.05$). Similar to the ELISA data, these data indicate that there is some induction of HBV knockdown with the pRS-31/5 construct at the concentrations of mifepristone which were investigated.

Similarly, knockdown of HBV gene expression by pri-miR-31/5/8/9 and dose dependence under the control of the inducible system was then assessed using the Dual Luciferase Assay. Unlike with pRS-31/5 (Figure 3.6A), significant knockdown in gene expression was observed with both induced and uninduced cells with all vector amounts of pRS-31/5/8/9 compared to pRS17 (Figure 3.7A). This supports the previous observations that pri-miR-31/5/8/9 expressed from the mifepristone inducible system results in greater silencing compared to pri-miR-31/5. Dose-dependent knockdown was observed (Figure 3.7B) although it was more variable than with pRS-31/5. Knockdown observed with uninduced cells further confirms that there is leaky expression of anti-HBV pri-miR sequences from the pRS17-derived constructs. Hence understanding the mechanism of leaky expression is important and will be beneficial in optimising the system.

A


10-8 M Mifepristone
Uninduced
Normalised ratio Renilla:Firefly
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pRS17
25 ng
50 ng
100ng
pCI-neo
pCI-pri-miR-31/5
pRS-31/5 amount added

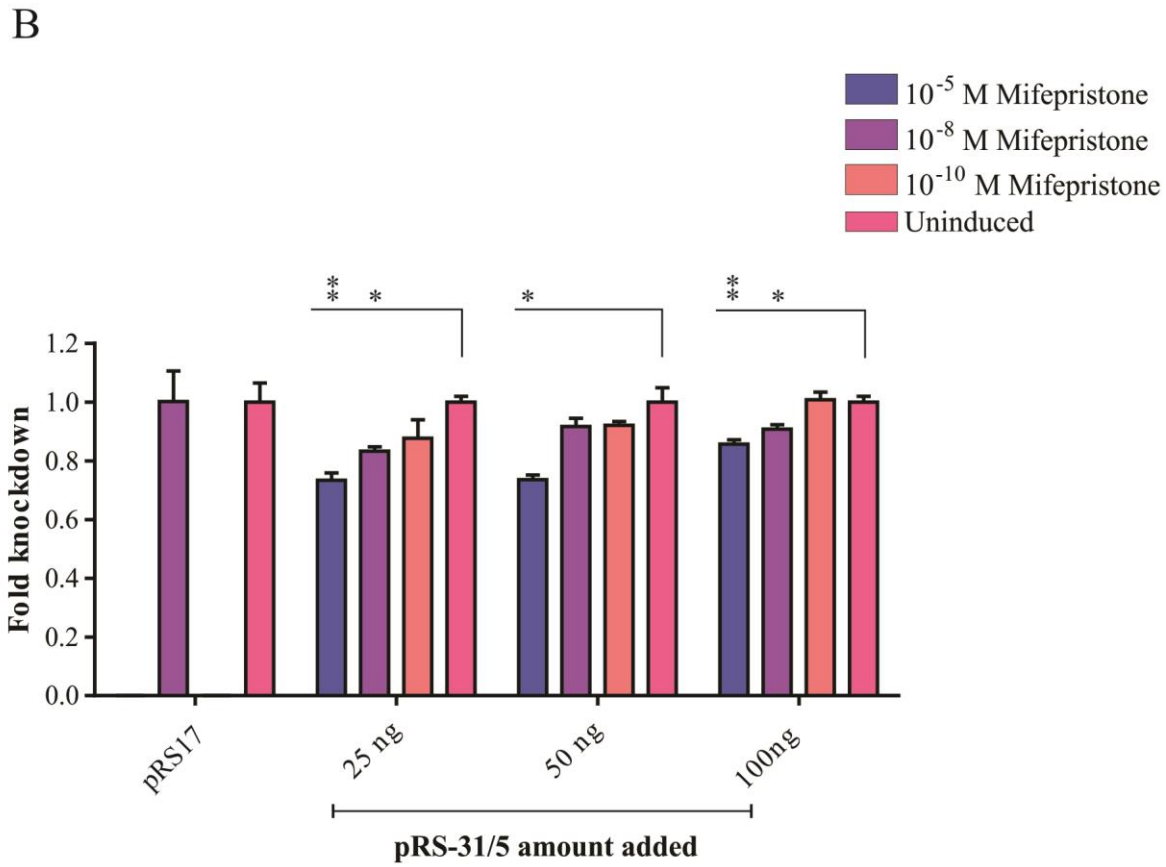
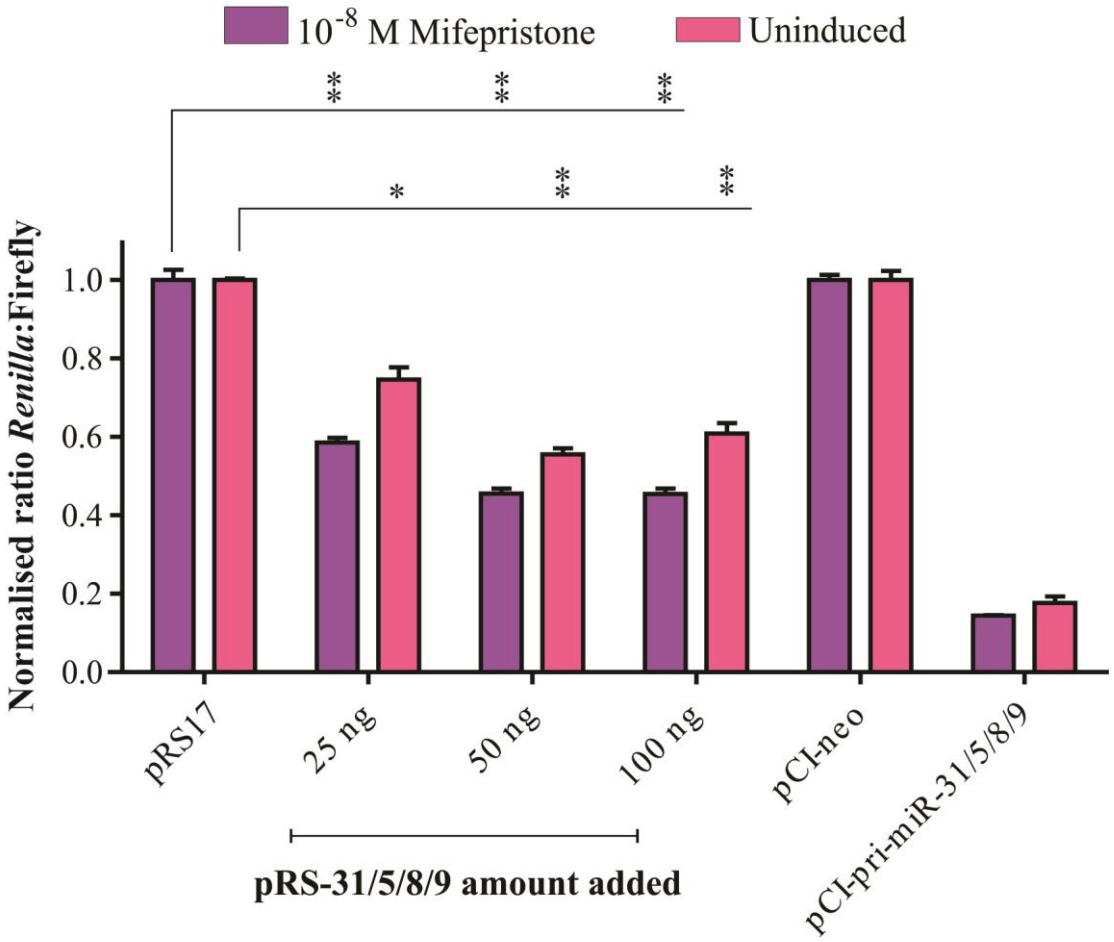


Figure 3.6: Assessment of HBV gene expression knockdown in cultured Huh7 cells expressing pri-miR-31/5 using the Dual Luciferase Assay.

Huh7 cells were transfected with 100 ng of psiCHECK-*HBx* and different amounts of pRS-31/5 or 100 ng of the empty vector pRS17 as a negative control. Cells were transfected with 100 ng of pCI-pri-miR-31/5 as a positive control or 100 ng of pCI-neo to normalise pCI-pri-miR-31/5. Five hours after transfection cells were induced with 10^{-8} M or 10^{-10} M, 10^{-8} M and 10^{-5} M mifepristone or ethanol for uninduced cells. Knockdown was assessed relative to pRS17 (A) and dose dependence was assessed relative to the uninduced cells (B). Fold knockdown is the ratio of the average knockdown obtained with each concentration of mifepristone and the average knockdown obtained with the uninduced cells. Error bars indicate the normalised standard deviation (n=3). *p< 0.05, **p<0.01, t test, relative to the pRS17 induced and uninduced cells (A) and the uninduced cells (B).

A


10-8 M Mifepristone
Uninduced
Normalised ratio Renilla:Firefly
1.0
0.8
0.6
0.4
0.2
0.0
pRS17
25 ng
50 ng
100 ng
pCI-neo
pCI-pri-miR-31/5/8/9
pRS-31/5/8/9 amount added

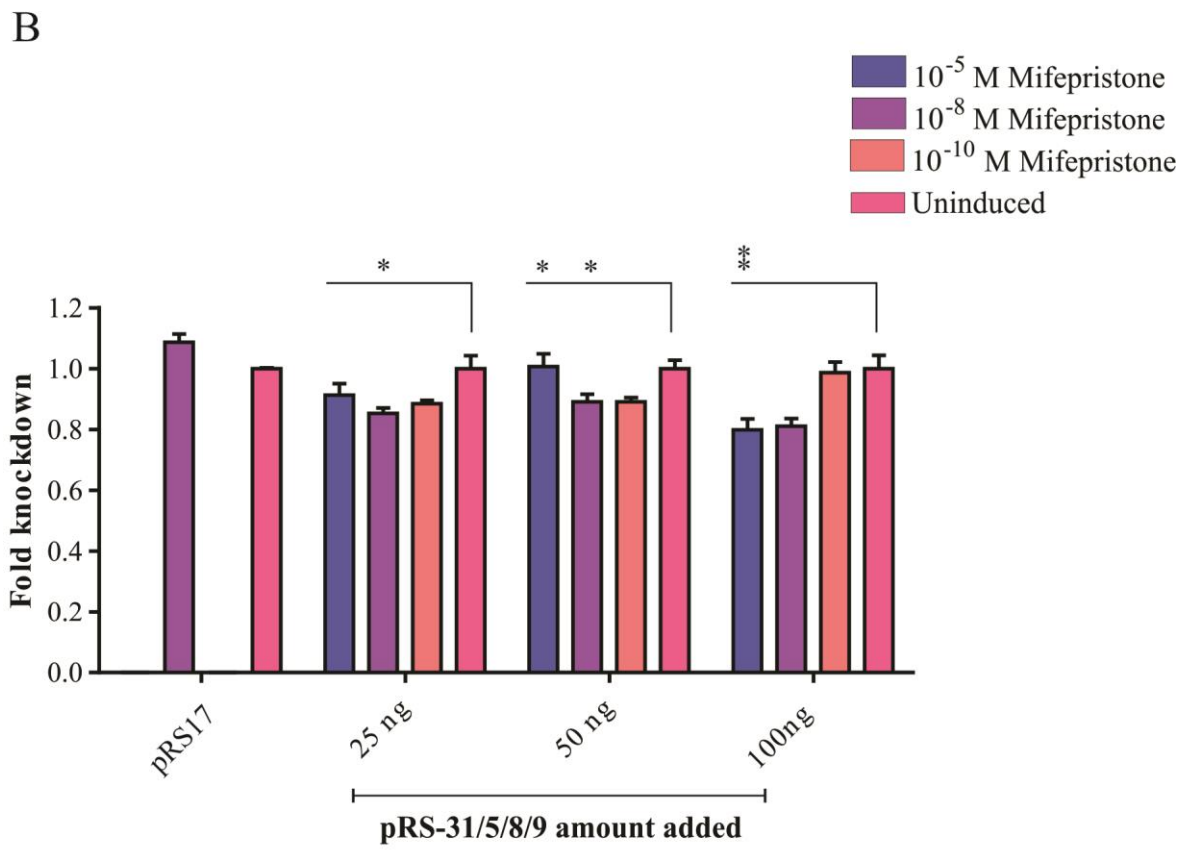


Figure 3.7: Assessment of HBV gene expression knockdown in cultured Huh7 cells expressing pri-miR-31/5/8/9 using the Dual Luciferase Assay.

Huh7 cells were transfected with 100 ng of psiCHECK-*HBx* and different amounts of pRS-31/5/8/9 or 100 ng of the empty vector pRS17 as a negative control. Cells were transfected with 100 ng of pCI-pri-miR-31/5/8/9 as a positive control or 100 ng of pCI-neo to normalise pCI-pri-miR-31/5/8/9. Five hours after transfection cells were induced with 10^{-8} M or 10^{-10} M, 10^{-8} M and 10^{-5} M mifepristone or ethanol for uninduced cells. Knockdown was assessed relative to pRS17 (A) and dose dependence was assessed relative to the uninduced cells (B). Fold knockdown is the ratio of the average knockdown obtained with each concentration of mifepristone and average knockdown obtained with the uninduced cells. Error bars indicate the normalised standard deviation (n=3). *p< 0.05, *p<0.01, t test, relative to the pRS17 induced and uninduced cells (A) and the uninduced cells (B).

3.4 HBV knockdown with the separated constituent cassettes of the mifepristone inducible system

A reduction in HBsAg secretion was observed with both pRS-31/5/8/9 (Figure 3.3) and pRS-31/5 (Figure 3.5) in the absence of mifepristone. These observations were confirmed with the Dual Luciferase Assay (Figure 3.6A & Figure 3.7A) and suggest that expression of the pri-miRs from the mifepristone inducible system is leaky. In this system, in the absence of mifepristone the GLp65 transactivator protein is constitutively expressed but remains inactive. Binding of mifepristone to the ligand-binding domain of GLp65 results in dimerisation of GLp65 and translocation to the nucleus. In the nucleus, the GAL4 DNA-binding domain interacts with the GAL4 UAS in the promoter of the target gene. This results in the p65 transactivation domain being in close enough proximity to activate target gene expression (173). Therefore there are two possibilities which may account for the leaky expression observed: 1) GLp65 dimerises in the absence of mifepristone and translocates to the nucleus resulting in activation of the *E1B* promoter driving the pri-miR expression or, 2) The *E1B* promoter is active in the absence of mifepristone-activated GLp65.

To investigate the activity of the *E1B* promoter in the absence of mifepristone-activated GLp65, the expression cassette which contains pri-miR-31/5/8/9 under the control of the *E1B* promoter was separated from the cassette which contains the transactivator, GLp65. Briefly, the transgene expression cassette containing the pri-miR-31/5/8/9 sequence was amplified using PCR from pRS-31/5/8/9 and ligated into the pTZ57R/T vector giving pTZ-pri-miR. To obtain a plasmid containing the GLp65 transactivator only, the cassette containing the pri-miR-31/5/8/9 sequence was deleted from pRS-31/5/8/9 by digestion

with *Asc*I and *Pme*I, creating the GLp65 plasmid. Cells were transfected with 100 ng of the target plasmid, psiCHECK-*HBx*, and different amounts of pTZ-pri-miR or 500 ng of pTZ57R/T as a negative control, GLp65, or pRS17 corresponding to the highest amount of pTZ-pri-miR investigated. Cells were transfected with 500 ng of pCI-pri-miR-31/5/8/9 as a positive control or 500 ng of pCI-neo to normalise pCI-pri-miR-31/5/8/9. Five hours after transfections cells were induced with 10^{-8} M mifepristone or left uninduced. The Dual Luciferase Assay was carried out 24 hours after induction.

Significant HBV gene expression knockdown was achieved with all vector amounts of the pTZ-pri-miR cassette compared to pTZ57R/T ($0.0004<p<0.03$) (Figure 3.8). In addition, there was no significant difference in knockdown achieved when comparing the induced and uninduced cells ($p>0.05$). This observation indicates that there was no induction and suggests that GLp65 is necessary for induction. The GLp65 cassette did not result in knockdown in the presence or absence of mifepristone indicating that the pri-miR-expressing cassette is required for knockdown. However, knockdown observed with the pri-miR-expressing cassette in the absence of the GLp65 transactivator confirms that expression of the pri-miR mimics from the mifepristone inducible promoter is leaky. This leakiness appears to be as a result of the *E1B* promoter switching on gene expression in the absence of mifepristone.

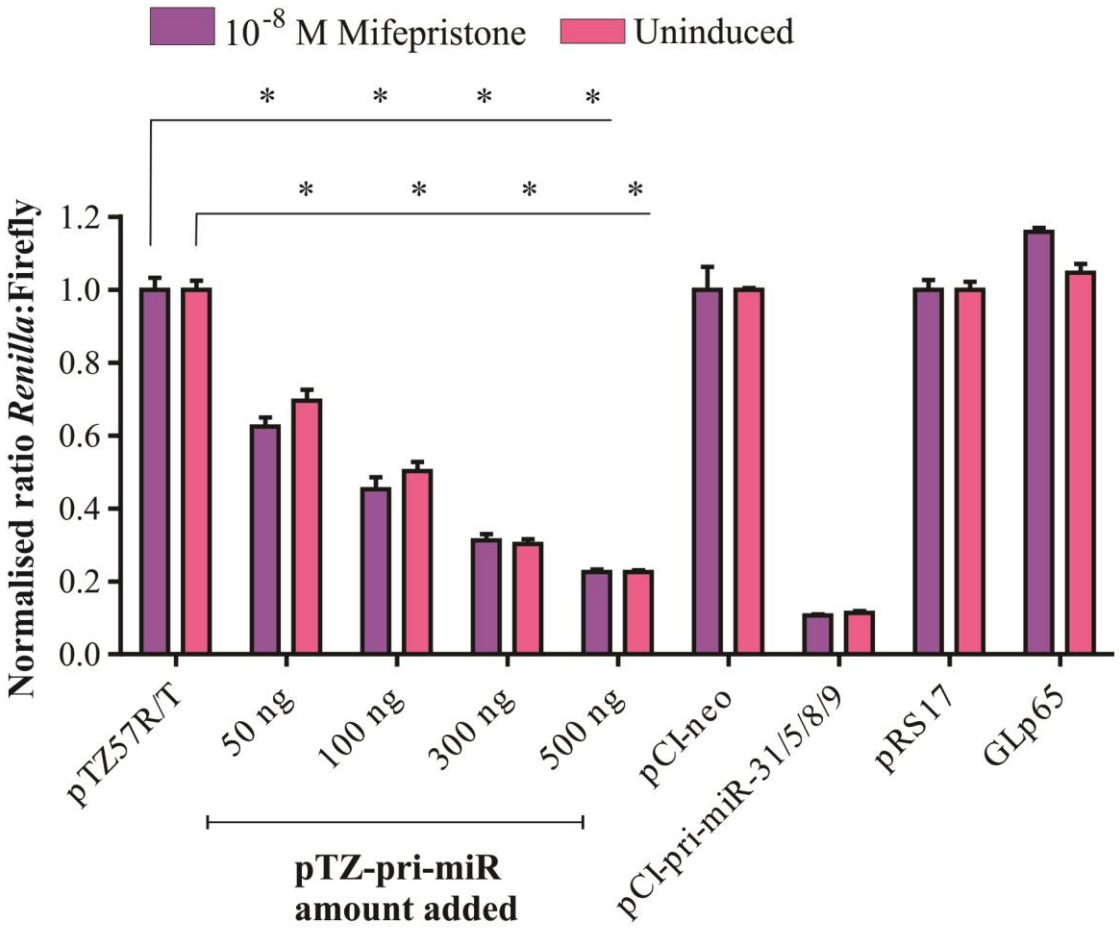


Figure 3.8: Assessment of HBV gene expression knockdown by the pri-miR-31/5/8/9 cassette in the absence of the transactivator cassette.

Huh7 cells were transfected with 100 ng of psiCHECK-*HBx* and different amounts of pTZ-pri-miR or 500 ng of pTZ57R/T as a negative control, GLp65, or pRS17. Cells were transfected with 500 ng of pCI-pri-miR-31/5/8/9 as a positive control or 500 ng of pCI-neo to normalise pCI-pri-miR-31/5/8/9. Five hours after transfection cells were induced with 10^{-8} M mifepristone or ethanol for uninduced cells. The Dual Luciferase Assay was carried out 24 hours after induction. Error bars indicate the normalised standard deviation (n=3). *p< 0.01, t test, relative to the pTZ57R/T induced and uninduced cells.

3.5 The expression of the *Firefly luciferase* gene with the mifepristone inducible system *in vivo*

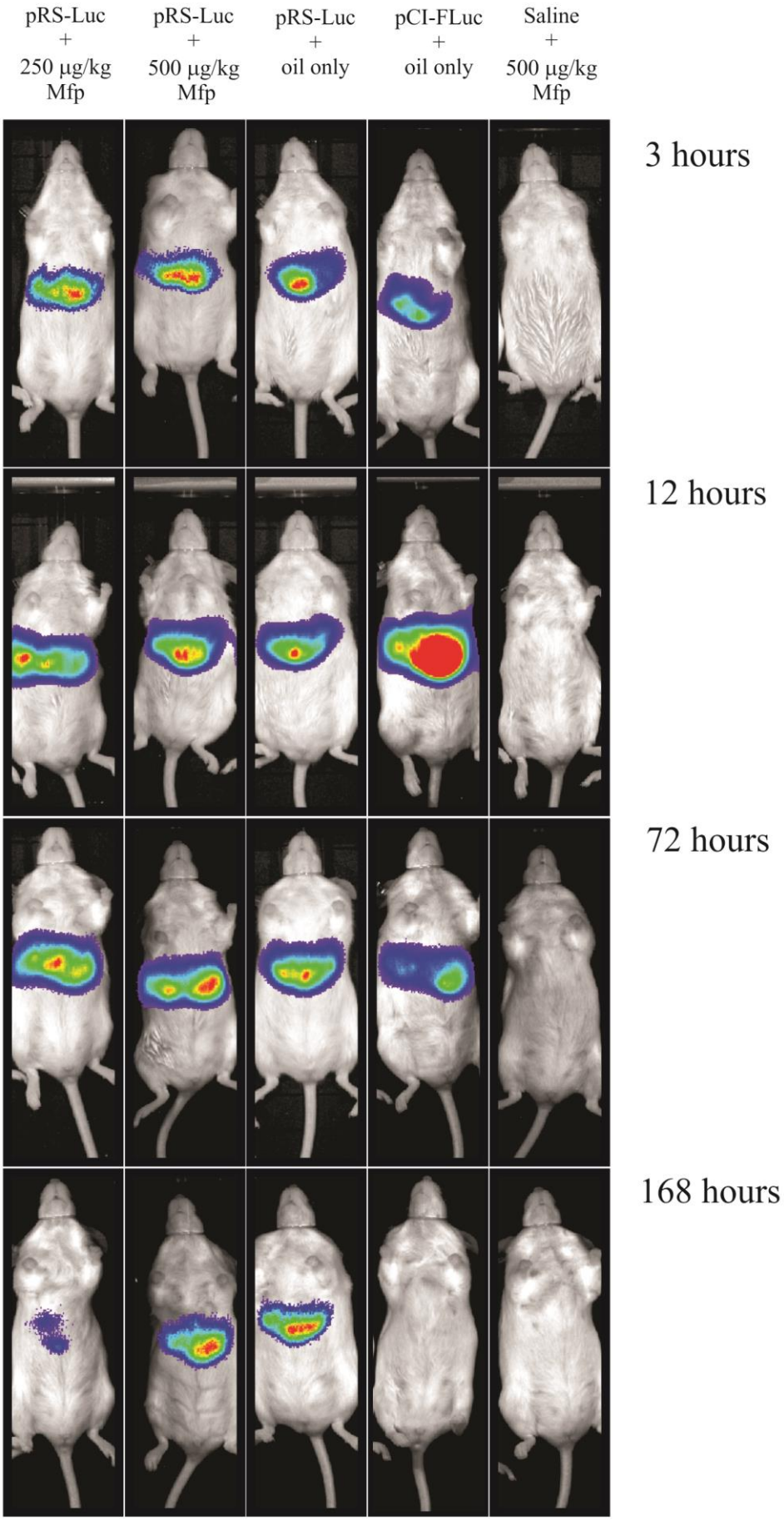
Despite the *in vitro* leakiness and marginal induction of anti-HBV pri-miR sequence expression from the mifepristone inducible system observed in this study, previous studies have shown lower basal expression and pronounced induction of expression using this system *in vivo* as compared to *in vitro* [Gonzalez-Aseguinolaza, G & Hernandez-Alcoceba, R (CIMA, Spain), personal communication]. We therefore continued to investigate the induction of gene expression by mifepristone in a mouse model.

We demonstrated that inducible expression of Firefly luciferase could be achieved with the mifepristone inducible system *in vitro* therefore we again used the luciferase-expressing construct as a starting point to assess the regulation of gene expression under the control of the inducible system *in vivo*. Briefly, mice were injected with the pRS-Luc construct and treated with varying concentrations of mifepristone. Mice received an intraperitoneal injection of mifepristone, 250 µg/kg or 500 µg/kg or oil only, in a total volume of 100 µl prior to plasmid delivery. The mifepristone inducible system has been shown to be responsive to concentrations of mifepristone as low as 10 µg/kg (188). However, 250 µg/kg and 500 µg/kg of mifepristone have frequently been used to induce gene expression *in vivo* therefore these were the starting concentrations investigated (180, 189, 202). Up to 20 µg of DNA has been previously administered when conducting *in vivo* studies with the pri-miRs expressed from a CMV promoter (78) therefore 20 µg of DNA was administered per mouse in this study. Mice were injected with 20 µg of pCI-FLuc and oil only as a positive control or with saline and 500 µg/kg of mifepristone as a negative control. The DNA was made up to a volume of 2.5 ml using normal saline and delivered to the mice

using the hydrodynamic injection. The luciferase substrate D-Luciferin was administered to the mice via intraperitoneal injection 5-10 minutes prior to imaging. The mice were anaesthetised with isofluorane and imaging carried out using the Xenogen IVIS® imaging system at different time points. Bioluminescence was quantified using the Living Image® software.

Sustained luminescence localised in the liver was observed in the mice that received the pRS-Luc construct at all time points (Figure 3.9A). However, there was no significant difference in the luminescence observed when comparing the induced and uninduced mice. In addition, no significant difference in luminescence was observed when comparing the mice that received 250 µg/kg and 500 µg/kg of mifepristone (Figure 3.9B). As previously observed with the pRS-31/5/8/9 construct *in vitro* (data not shown), high amounts of the vector result in high basal expression and masked induction. Therefore lower amounts of pRS-Luc were investigated in the subsequent experiment. Interestingly, pCI-FLuc which contains the *Firefly luciferase* gene under the control of the CMV promoter, resulted in high expression but was quickly cleared in comparison and luminescence could no longer be detected at the 168 hour time point.

A


pRS-Luc + 250 µg/kg Mfp
pRS-Luc + 500 µg/kg Mfp
pRS-Luc + oil only
pCI-FLuc + oil only
Saline + 500 µg/kg Mfp
3 hours
12 hours
72 hours
168 hours

B

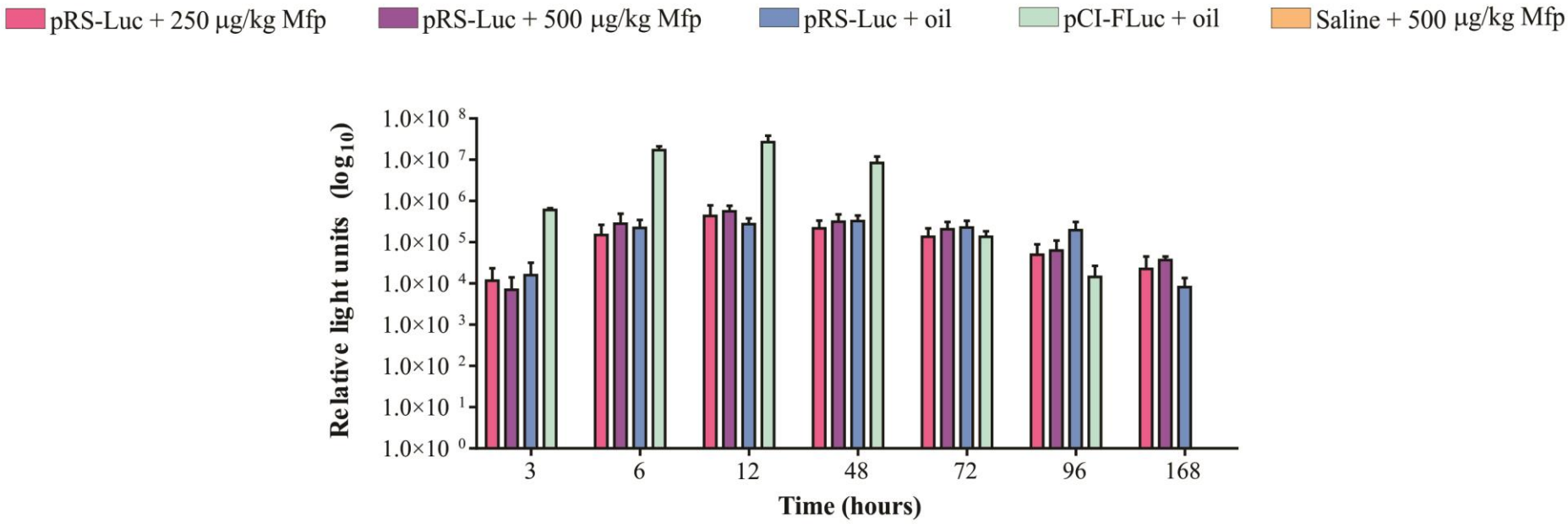


Figure 3.9: Assessment of *Firefly luciferase* gene expression with the mifepristone inducible promoter *in vivo* using bioluminescence imaging.

(A) Images were captured using the Xenogen IVIS® imaging system at 5 seconds exposure. (B) Bioluminescence was quantified using the Living Image® software. Mice received an intraperitoneal injection of mifepristone, 250 µg/kg or 500 µg/kg, or oil only and 20 µg of DNA was administered per mouse. Mice were injected with 20 µg of pCI-FLuc and oil only as a positive control or with saline and 500 µg/kg of mifepristone as a negative control. Error bars indicate the normalised standard deviation (n=3).

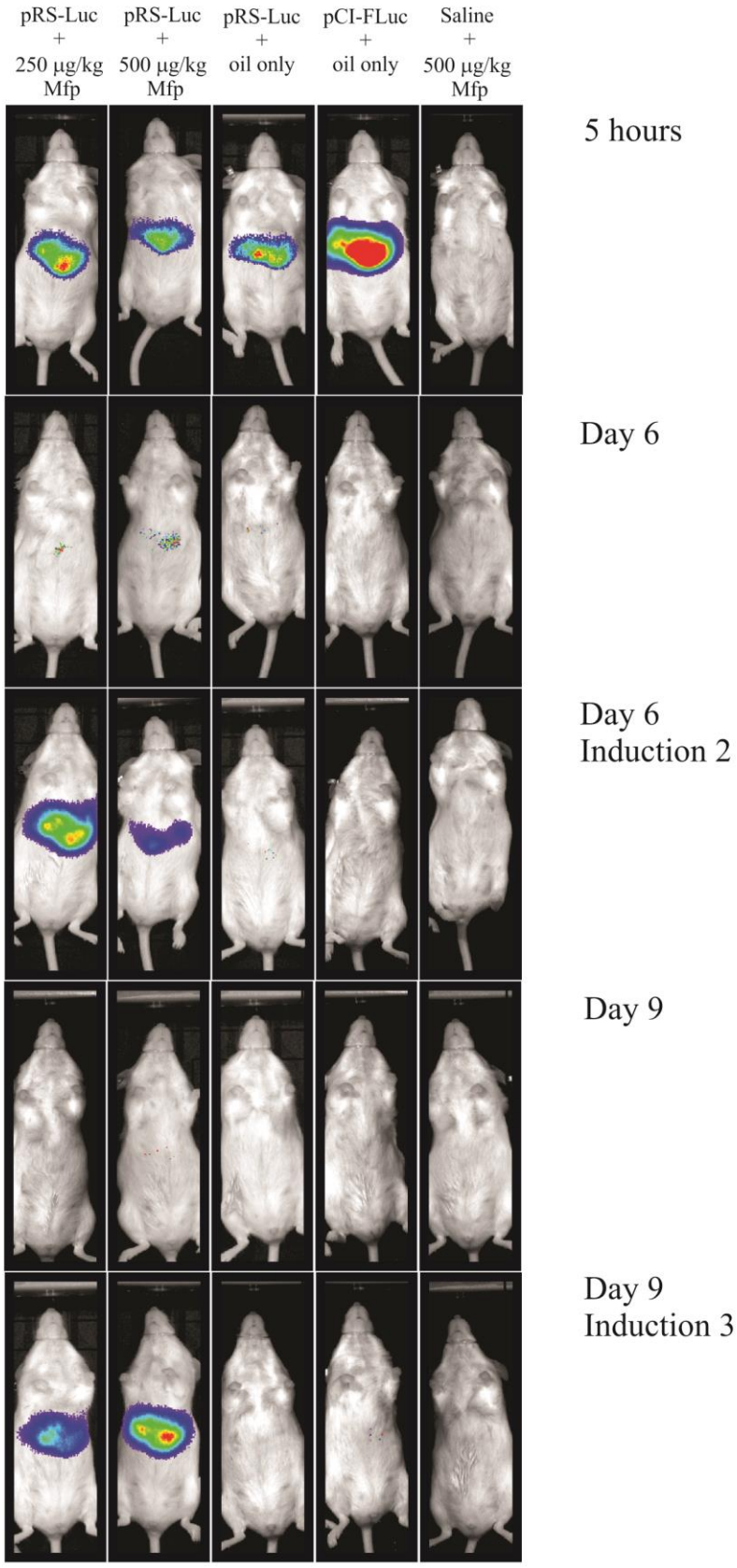
3. 6 Induction of *Firefly luciferase* gene expression with mifepristone *in vivo* at a lower vector amount

To assess induction of Firefly luciferase expression under the control of the mifepristone inducible system with a lower amount of pRS-Luc, 5 µg of pRS-Luc was used for injections. Briefly, mice received an intraperitoneal injection of mifepristone, 250 µg/kg or 500 µg/kg or oil only prior to plasmid delivery. Mice received 5 µg of the pRS-Luc construct or 5 µg of pCI-FLuc and oil only as a positive control or saline and 500 µg/kg of mifepristone as a negative control. Re-induction was carried out on day 6 and day 9 when bioluminescence had almost completely disappeared and basal expression was almost undetectable. Imaging was carried out using the Xenogen IVIS® imaging system at different time points. Bioluminescence was quantified using the Living Image® software.

Five hours after induction and injection, luminescence was observed in the mice that received the pRS-Luc construct (Figure 3.10A). However, similar luminescence was observed in the induced and uninduced mice indicating high basal expression of pRS-Luc. After 6 days the amount of luminescence had greatly decreased indicating reduced Firefly luciferase expression. Following re-induction on day 6, there was a significant increase in luminescence observed in the mice that received 250 µg/kg of mifepristone compared to the mice that received oil only ($p= 0.0053$) (Figure 3.10B). By day 9 Firefly luciferase expression had again decreased and re-induction was carried out. Following re-induction, there was a significant increase in luminescence observed in the mice that received 500 µg/kg of mifepristone compared to the mice that received oil only ($p= 0.034$) (Figure 3.10B). These data demonstrate that Firefly luciferase expression with the mifepristone inducible system is inducible *in vivo*. There was a difference in the luminescence observed

when comparing mice that received 250 µg/kg and 500 µg/kg of mifepristone, however, it was not statistically significant. This necessitates further studies to identify the optimum mifepristone concentration for induction *in vivo*.

A


pRS-Luc + 250 µg/kg Mfp
pRS-Luc + 500 µg/kg Mfp
pRS-Luc + oil only
pCI-FLuc + oil only
Saline + 500 µg/kg Mfp
5 hours
Day 6
Day 6 Induction 2
Day 9
Day 9 Induction 3

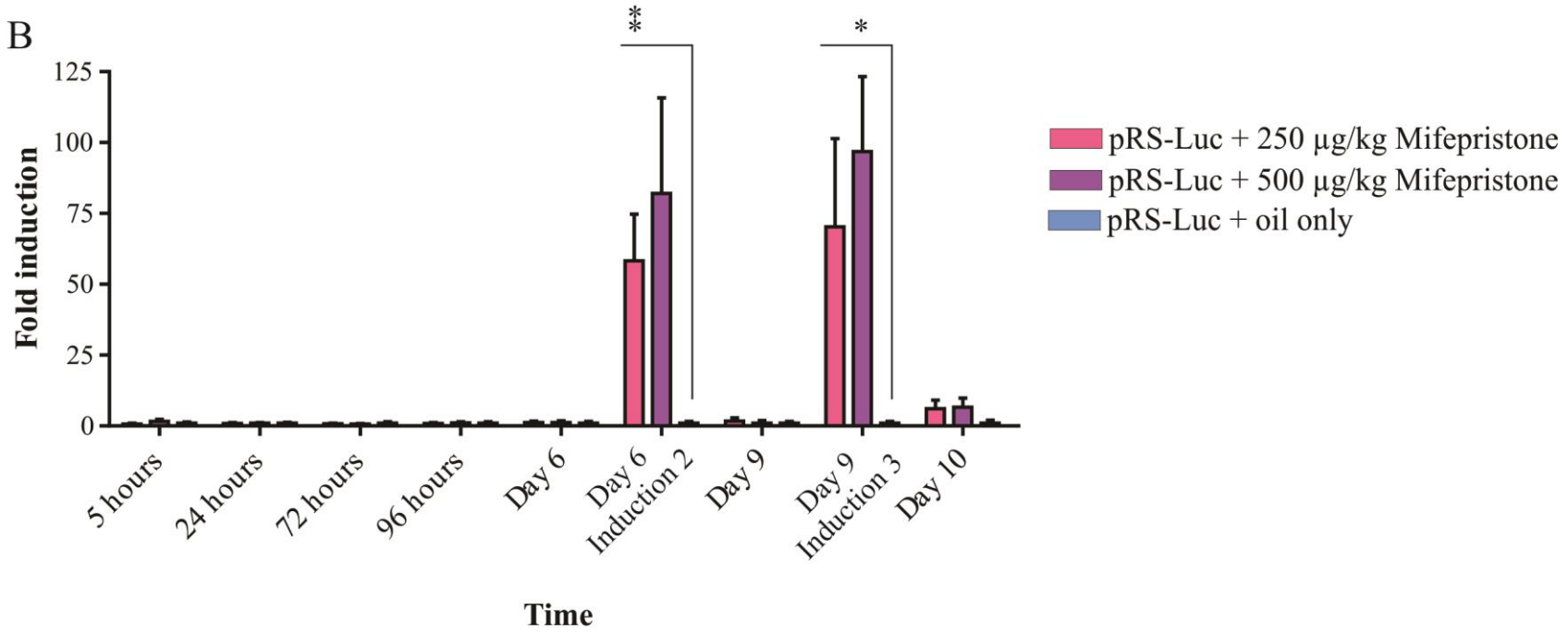


Figure 3.10: Induction of *Firefly luciferase* gene expression under the control of the mifepristone inducible promoter *in vivo*.

(A) Images were captured using the Xenogen IVIS® imaging system at 5 seconds exposure. (B) Bioluminescence was quantified using the Living Image® software. Mice received intraperitoneal injections of mifepristone, 250 µg/kg or 500 µg/kg or oil only and 5 µg of DNA was administered per mouse. Re-induction was carried out on day 6 and day 9. Mice were injected with 5 µg of pCI-FLuc and oil only as a positive control or with saline and 500 µg/kg of mifepristone as a negative control. Error bars indicate the normalised standard deviation (n=5). *$p < 0.05$, **$p<0.01$, t test, relative to the uninduced mice.

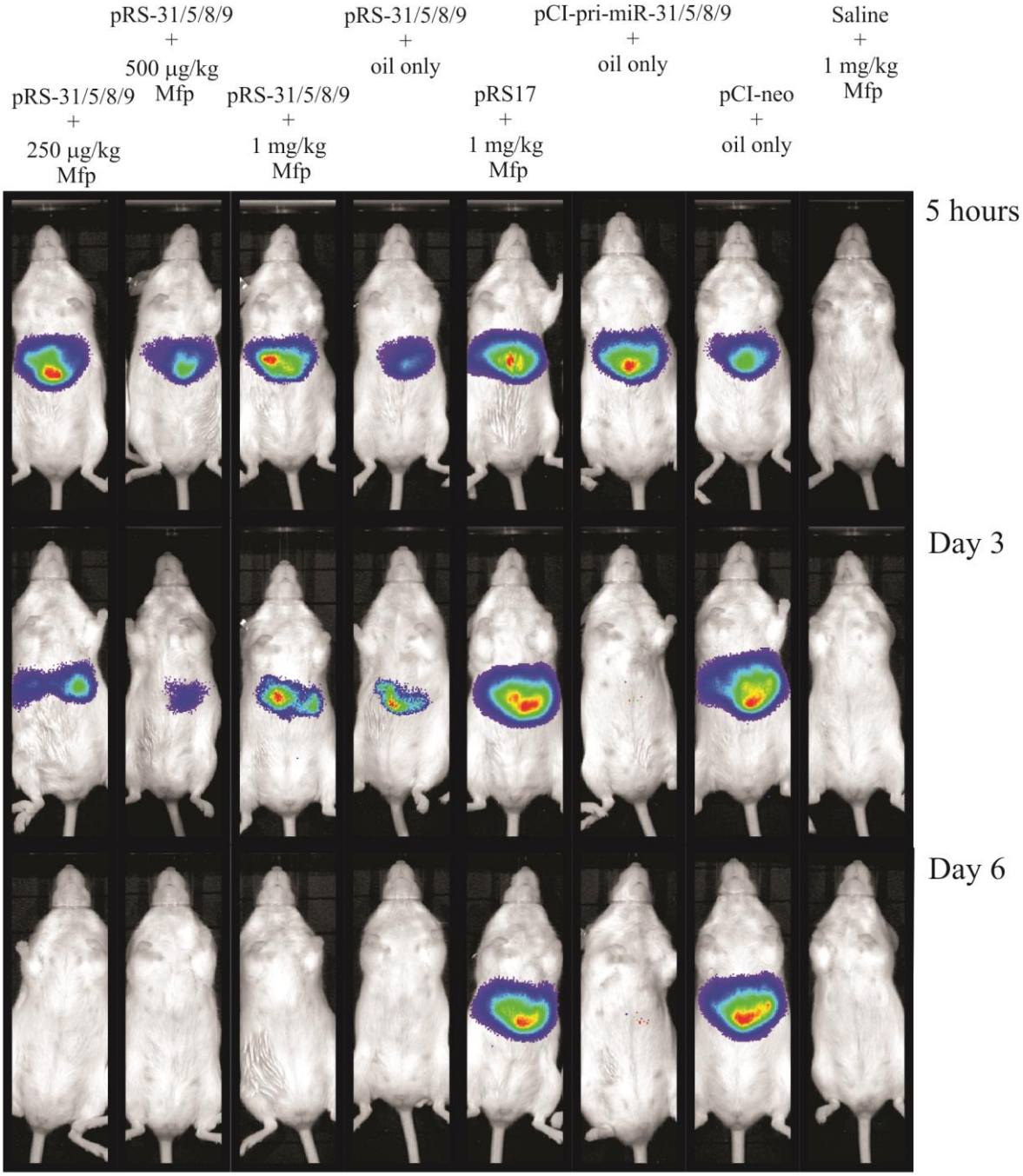
3.7 Assessment of knockdown of HBV gene expression following induction of pri-miR expression with mifepristone *in vivo*

We demonstrated that HBV gene expression knockdown could be achieved with pri-miR-31/5 and pri-miR-31/5/8/9 expressed from the mifepristone inducible system *in vitro*. However, the inducibility of the system was decreased with high amounts of the pri-miRs. To assess HBV knockdown following induction with mifepristone *in vivo*, the pri-miR-31/5/8/9-expressing construct was used. Briefly, mice received an intraperitoneal injection of mifepristone, 250 µg/kg, 500 µg/kg or 1 mg/kg or oil only, in a total volume of 100 µl prior to plasmid delivery. Subsequent inductions were carried out daily. Mice were injected with 5 µg of pRS-31/5/8/9 and 5 µg of the target plasmid, pCH Firefly Luc, in which the *preS2/S* ORF of pCH-9/3091 is substituted with the *Firefly luciferase* sequence. As controls, mice were injected with 5 µg of the empty vector pRS17 and 5 µg of pCH Firefly Luc then induced daily with 250 µg/kg, 500 µg/kg or 1 mg/kg of mifepristone or oil only. Mice were injected with 5 µg of the target plasmid pCH Firefly Luc and either 5 µg of pCI-pri-miR-31/5/8/9 and oil as a positive control, 5 µg of pCI-neo and oil to normalise for pCI-pri-miR-31/5/8/9 or saline and 1 mg/kg of mifepristone as a negative control. The mice were anaesthetised with isofluorane and imaging carried out using the Xenogen IVIS® imaging system at 5 hours, day 3 and day 6. Bioluminescence was quantified using the Living Image® software.

Five hours after induction and injection, luminescence was observed in the mice that received the pRS-31/5/8/9 construct and pCH Firefly Luc (Figure 3.11A). On day 3, significant HBV expression knockdown had been achieved in the mice that received pRS-31/5/8/9 and 250 µg/kg or 500 µg/kg of mifepristone compared to the controls (p= 0.047

and p= 0.012 respectively) (Figure 3.11B). On day 6, a significant decrease in luminescence was observed in the mice that received pRS-31/5/8/9 and 500 µg/kg of mifepristone or oil only compared to the controls (p= 0.041 and p= 0.023 respectively) (Figure 3.11B). The reduction observed with mice that received oil only suggests that there was leaky expression of pRS-31/5/8/9. These data are similar to the *in vitro* observations which demonstrate that HBV gene expression knockdown is achieved with the pri-miRs under the control of the mifepristone inducible system but there is leaky expression. There was no significant difference in the luminescence observed when comparing mice that received 250 µg/kg, 500 µg/kg or 1 mg/kg of mifepristone. This necessitates further studies to identify the optimum mifepristone concentration for the induction of pri-miR expression *in* vivo. Although only 5 µg of the pri-miR-31/5/8/9-expressing construct was used, further investigations would be necessary to determine whether lower amounts of the vector can be used to reduce leaky expression. However, we have previously observed that 2.5 µg of plasmid DNA is not effectively delivered to the liver using the hydrodynamic injection method (data not shown).

A


pRS-31/5/8/9 + 250 µg/kg Mfp
pRS-31/5/8/9 + 500 µg/kg Mfp
pRS-31/5/8/9 + 1 mg/kg Mfp
pRS-31/5/8/9 + oil only
pRS17 + 1 mg/kg Mfp
pCI-pri-miR-31/5/8/9 + oil only
pCI-neo + oil only
Saline + 1 mg/kg Mfp
5 hours
Day 3
Day 6

B

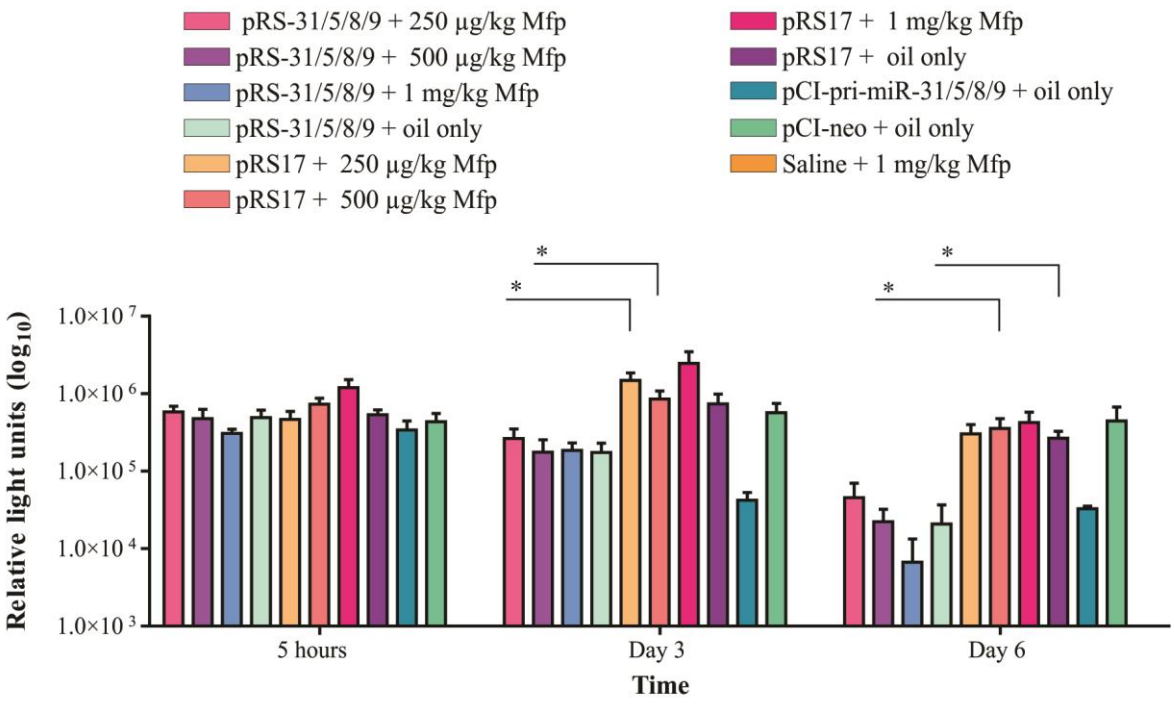


Figure 3.11: HBV gene expression knockdown with pri-miR-31/5/8/9 expressed from the mifepristone inducible system *in vivo*.

(A) Images were captured using the Xenogen IVIS® imaging system at 10 seconds exposure. (B) Bioluminescence was quantified using the Living Image® software. Mice were injected with 5 µg of pRS-31/5/8/9 and 5 µg of the target plasmid, pCH Firefly Luc, and received daily intraperitoneal injections of mifepristone, 250 µg/kg, 500 µg/kg or 1 mg/kg or oil only. As controls, mice were injected with 5 µg of the empty vector pRS17 and 5 µg of pCH Firefly Luc then induced daily with 250 µg/kg, 500 µg/kg or 1 mg/kg of mifepristone or oil only (only images for 1 mg/kg induction shown). Mice were injected with 5 µg of the target plasmid pCH Firefly Luc and either 5 µg of pCI-pri-miR-31/5/8/9 and oil as a positive control, 5 µg of pCI-neo and oil to normalise pCI-pri-miR-31/5/8/9 or saline and 1 mg/kg of mifepristone as a negative control. Error bars indicate the normalised standard deviation (n=5). *$p < 0.05$, t test, relative to the control mice.

CHAPTER 4

4 DISCUSSION

HBV is a major global health burden and chronic carriers are at high risk of developing hepatocellular carcinoma (HCC) and cirrhosis (1-3). Current treatment strategies are only partially effective, thus emphasising the need for improved treatment strategies. Exploiting the RNA interference (RNAi) pathway to silence HBV gene expression has shown promise as a therapeutic approach. However, there are obstacles that need to be overcome before RNAi-based treatment of HBV infection can advance to the clinic. These include problems of liver tissue targeting and dose regulation. RNA polymerase (Pol) III promoters are most commonly used to express RNAi activators. However, they are constitutively active and may cause saturation of the endogenous RNAi pathway and resultant toxicity (67). Pol II promoters are inducible and have the added benefit of compatibility with anti-HBV pri-miR shuttles (120). This study investigated the use of a liver specific and mifepristone inducible Pol II promoter system for the specific and precise regulation of anti-HBV sequence expression.

4.1 Gene expression under the control of the mifepristone inducible system is regulatable and dose-dependent

The mifepristone inducible system has been used to achieve inducible and tissue specific gene expression and is therefore a valuable tool for gene therapy [reviewed in (173, 203)].

To assess gene regulation under the control of the mifepristone inducible system in our hands, the pRS-Luc construct, which expresses Firefly luciferase from a mifepristone inducible promoter, was used. A 10-30-fold induction of *Firefly luciferase* gene expression was observed with the two concentrations of mifepristone (10^{-8} M and 10^{-6} M) investigated when 100-500 ng of the vector was used. The lower induction (10-fold) observed at higher vector amounts (500 ng) is as a result of higher basal expression. This effect of basal expression on induction has previously been demonstrated by Abruzzese and colleagues. The authors observed a modest induction (10-fold) of secreted human placental alkaline phosphatase (SEAP) expression as a result of high basal expression with the mifepristone inducible system (183). Our observations are also similar to previous data in which a 10-20-fold induction of Firefly luciferase expression is observed in Huh7 cells [Hernandez-Alcoceba, R (CIMA, Spain), personal communication]. This confirms that gene expression under the control of the mifepristone inducible system is inducible and that the system was functioning as intended in our hands.

However, variations in the levels of gene expression and induction have been observed with the mifepristone inducible system *in vitro*. This variation seems to be dependent on factors such as the particular gene being expressed, the cell lines being used and the concentration of the inducer. As previously mentioned, the vector amounts transfected and the resulting basal expression also have an influence on induction (183). This variation was particularly well demonstrated in a study by Abruzzese and colleagues. In this study, the African green monkey kidney fibroblast-like COS-1 cell line was transfected with a plasmid containing the GLp65 transactivator under the control of a CMV promoter and inducible plasmids encoding different genes. Upon induction with 10^{-8} M mifepristone, a

10-fold increase in SEAP activity was observed. In contrast, the authors observed a 33-fold induction in vascular endothelial growth factor (hVEGF) protein levels and a 16-fold increase in murine erythropoietin (mEpo) protein levels (183). This highlights the versatility of this system but also the importance of optimising the system for each application.

Therefore, in this study, attempts were made to optimise induction conditions by investigating dose dependence with the mifepristone inducible system *in vitro*. Huh7 cells were transfected with pRS-Luc and induced with different concentrations of mifepristone. The greatest induction was achieved with 10^{-9} M mifepristone while the reduced induction observed with the highest mifepristone concentrations investigated (10^{-5} M and 10^{-6} M) was possibly as a result of a toxic effect of mifepristone on the cells at these concentrations. Further studies are necessary to fully investigate the latter observation. Our observations are in accordance with several previous studies. A study in which the system was incorporated into a lentiviral vector resulted in the maximal activation of DsRed2 red fluorescent protein marker expression with 10^{-9} M and 10^{-8} M mifepristone. The authors also demonstrated the optimal expression of human α_1-antitrypsin (hAAT) after induction with 10^{-8} M mifepristone (186). Another study in which human interleukin-12 (hIL-12) was expressed in liver-derived cells showed a dose-dependent increase in expression from 10^{-10} M to 10^{-7} M mifepristone (202). Based on our observations and those of others we can conclude that gene expression under the control of the mifepristone inducible system *in vitro* is dependent on the concentration of the inducer, mifepristone. Despite the aforementioned evidence of variation in expression with the mifepristone inducible system, the data obtained with the luciferase-expressing construct *in vitro* were valuable as they

provided a starting point for the expression of the pri-miRs from the inducible system *in vitro*.

To assess the *in vivo* functionality of the inducible system, the expression of Firefly luciferase from pRS-Luc was investigated in a mouse model. Firefly luciferase expression was successfully demonstrated *in vivo*, however, with a high vector amount of pRS-Luc (20 µg), induction was masked as a result of high basal expression. In addition, for the initial experiments, induction was only carried out once, which is not ideal. In humans, after a single oral administration, mifepristone concentration in the serum reaches its peak in about 2 hours and has a half-life of 20-30 hours (205-207). Therefore multiple inductions with mifepristone would be required to ensure sustained expression of the transgene. In our case, the combination of high basal expression and a single induction explain the similar luminescence observed in the induced and uninduced mice. In addition, the hydrodynamic injection itself has been proven to be hepatotoxic (45) therefore it might be beneficial to allow some time for the animals to recover to exclude any effects of liver damage on gene expression. Interestingly, the construct which contains the *Firefly luciferase* gene under the control of the CMV promoter resulted in high expression but was rapidly cleared from the liver. Expression under the control of the CMV promoter has been shown to be variable and susceptible to silencing in certain tissues (208-210), specifically in hepatocytes (211), which may explain our observations.

In the subsequent study, the vector amount administered to the mice was decreased from 20 µg to 5 µg. In addition, re-induction was carried out only when basal expression was no longer detectable and therefore negligible to prevent masking of induction. As expected,

reducing the vector amount administered to the mice as well as inducing in the presence of minimal basal expression significantly improved the inducibility of the system *in vivo*. This was demonstrated by the 50 to 100-fold induction observed upon re-induction with 250 µg/kg or 500 µg/kg of mifepristone. This was again in agreement with previous studies in which improved induction is achieved with the inducible system *in vivo* compared to *in vitro* [Gonzalez-Aseguinolaza, G & Hernandez-Alcoceba, R (CIMA, Spain), personal communication]. A difference was observed in the induction by 250 µg/kg compared to 500 µg/kg of mifepristone, however, this difference was not statistically significant. This indicates that further studies need to be carried out to determine the optimum mifepristone concentration for the induction of Firefly luciferase expression. Nonetheless, these data confirm that inducible gene expression with the mifepristone inducible system can be achieved *in vivo*.

As is the case with the *in vitro* expression of a transgene, gene expression under the control of the inducible system *in vivo* has also been demonstrated to be variable. In this case, the determinants of variation seem to be more complex than *in vitro* and include factors such as the particular gene being expressed as well as vector amounts, the site of expression, the method of delivery and the concentration of the inducer. A study by Burcin and colleagues incorporated the inducible system into an adenoviral vector and delivered the construct to mice by tail vein injection. Mice received intraperitoneal (i.p) injections of 250 µg/kg of mifepristone and a 50 000-fold induction of human growth human (hGH) serum levels was detected (180). In another study, a plasmid containing the transactivator and an inducible reporter plasmid expressing SEAP were delivered to the hind-limb muscles of mice by direct injection followed by electroporation. The mice received a single i.p dose of mifepristone (0.33 mg/kg) resulting in a 14-fold induction of SEAP serum levels. The

authors also demonstrated mifepristone dose dependence by assessing the responsiveness of the system with a range of mifepristone concentrations from 0 to 1 mg/kg. A 0.01 mg/kg dose was sufficient to elicit a partial response while a maximal response was observed with 0.33 mg/kg of mifepristone (188). A third study by Wang and colleagues demonstrated both vector and inducer dose dependence. The inducible system expressing hIL-12 was incorporated into a gutless adenoviral vector and delivered to the mice at two different concentrations [1×10^9 or 3×10^9 infectious units (iu)] by tail vein injection. Mifepristone was then administered at three different concentrations (125, 250 or 500 µg/kg) via i.p injection. hIL-12 serum levels were highest when the highest vector (3×10^9 iu) and mifepristone concentrations (500 µg/kg) were combined (189). This is in contradiction with our observations in which high vector amounts result in reduced induction. However, it is difficult to make a comparison of results as the methods of delivery in the two studies were different. The authors used an adenoviral vector for delivery whereas we administered plasmid DNA to the mice in our study. This, in addition to the other aforementioned factors, may have contributed to the differences observed.

Gene expression under the control of an inducible system *in vivo* is further complicated by considerations such as the duration of inducibility and the effect of repeated inductions, particularly in a therapeutic context. Loss of inducibility has typically been found to be as a result of the loss of plasmid. (188). However, a study by Abruzzese and colleagues in which SEAP was expressed from the inducible system demonstrated that antibodies were generated against the foreign protein, SEAP. They went on to show that the induction of these anti-SEAP antibodies was sufficient to inhibit the function of the inducible system (188). The effect of chronic administration of mifepristone *in vivo* has also been investigated by several groups. An example is the study by Wang and colleagues in which

the inducible system expressing hIL-12 was incorporated into a gutless adenoviral vector and induction was carried out every 12, 24 or 48 hours. They observed that induction every 48 hours resulted in a saw-like pattern of expression whereas more sustained expression was observed with induction every 12 or 24 hours (189). Evidently the half-life of the transgene product itself is an important consideration and will likely dictate the schedule of induction. The pharmacokinetics and pharmacodynamics of the inducer, which have been established for mifepristone (165, 167), will also influence induction. This evidence indicates that there are complex interactions which must be elucidated and many factors to consider when this system is used *in vivo*. Therefore, similar to the observations *in vitro* and taken together with observations from previous studies, these data demonstrate that optimisation of induction conditions must also be carried out for each application of this system *in vivo*.

4.2 Effective HBV gene expression knockdown can be achieved with pri-miRs expressed from the mifepristone inducible system

The pri-miR mimics used in this study have been found to result in significant HBV gene expression knockdown *in vitro* and *in vivo* when expressed from a CMV promoter (57, 78). The mifepristone inducible system was used to regulate the expression of the pri-miRs and achieve liver-specific expression, thus targeting the primary site of HBV infection. The ability to achieve inducible expression of RNAi effectors will address the toxicity concerns associated with RNAi-based therapeutics and will contribute to the advancement of RNAi to the clinic.

Assessment of HBsAg secretion, a marker of HBV gene expression, using ELISA was the first approach used to determine the efficacy of the pri-miRs under the control of the inducible system *in vitro*. Both the mono- and polycistronic anti-HBV pri-miR-31/5 and pri-miR-31/5/8/9 sequences expressed from the inducible system resulted in significant reduction of HBsAg. However, similar reduction observed with induced and uninduced cells with both constructs suggests that pri-miR expression from the mifepristone inducible system is leaky *in vitro*. In addition, the high vector amounts investigated appeared to mask induction, particularly with the pri-miR-31/5/8/9-expressing construct (pRS-31/5/8/9). Similar to the luciferase-expressing construct, we have observed that high vector amounts of pRS-31/5/8/9 result in high basal expression (data not shown).

Therefore, attempts were made to optimise the amount of vector added such that induction of gene expression can be achieved with minimal basal expression. We established the vector amounts of pRS-31-5/8/9 at which there is negligible basal expression (10-30 ng) as observed by no significant reduction in HBsAg. However, upon induction there was no significant induction observed. These optimisation studies revealed that for the expression of pri-miRs from the inducible system some basal expression in the absence of the inducer must be tolerated. However, ideally, basal expression and induction must be at acceptable levels such that tight regulation of gene expression can still be achieved. The requirement for this balance was illustrated in a study by Abruzzese and colleagues in which the inducible system was transformed into an auto inducible system such that induction with mifepristone results in the activation of the transgene as well as the regulator itself. Although this increased the “tightness” of the system, i.e. reduced basal expression of the transgene, this came at the expense of reducing the level of induced transgene expression.

A 10-fold reduction in basal expression was accompanied by a 2-fold reduction in induction (183).

To corroborate the data obtained with ELISA, the Dual Luciferase assay was used to assess HBV gene expression knockdown with the pri-miRs expressed from the mifepristone inducible system. The starting vector amounts taken from the *in vitro* studies with the luciferase-expressing construct were too high when it came to the expression of the pri-miRs from the inducible system as demonstrated by the high basal expression and masked induction. This is not surprising given the variable expression that has been observed with the inducible system. Therefore, lower vector amounts (25-100 ng) were investigated to minimise basal expression. However, there was no significant HBV gene expression knockdown with the pri-miR-31/5-expressing construct (pRS-31/5) with the lower vector amounts investigated. It appears that attempting to reduce the basal expression came at the expense of reducing knockdown efficacy. Nonetheless, induction in the form of knockdown was observed with 10^{-8} M and 10^{-5} M mifepristone when comparing the induced cells to the uninduced cells. The knockdown was marginal, reaching a maximum of about 30%. In contrast to pRS-31/5, pRS-31/5/8/9 resulted in significant HBV gene expression knockdown with all vector amounts investigated with and without induction. In addition, induction in the form of knockdown was observed with all three concentrations of mifepristone investigated suggesting more variation compared to the pri-miR-31/5-expressing construct. The knockdown observed was lower than with pRS-31/5, reaching a maximum of about 20%. However, similar knockdown was again observed with induced and uninduced cells confirming the leaky expression of anti-HBV pri-miR sequences from the inducible system.

Interestingly, overall, we observed more potent silencing with pRS-31/5/8/9 than with pRS-31/5. This may be as a result of its polycistronic nature which allows for the silencing of more than one target simultaneously. Previous studies have shown that the efficacy and processing of the three pri-miRs is not equal owing to their specific sequences and position within the polycistron. In addition, *in vivo*, the difference in silencing achieved with pri-miR-31/5/8/9 compared to pri-miR-31/5 is marginal (78). Nonetheless, the presence of three silencing RNAi miRs versus one may still have contributed to increased efficacy, particularly in the context of this specific expression system. However, it appears that the more potent silencing by pRS-31/5/8/9 came at the expense of reducing the inducibility of gene expression.

Despite the marginal induction and leakiness, as previously observed in this study with the luciferase-expressing construct, this system exhibits improved inducibility *in vivo.* Therefore, we went on to assess HBV gene expression knockdown with pri-miR-31/5/8/9 expressed from the mifepristone inducible system in a mouse model. Each mouse received 5 µg of the target plasmid pCH Firefly Luc, in which the *preS2/S* ORF of pCH-9/3091 is substituted with the *Firefly luciferase* gene, and 5 µg of pRS-31/5/8/9. Induction was carried out daily to ensure sustained expression of the pri-miR. HBV gene expression knockdown (observed as decreased bioluminescence) up to two orders of magnitude was observed by pRS-31/5/8/9 compared to the controls. However, similar knockdown was observed with both induced and uninduced mice. This is in agreement with the observations *in vitro* and demonstrates that HBV gene expression knockdown is achieved with the pri-miRs under the control of the mifepristone inducible system *in vivo* but there is leaky expression. It might be useful to reduce the amount of plasmid administered to the

mice to reduce leaky expression. Although only the administration of 5 µg per mouse was reported in this study, we have also observed that 2.5 µg of plasmid is not effectively delivered to the liver using the hydrodynamic injection. From these observations it is evident that understanding the mechanism of leaky expression is important and will be beneficial in optimising the system both *in vitro* and *in vivo*.

4.3 The mechanism of leaky expression of anti-HBV pri-miRs under the control of the mifepristone inducible system

The observations from knockdown studies with both pRS-31/5 and pRS-31/5/8/9 strongly suggest that there is leaky expression of the pri-miRs from the inducible system. The modifications made to this system from the first generation derivatives to the version currently used have generally resulted in lower basal expression, and therefore better inducibility of gene expression, and responsiveness to lower concentrations of mifepristone (178, 180). In this system, the GLp65 transactivator protein is constitutively expressed but remains inactive in the absence of mifepristone. Binding of mifepristone to the ligand-binding domain of GLp65 results in dimerisation of GLp65 and translocation to the nucleus. In the nucleus, the GAL4 DNA-binding domain interacts with the GAL4 UAS in the promoter of the target gene. This results in the p65 transactivation domain being in close enough proximity to activate target gene expression (173). Therefore in the event of leaky expression such as we have observed, this may either be as a result of the GLp65 dimerising in the absence of mifepristone and activating the *E1B* promoter driving the expression of the pri-miRs or the activity of the *E1B* promoter in the absence of mifepristone-activated GLp65.

To investigate these possibilities, knockdown studies were conducted with the two separated constituent cassettes of the mifepristone inducible system. Significant HBV gene expression knockdown was observed with the pri-miR-31/5/8/9-expressing cassette separated from the transactivator. This observation confirms that expression of the pri-miR mimics from the mifepristone inducible promoter is leaky. This leakiness appears to be as a result of the *E1B* promoter switching on gene expression in the absence of mifepristone. Furthermore, upon addition of mifepristone there was no induction observed indicating the role of GLp65 for this function. The GLp65 transactivator alone did not result in knockdown of HBV gene expression indicating the role of the pri-miR-31/5/8/9-expressing cassette for this function.

Several studies have previously demonstrated the expression of RNAi activators from inducible systems. Most commonly, tetracycline-responsive derivatives of Pol III promoters have been used. The design strategy usually involves introducing tetracycline operator (*tet*O) sequences between the TATA box and transcription start site of the promoter (212). Several groups have developed inducible siRNA and shRNA expression systems based on the tetracycline repressor and the U6 Pol III promoter (212-214). Variants using the H1 Pol III promoter have also been designed (215, 216). Yu and McMahon subsequently demonstrated that a U6 promoter with stuffer sequences flanked by LoxP sites inserted at three different sites within the promoter was able to drive shRNA expression in a Cre-recombinase-dependent manner (217). U6 and H1 promoters have also been used in conjunction with the ecdysone (218) and bacterial *lac* (219) repressors respectively for the inducible expression of RNAi activators. A few groups have also used the tetracycline inducible system to generate conditional RNAi cell lines for miR-mediated

gene inactivation (220, 221). The use of the tetracycline system has also been investigated for the expression of pri-miR and miR-based shRNAs (66, 222).

To our knowledge, this is the first study to demonstrate pri-miR expression under the control of the mifepristone inducible system. Therefore our observations are not particularly surprising given the variable gene expression and induction that have already been demonstrated with this system. A study by Sirin and Park demonstrated impaired induction of hAAT expression compared to red and green fluorescent protein markers by the inducible system incorporated in a lentiviral vector. They suggested that there may be transgene-specific effects in the inducibility of this system when comparing the expression of secreted and intracellular proteins (186). In particular, improved inducibility of the system *in vitro* as well as *in vivo* has been observed with hIL-12, a secreted protein, compared to intracellular proteins such a Firefly luciferase with which there is also high basal expression [Hernandez-Alcoceba, R (CIMA, Spain), personal communication]. If this is the case, this may explain our observations with the pri-miRs and their expression in this novel context. Therefore the impaired expression and induction with the pri-miRs may, in fact, be attributed to the inherent nature of the inducible system and its constituent elements.

Similar to our approach in this study, Abruzzese and colleagues used separate plasmids containing the transactivator (GLp65) or transgene to further investigate the induction potential of the inducible system *in vitro* and *in vivo*. In particular, they sought to investigate the effects of the possible accumulation of GLp65 owing to expression under the control of the strong CMV promoter. They found that when they kept the amount of

transgene plasmid constant and increased the amounts of the CMV-driven transactivator plasmid, they observed increasing levels of basal expression. They proposed that GLp65 may be able to bind to GAL4 sites and partially activate transcription in the absence of the inducer, and that this ability is greater when higher levels of GLp65 are produced. However, they also pointed out that the effect on the magnitude of induction was not significant (188). Similarly, we have also investigated the expression and induction of pRS-31/5/8/9 in the non-liver HEK293 cell line derived from human embryonic kidney cells in an attempt to reduce the activity of the TTR liver-specific promoter and therefore the accumulation of GLp65. In this way we hoped to reduce leakiness from the inducible system. However, this did not significantly improve induction (data not shown).

In a second study, Abruzzese and colleagues investigated the activity of two inducible promoters; one consisted of six copies of the GAL4 DNA binding sites linked to a TATA box and the other consisted of four copies of the GAL4 DNA binding sites linked to a minimal thymine kinase gene (*tk*) promoter. They found that both promoters exhibited activity in the absence of GLp65 and mifepristone. They went on to propose that if it is assumed that the GAL4 sites are silent in the absence of the transactivator, then the core elements of the inducible promoter contribute a substantial level of expression. Therefore modifying the core elements of the inducible promoter may result in reduced basal expression (183, 188). Based on our observations and those of others it appears that both the *E1B* promoter which drives the expression of the transgene as well as the transactivator contribute to the leakiness observed. However, it is evident that the mechanism of gene expression and induction under the control of the mifepristone inducible system is complex and the exact function of both the inducible promoter and the transactivator under different circumstances needs to be further elucidated.

4.4 Potential improvements to the mifepristone inducible system

While optimisation studies have proven valuable in the use of the mifepristone inducible system, it is also worth considering modifications to the system itself and its constituent elements to improve the suitability of the system for a specific application. With regards to this study, substituting the pri-miR expressed from the inducible system may improve its performance. We have observed that the more potent pri-miR-31/5/8/9 expressed from the inducible system results in effective HBV gene expression knockdown but impaired induction. However, the ability to achieve knockdown with lower vector amounts is an advantage particularly in a therapeutic context. In contrast, pri-miR-31/5 exhibits less potent silencing but better inducibility. However, higher vector amounts are required to achieve significant HBV gene expression knockdown which may lead to toxicity (67). Therefore it may be useful to screen the performance of different pri-miRs incorporated into the inducible system. An ideal pri-miR could possibly exhibit moderate HBV gene expression silencing compared to pri-miR-31/5 and pri-miR-31/5/8/9 and adequate levels of induction, for example.

Promoter choice is another important consideration that may affect the performance of an inducible system. Wang *et al.* (174) as well as Burcin *et al.* (180) investigated promoter dependence on the performance of the first and second generation mifepristone inducible systems respectively. Both groups found that optimal mifepristone-dependent transgene regulation was achieved when the target gene was under the control of a TATA promoter compared to a *tk* promoter. Therefore, the TATA promoter can be used where low basal activity is preferred whereas the *tk* promoter can be used where overall higher expression is

required and higher basal activity can be tolerated (174). Expressing the transactivator under the control of a weaker promoter, such as the liver-specific transthyretin promoter, instead of the more active CMV promoter has also been proposed as a method to reduce basal expression (188).

Various modifications have also been carried out on the CMV promoter itself creating improved tet-responsive promoters with reduced background expression (223). The use of an autoinducible promoter which results in the activation of the transgene as well as the regulator itself when mifepristone is added has also been investigated with the mifepristone inducible system. This induces the synthesis of more of the regulator and results in lower basal activity and thus tighter regulation of gene expression (183). Other modifications which have been proposed to improve performance and reduce leakiness include the use of insulators to shield the regulated promoter from interference by neighbouring chromatin and the site-specific integration of conditionally controlled transgenes into certified loci (198). While some of the aforementioned modifications have already been incorporated into the current version of the mifepristone inducible system, this evidence demonstrates the vast potential for modifications to the system and the benefits they may yield.

Despite the shortcomings observed in this study, the mifepristone inducible system performs well in direct comparison to the other widely used inducible systems and has no prominent drawbacks (224). The limitations associated with the tetracycline inducible system include the toxicity of the tetracyclines themselves (158) and toxicity of the tTA transactivator protein (157). Whereas rapamycin, the inducer in the rapamycin inducible system, has immunosuppressive effects (157) and the insect-derived proteins expressed in

the ecdysone system may be immunogenic (159). The constituent components of the mifepristone inducible system and its inducer have also been found to be less toxic in comparison with other commonly used systems and inducers (158, 181). Future studies with this system are warranted and may lead to a greater understanding of the mechanism of gene expression and induction and thus improve its performance in a wide range of applications.

4.5 Future studies

In this study, we used HBV gene expression knockdown as a means of determining the expression of the pri-miRs under the control of the inducible system, however, this is not ideal. Several studies involving DNA microarray analysis have demonstrated that phenotype, in this case HBV knockdown by the pri-miRs, does not necessarily correlate with genotype, especially in human disease (225, 226). For example, Miklos and Maleszka found that genes whose expression changes are deemed to be of importance in microarrays are rarely those classified as of importance from clinical, *in situ*, molecular single nucleotide polymorphism (SNP) association, knockout and drug perturbation data. They thus highlighted the inconsistencies that can exist between microarray data and genome-wide phenotypic data (227).

With reference to this study, it is possible that induction of gene expression occurred at the level of transcription but did not necessarily translate to visible induction at the level of HBV gene expression knockdown. Therefore the accurate way of measuring pri-miR gene

expression would be to determine the abundance of mRNA transcripts under specified conditions. The first strategy commonly used, RT-PCR, was used by Abruzzese and colleagues to assess gene expression under the control of their derivative of the mifepristone inducible system which contains an autoinducible promoter. The authors used qRT-PCR to enable quantification of the mRNA copy numbers of the transctivator and target genes in the presence and absence of mifepristone (183). The design of a PCR assay for miRs proved challenging for several years, particularly because the miR precursor consists of a stable hairpin and because of the small size of the mature miR, roughly the size of a standard PCR primer (228). The development of techniques such as stem-loop RT-PCR has since enabled accurate and sensitive miR expression profiling (229). In addition, RT-PCR requires small amounts of starting material and this can prove to be advantageous. The limitations associated with this technique include high cost and the requirement for very sophisticated primer design and special expertise. Microarray analysis of miR accumulation has also been used and allows for high throughput but requires special tools and expertise (230).

Another widespread strategy used to assess the accumulation of target miRs is polyacrylamide gel electrophoresis of RNA samples combined with northern blot analysis. This technique allows for the quantification of the expression level of miRs as well as determination of the size of RNA (230). A potential limitation of northern blotting that we observed in this study is the high amounts of DNA that must be transfected to then be able to detect the expressed and processed pri-miRs. We found that the optimised amount of plasmid that is required to reduce basal expression from the inducible system is too low to be detected with northern blotting (data not shown).

Therefore in future studies we will use RT-PCR to quantify miR abundance and thus gene expression and induction under the control of the inducible system. We also intend to shed light on the contribution of GLp65 on gene expression and determine whether further optimisation of pri-miR expression and induction with the mifepristone inducible system is possible.

CHAPTER 5

5 CONCLUSION

In this study, the expression of anti-HBV primary micro-RNA (pri-miR) mimics under the control of a liver-specific and inducible RNA polymerase (Pol) II promoter system is demonstrated. This is the first study to incorporate RNAi activators into the mifepristone inducible system.

The mono- and polycistronic pri-miR sequences used in this study have previously been demonstrated to effectively silence HBV gene expression *in vitro* and *in vivo* when expressed from a CMV promoter. Nonetheless, the difficulty of achieving dose regulation and liver tissue targeting remains an obstacle preventing this therapy from advancing to the clinic. Typically, RNAi activators are expressed from Pol III promoters; however, they are constitutively active and as such may lead to toxicity as a result of the saturation of the endogenous RNAi machinery. Pol II promoters are more easily regulated and have therefore been favoured for the expression of therapeutic RNAi sequences. In addition, it is important to ensure that expression of RNAi sequences is confined to the tissues and organs of interest to avoid off-target effects brought about by non-specific binding.

In this study, liver-specific expression of the pri-miRs and the *Firefly luciferase* gene was demonstrated *in vitro* and *in vivo*. Firefly luciferase expression was shown to be inducible and mifepristone dose-dependent in agreement with previous studies. Both the pri-miR-

31/5 and pri-miR-31/5/8/9 sequences expressed from the mifepristone inducible system resulted in effective HBV gene expression knockdown *in vitro* and *in vivo*. In addition, induction *in vitro* was demonstrated to be dependent on the concentration of mifepristone. Although leaky expression of the pri-miRs was observed from the inducible system, reducing the vector amounts decreased basal expression and improved the inducibility of the system in cell culture studies. The mechanism of leaky expression was investigated and found to be as a result of the *E1B* inducible promoter driving pri-miR expression in the absence of mifepristone. Future studies are necessary to investigate the contribution of the GLp65 transactivator towards leaky expression.

The leaky expression of the pri-miRs from the inducible system and evidence from previous studies highlight the variation in expression with the inducible system and necessitates careful optimisation of vector amounts and mifepristone concentrations for effective transgene expression. However, this variation seems to be as a result of the intrinsic nature of the system and its constituent elements. A balance between basal expression and induction must therefore be achieved to optimise the performance of the system as demonstrated in this study. Nevertheless, the mechanism of leaky expression must be further elucidated to improve the performance of the system and expand its use in different applications.

This study demonstrates the exciting potential for the regulated and tissue-specific expression of therapeutic RNAi activators. In this way, our findings may contribute to the development of safe and clinically feasible RNAi-based antiviral treatment strategies.

APPENDIX

6 APPENDIX

6.1 Bacterial methods

6.1.1 Luria Bertani medium

Ten grams of bactotryptone, 5 g of yeast extract and 5 g of NaCl were dissolved in 1 litre of deionised water. The medium was autoclaved for 20 minutes at 121°C and 1 kg/cm^2.

6.1.2 Ampicillin stock solution (100 mg/ml)

One gram of ampicillin was added to 5 ml of deionised water and 5 ml of absolute ethanol added. The solution was filter sterilised and stored at -20°C.

6.1.3 Luria Bertani agar plates

Ten grams of bacteriological agar, 10 g of bactotryptone, 5 g of yeast extract and 5 g of NaCl were dissolved in 1 litre of deionised water. The solution was autoclaved for 20 minutes at 121°C and 1 kg/cm^2. Ampicillin was added to a final concentration of 100 µg/ml. The agar was poured into petri dishes and allowed to solidify at room temperature.

6.1.4 Transformation buffer

One hundred millimolar $CaCl_2$ and 10 mM PIPES-HCl were added to 15 ml of glycerol.

Deionised water was added to make up the volume to 80 ml. The pH was adjusted to 7.0 with NaOH and deionised water added to make up the final volume of 100 ml. The solution was autoclaved for 20 minutes at 121°C and 1 kg/cm^2 then stored at -20°C.

6.1.5 DNA isolation solutions

Resuspension buffer (Buffer P1)

Tris base (tris(hydroxymethyl)aminomethane) (6.06 g) was added to 3.72 g of $Na_2EDTA._2H2O$ and deionised water used to make the volume up to 800 ml. The pH was adjusted to 8.0 using HCl then the volume made up to 1 litre with deionised water. The solution was autoclaved for 20 minutes at 121°C and 1 kg/cm^2 then allowed to cool and RNAse A (100 mg) added. The buffer was mixed thoroughly and stored at 4°C.

Lysis buffer (Buffer P2)

Eight grams of NaOH pellets were added to 500 ml of deionised water. Ten grams of SDS was added and the volume made up to 1 litre with deionised water. The buffer was stored at room temperature.

Neutralisation buffer (Buffer P3)

Potassium acetate (294.5 g) was added to 500 ml of deionised water. The pH was adjusted to 5.5 using acetic acid then the volume made up to 1 litre with deionised water. The buffer was stored at 4°C.

6.1.6 0.5M EDTA

To make 1 litre, 146.12 g of EDTA was added to 800 ml of deionised water. The pH was adjusted to 7.5 with NaOH pellets then to 8 with 10M NaOH. The volume made up to 1 litre with deionised water.

6.1.7 50 × TAE (Tris-acetate-EDTA) buffer

Tris base (242g) was added to 57.1 ml of glacial acetic acid then 100 ml of 0.5M EDTA (pH 8) was added. The volume was made up to 1 litre with deionised water. One litre of 1× TAE buffer was made by adding 20 ml of 50 × TAE buffer to 980 ml of deionised water.

6.1.8 X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside)

Twenty milligrams of X-gal was dissolved in 1 ml of dimethyl formamide. The tube was covered in foil and the solution stored at -20°C.

6.1.9 IPTG (isopropyl-beta-D-thiogalactopyranoside)

One hundred milligrams was dissolved in 1 ml of deionised water. The solution was filter sterilised and stored at -20°C.

6.2 Cell culture studies

6.2.1 DMEM cell culture medium (DMEM)

DMEM was made according to the manufacturer's instructions (Life Technologies, CA, USA).

6.2.2 0.5× Trypsin

Trypsin (1×) (Life Technologies, CA, USA) was diluted 2 fold with saline containing 0.01% EDTA. The mixture was filter sterilised and stored at 4°C.

6.2.3 Mifepristone for cell culture studies

A 20 mM stock solution was prepared by dissolving 80 mg of mifepristone in 9.3 ml of 100% ethanol. The stock solution was stored at -20°C. Working solutions were prepared by dissolving the stock solution in ethanol.

6.3 Mouse studies

6.3.1 Mifepristone for *in vivo* studies

A stock solution of mifepristone was prepared by dissolving 4.5 mg of mifepristone in 18 ml of sesame oil (Sigma-Aldrich, MO, USA). The solution was left shaking overnight, in the dark at 4°C. Working solutions of mifepristone were prepared by diluting the stock solution in sesame oil.

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