Liver Specific MicroRNA Control of Adenovirus Serotype Five

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Abstract

MicroRNAs are small non-coding RNA molecules that regulate mRNA translation by binding to complementary sequences usually within the 3' un-translated region (UTR). By inserting four perfectly complementary binding sites for the hepatic specific microRNA mir122 into the 3' UTR of adenovirus wild type 5 (Ad5 WT) E1A mRNA I show that the acute liver toxicity caused by Ad5 WT in mice can be significantly reduced. This virus, termed Ad5-mir122, is a promising virotherapy candidate and causes no obvious liver pathology whilst maintaining Ad5 WT replication in mir122 negative cells.

Data shows that repeat intravenous administration of Ad5-mir122 ($2x10^{10}$vp) to HepG2 tumour bearing mice mediated significant anti-cancer efficacy. RT-QPCR for E1A mRNA demonstrated a 29-fold reduction when compared to Ad5 WT in murine liver whilst western blot confirmed that all E1A protein variants were knocked down. Viral genomic replication was also reduced in mouse liver by 25-fold compared to Ad5 WT. This control of virus activity reduced alanine and aspartate transaminase release by >15-fold and histological analysis showed little to no pathology in Ad5-mir122 infected livers.

Measurement of mature mir122 levels in Ad5-mir122 infected livers by RT-QPCR showed that the quantity of mir122 remained unaffected at therapeutic doses. Complete genome mRNA array profiling of infected livers showed that the transcript levels of >3900 different mRNAs were changed more than 2-fold following Ad5 WT infection whilst less than 600 were changed by Ad5-mir122. A non-replicating control adenovirus vector altered >550 mRNAs. No known mir122 target mRNAs were affected following infection with Ad5-mir122. Western blot analysis of a known mir122 regulated target (Aldolase A) confirmed these results, demonstrating no change in protein level despite infection with Ad5-mir122. These data combined demonstrate that the exploitation of microRNA mir122 regulation to control adenovirus replication is a safe method of control and does not alter the endogenous level or activity of the microRNA or its endogenous mRNA targets. Ad5-mir122 is a potent anti-cancer agent that replicates to wild-type levels in microRNA mir122 negative cells but is specifically and safely attenuated in hepatocytes.

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List of Abbreviations

/	Per
3’ and 5’	3 prime and 5 prime ends of a DNA/RNA molecule
µ	Micro
ρ	Density
Ad5	Adenovirus serotype five
AdLuc	Ad5 expressing luciferase under CMV control (non-replicating)
Ad5-mir122	Ad5 containing four mir122 binding sites in the E1A 3’UTR
Ad5-mir122-Luc	Ad5 containing four mir122 binding sites with E1A fused to luciferase
Ad5 WT	Ad5 wild type strain (Accession AC000008)
Ad5-WT-Luc	Ad5 wild type strain with E1A fused to luciferase
cDNA	Complementary DNA
CMV	Human cytomegalovirus
dH_2O	de-ionised water
DNA	Deoxyribonucleic acid
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,Ntrimethylammonium methylsulfate
EDTA	Ethylenediamine tetra-acetic acid
g	Gravitational force
GFP	Green fluorescent protein from *Aequorea victoria*
h	Hour(s)
HCl	Hydrogen Chloride
iv	Intravenous
Kb	Kilobases
L	Litre
m	Milli
M	Mole/Molar
min	Minute(s)

mRNA	Messenger RNA
n	Nano
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Second(s)
TCID50	Tissue Culture Infectious Dose 50
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
vp	Virus particles
v	Volume
w	Weight
WT	Wild-type
w/v	Weight over volume
w/w	Weight over weight
x	Multiplied by

Chapter 1: Introduction

General Introduction:

Oncolytic virotherapy

In 1910 the Italian physician N.G. De Pace presented findings that one of his female patients suffering from cervical carcinoma had undergone a complete spontaneous remission after receiving an inoculation of the live attenuated Pasteur-Roux Rabies virus vaccine (DePace 1912). This was the first documented case of a tumour remission that was attributed to the lytic effects of a virus and began the field of oncolytic virotherapy.

This early observation was followed by many attempts to reproduce the same result using different virus species in the treatment of many different forms of cancer. Unfortunately, most clinical results were disappointing with only variable, transient and limited remissions observed (Southam *et al.* 1952). The preparations used in these early studies were in many cases crude and poorly quantified or defined. From the 1950s to the 1980s many viruses were tested as candidate anti-neoplastic agents in humans including live attenuated strains of measles virus, mumps virus, Newcastle disease virus, West Nile virus, and adenovirus (Liu *et al.* 2007). In the 1990s, the advent of genetic engineering and an increased understanding of cancer genetics and cancer biology rapidly led to the development of more refined genetically modified virus strains and the establishment of the field of oncolytic virotherapy as a major area of biomedical research.

The principle of oncolytic virotherapy is to deliver a therapeutic virus into a tumour either following systemic or local administration where the virus is able to replicate and lyse the cancer cells until the tumour is cleared. This is the only form of anti-cancer therapy that is designed to amplify at the therapeutic site.

Viruses as cancer therapeutics and clinical progress

In recent decades the development of novel therapies that are targeted to exploit specific genetic alterations within tumours has expanded significantly. Included within these therapies are many oncolytic viruses which have demonstrated efficacy in pre-clinical models and have progressed into human clinical trials (Liu *et al.* 2007). Viruses tested in humans within the last 15 years include genetically modified adenoviruses, herpesviruses, poxviruses and retroviruses as well as vaccine strains of reoviruses, paramyxoviruses and flaviviruses (Table 1). Wild type strains of picornaviruses have also been assessed (Table 1).

Until 1997 all viruses tested in human clinical trials in the treatment of cancer were either wild-type strains, tissue culture adapted strains or vaccine strains. The genetic cause of attenuation in many of these viruses was poorly defined. This changed with the development of an oncolytic serotype five/two hybrid adenovirus, called dl1520 (ONYX-015, Onyx Pharmaceuticals, Emeryville, CA), which contained deletions within the genes encoding both the E1B-55K protein and the E3B protein (Bischoff *et al.* 1996). The E1B-55K protein interacts with cellular p53 protein and inactivates it (Blackford *et al.* 2009). Therefore, the deletion of this gene was designed to allow the virus to replicate only within cells in which a p53 mutation was already present. More recent research has since shown that deletion of E1B-55K influences other components of viral replication and the method of attenuation is not p53 specific (Rothmann *et al.* 1998). In total the results of 15 clinical trials with dl1520 have been published from multiple clinical testing centres (Kirn 2001). Trials have tested dl1520 in a variety of neoplasias including glioblastoma, pancreatic cancer, head and neck cancer, ovarian carcinoma and colorectal metastasis to

the liver (Liu *et al.* 2007). Dl1520 has also been tested in conjunction with the chemotherapeutic agents cisplatin and 5-fluorouracil in the treatment of head and neck cancer (Khuri *et al.* 2000). Toxicities from trials of Dl1520 included flu-like symptoms following systemic virus delivery and pain at the injection site following intra-tumoural injections (Liu *et al.* 2007). No additional toxicities were observed following injection of virus and chemotherapy in combination (Khuri *et al.* 2000). Transient efficacy was demonstrated in the treatment of head and neck cancer only, with an objective response rate of 14% (Kirn 2001).

More recent clinical trials of adenoviruses include the use of an E1B-55K deleted, E3 intact serotype five adenovirus (H101, Sunway, Shanghai, China) (Yuan *et al.* 2003) and the transcriptionally targeted prostate specific serotype five adenoviruses CG7870 and CG7060 (Cell Genesys, San Francisco, CA)(DeWeese *et al.* 2001; Small *et al.* 2006). Objective anti-cancer responses were not observed with any of these agents and toxicities were similar to those with Dl1520 (Yuan *et al.* 2003).

Genetically modified viruses from three other viral families have also entered clinical trials and published data shows some objective responses. The vaccinia virus JX549 is an oncolytic virus based on the Wyeth vaccine strain of vaccinia which has been genetically modified to make it both cell cycle selective by deleting the gene encoding thymidine kinase and immune stimulatory by expressing the human gene encoding granulocyte macrophage colony-stimulating factor (GM-CSF) (Kim *et al.* 2009). Trials have been conducted with JX594 against hepatoma, colorectal, lung and melanoma cancers in multiple phase one and two studies (Kim *et al.* 2009). Some patients demonstrated objective transient responses following JX594 local injection.

Human Herpes Simplex virus 1 (HSV-1) has also been tested in clinical trials. In total, four viruses have been studied in which the neuro-virulence gene gamma 34.5 (ICP34.5) has been deleted. These viruses include G207 (Markert *et al.* 2000), 1716 (Harrow *et al.* 2004; Harrow *et al.* 2005), NV1020 (Kemeny *et al.* 2006) and OncoVEX^{GM-CSF} (Biovex Ltd, Abingdon, UK) (Senzer *et al.* 2009). All four strains have been tested in human clinical trials but only OncoVEX^{GM-CSF} has proceeded past phase one testing. OncoVEX^{GM-CSF} is a wild type isolate of HSV-1 in which the ICP34.5 gene has been replaced with the human gene encoding GM-CSF (Liu *et al.* 2003). This virus has progressed through several phase two clinical trials and is currently undergoing phase three multi-centre analysis for both squamous cell head and neck cancer and metastatic melanoma.

More recently a replication competent retrovirus (murine leukemia virus, MLV) encoding the yeast suicide enzyme cytosine deaminase has entered clinical trial for the treatment of glioblastoma. Results have yet to be published.

The genetic engineering of viruses has allowed the development of many new oncolytic strains that are designed to reduce toxicity and enhance therapy. The clinical trial results of all oncolytic viruses have consistently shown that whilst virotherapy is a safe, well tolerated treatment the efficacy mediated by these viruses is usually transient and of limited therapeutic benefit. This may reflect the fact that many of the programmed genetic modifications that have been made attenuate the ability of the virus to replicate in cancer cells, despite showing enhanced cancer cell selectivity (Bazan-Peregrino *et al.* 2008).

Table 1 (below): Oncolytic viruses tested in human clinical trials. Many viruses have been tested in human clinical study and details where known are presented in the table below. Rabies virus is excluded because details of early clinical studies are not known. The highest dose in each trial is shown and the strain of each virus, or the genetic modifications made, are also highlighted where possible. Delivery methods are as follows OR-Oral, IV-intravenous, IT-intratumoural, IH-inhalation, ID-intradermal, Ipros-Intraprostatic, RT-rectal, IA-intrarterial, IM-intramuscular, IP-intraperitoneal. Virus quantity units are as follows VP-viral particles, PFU-plaque forming units, IU-infectious units, ND-not determined.

Virus	Strain	Genetic Changes	Complementation	Armed	Delivery Method	Highest Dose	Ref
Newcastle Disease virus	PV101	Unknown	Vaccine Strain	N/A	IV	$1.2x10^{11}/m^2$ PFU	20
Newcastle Disease virus	OV001 (HUJ)	Unknown	Vaccine Strain	N/A	IV	$1x10^{10}$ IU	21
Newcastle Disease virus	MTH/68-H	Unknown	Vaccine Strain	N/A	IV/IH	IH 4000 PFU/ twice weekly. IV $2.5x10^8$	22
Reovirus	Serotype 3 (Dearing)	Unknown	PKR/Interferon Defects	N/A	IT	$1x10^{10}$ PFU	23
Mumps virus	Urabe	Unknown	Vaccine Strain	N/A	IT/IV	$1x10^{10}$ PFU	24
Mumps virus	Clinical Isolate	None		N/A	IT/IV/OR/RT/IH	$3x10^7$ PFU	25
West Nile virus	Egypt 101	Early Isolate (3rd passage)	Unknown	N/A	IM/IP	ND	2
West Nile virus	Wild type	None	Unknown	N/A	IM/IV/IT	ND	26
Ilheus Virus	Wild type	None	Unknown	N/A	IM/IV/IP	ND	26
Bunyamwera virus	Wild type	None	Unknown	N/A	IV/IM	ND	26
Parvovirus	H-1	None	Unknown	N/A	Unknown	ND	27
Vaccinia Virus	Lister	Unknown	Vaccine Strain	N/A	IT/IV	$2x10^8$ PFU	28
Vaccinia Virus	Wyeth	Unknown	Vaccine Strain	N/A	IT/ID	$1x10^8$ PFU	29, 30
Vaccinia Virus	BP	Unknown	Vaccine Strain	N/A	IT	$1x10^7$ PFU	31
Vaccinia Virus	AS	Unknown	Vaccine Strain	N/A	IV	$2x10^{10}$ PFU/total treatment	32
Vaccinia Virus	JX594 (Wyeth)	Thymidine Kinase deleted	E2F pathway	GM-CSF	IT/IV	$3x10^9$ PFU	33
Vaccinia Virus	DryVax	Unknown	Vaccine Strain	N/A	IT	$1x10^8$ PFU	34
Adenovirus	Wild Type (10 Serotypes)	None		N/A	IT/IV/IA	$1x10^{10}$ PFU	35
Adenovirus	CV7060	Prostate specific Promoter E1A/ΔE3B	Prostate-specific	N/A	Ipros	$1x10^{13}$ vp	10
Adenovirus	CG7870	Prostate specific Promoter E1A & E1B	Prostate-specific	N/A	IV	$6x10^{12}$ vp	11
Adenovirus	dl1520 (Onyx-015)	ΔE1B-55K/E3B	p53 defects	N/A	IT/IV/IP/HAI	$2x10^{12}$ vp	7
Adenovirus	H101	ΔE1B-55K	p53 defects	N/A	IT	$1.5x10^{12}$ vp	9
Adenovirus	Ad5-CD-TK	ΔE1B-55K/E3 ADP/E3B	p53 defects	Thymidine Kinase/ Cytosine Deaminase	Ipros	$1x10^{12}$ vp	36, 37
HSV-1	1716	ΔICP34.5	PKR/Interferon Defects	N/A	IT	$1x10^5$ PFU	14, 15, 38
HSV-1	G207	ΔICP34.5/ICP6	PKR/Interferon Defects	N/A	IT	$3x10^9$ PFU	13
HSV-1	NV1020 (R7020)	ΔICP34.5	Vaccine Strain	N/A	HAI	$1x10^8$ PFU	16
HSV-1	$OncoVEX^{GM-CSF}$	ΔICP34.5/UL24/UL56		GM-CSF	IT	$4x10^8$ PFU	17
Measles	Edmondson	CEA Expression	Vaccine Strain	CEA	IP	$1x10^9$ TCID50	39
Murine Leukemia Virus	Wild type strain	Addition of Cytosine deaminase under IRES control	Replicating Cells	Cytosine/ Deaminase	IT	Unknown	
Seneca Valley virus	Wild type strain	None	Unknown	N/A	IV	$1x10^{11}$ / kg	40

Therapeutic targeting modalities

Many wild type and vaccine strains of viruses have been selected as oncolytic agents because of their intrinsic or tissue culture acquired ability to undergo restricted replication in cancerous cells (Southam *et al.* 1952; Smith *et al.* 2000). This selectivity is desirable in order to reduce toxicity and allow replication of the therapeutic virus specifically at the tumour site. Whilst many viruses naturally show some level of cancer selectivity, many have been genetically engineered to replicate only in cancer cells by exploiting specific tumour-associated changes. There are several published examples of virus modifications that allow such selectivity. They normally include one or more of the following three forms of alteration:

1. *Transductional targeting:* The alteration of the virus surface to allow specific infection of neoplastic cells.
2. *Transcriptional targeting:* The alteration of DNA elements (e.g. promoters) so that key viral genes are only active in cancer cells.
3. *Translational targeting:* The alteration of viral mRNAs and proteins to allow them to be active only in cells with transforming or complementing mutations.

These methods of regulation have produced many new viral vectors which offer reduced toxicity and enhanced cancer selectivity. However, many of these changes also attenuate these viruses when compared to the wild-type strain (Bazan-Peregrino *et al.* 2008). For cancer virotherapy it would therefore be highly attractive to produce viruses that show wild type replication activity at the therapeutic site but are specifically attenuated at sites of potential pathology.

Transductional targeting

The outer surfaces of viruses have evolved to allow specific, often high affinity, interactions with cell surface components. In some cases these cell surface components are fortuitously up-regulated on cancer cells. Examples include CD46 (Measles virus receptor) (Russell *et al.* 2009) and the high-affinity laminin receptor (Sindbis virus receptor) (Venticinque *et al.* 2010). In some cases such viruses are found to preferentially infect and replicate in receptor positive cancer cells naturally. However, not all cancer types express these receptors and most viruses do not exploit cell surface receptors which are up-regulated on cancers. For this reason viruses have been genetically or chemically engineered to allow cancer-selective infection.

Adenoviruses have been extensively modified by the chemical addition of polymers which bind to the virus surface to allow re-targeting to cancer cell populations. Adenovirus preparations are readily amenable to such changes because they are non-enveloped, do not easily aggregate and remain active even after significant surface modification. Coating of adenovirus has been attempted with polymers based on poly-ethylene glycol (PEG) (Doronin *et al.* 2009) and N-(2-hydroxypropyl) methacrylamide (HPMA) (Fisher *et al.* 2009). HPMA based coating strategies allow re-targeting of viral infection through a range of different cell surface receptors and also afford some level of protection against antibody neutralization. The chemical alteration of the surface of viruses, whilst promising for initial delivery, is not heritable and therefore any new virus particles produced from replication *in vivo* will not demonstrate the same re-targeting properties. This may allow the hurdle of initial delivery to the tumour site to be overcome

by increasing virus blood stream kinetics and as such it represents a novel method of enhancing therapeutic virus delivery.

The genetic modification of the adenovirus surface proteins has also been attempted (Boulanger *et al.* 2001; Magnusson *et al.* 2007). This involves the alteration of the virus capsid genes to produce proteins that allow binding to the cancer cell surface via a cancer selective receptor. These changes are difficult to achieve without destabilizing the virus structure and it is also difficult to predict the necessary changes needed to achieve a level of avidity which allows successful virus infection. This approach has also been employed to attempt to make virus strains which are not recognized by pre-existing antibodies (Seregin *et al.* 2009).

Enveloped viruses have also been surface modified genetically to endow them with new infectious tropisms. The addition of targeting moieties (e.g. Epidermal growth factor) into the surface glycoproteins of Herpes Simplex virus (HSV-1) (Lollini *et al.* 2009), Vesicular Stomatitis virus (VSV) (Bergman *et al.* 2004) and Measles virus (MV) (Paraskevakou *et al.* 2007) have all shown that it is possible to alter the tropism of enveloped viruses to allow cancer specific infection. This is a promising technique for development of cancer selective viruses but is limited by antibodies against the remaining portions of the virus glycoprotein and poor blood stream kinetics following intravenous injection.

Transcriptional targeting

The principle of transcriptional targeting is to exploit the activation or over-expression of transcription factors within cancer cells which are either present at low levels, absent or inactive in most tissues or non-replicating cells. This technique is therefore limited to viruses which exploit the nuclear protein complement in order to replicate, primarily DNA nuclear replicating viruses. These include members of the adenoviridae family and the herpesviridae family.

This area of research has been primarily investigated using adenovirus serotype five where the promoter region upstream of the E1A gene has been replaced by a promoter which is activated by cancer or tissue associated transcription factors. These have included the insertion of a prostate specific promoter for the Probasin gene or the addition of prostate specific enhancer elements into E1A promoter region in order to allow prostate specific E1A transcription (DeWeese *et al.* 2001; Small *et al.* 2006). These modifications are prostate-selective rather than cancer-selective but no other human tissues should support viral replication which limits virus spread and pathology. Other adenoviruses have been produced that contain hypoxia responsive elements (HREs) or estrogen responsive elements in the E1A promoter (Bazan-Peregrino *et al.* 2008). These types of viruses show low-oxygen or tissue dependent replication, respectively.

The use of DNA elements and promoters to drive transcription of a viral gene may allow selectivity in desired cells but the inadequate strength of these promoters is a major limiting factor. Virus replication is often decreased compared to wild type virus strains even in cells in which the DNA elements are highly active. The promoters that have been inserted may not be sufficiently active when compared to the original strong viral

promoter that has evolved to rapidly transcribe significant quantities of mRNA to initiate viral replication. Alternatively, the decrease in virus fitness may reflect subtleties in the activity of the deleted viral promoters that allow the level of transcription to be linked directly with virus replication.

Translational targeting

The complementation of changes in both tumour and virus proteins has long been recognised as a useful method of control for virus replication. Many viruses that have natural oncolytic capabilities exploit the alteration of proteins in cancer cells which are functional in normal cells (Smith *et al.* 2000). These proteins include interferon-α, p53, pRb, PKR and Ras (Smith *et al.* 2000). Whilst some viruses demonstrate natural selectivity due to tumour-specific genetic alterations, many viruses have been genetically modified to either introduce or enhance this phenotype.

VSV is a negative stranded RNA virus which is capable of shutting down host cell protein synthesis and preventing interferon type one production (Hoffmann *et al.* 2010). Interferon is known to hinder VSV replication significantly and it is therefore of critical importance for the virus to ensure that little to no interferon is stimulated during infection. To ensure this, VSV encodes a Matrix protein which efficiently shuts down host-protein synthesis and prevents the production of interferon. A VSV variant has been developed which encodes a deletion of a methionine amino acid at the 51st residue in the matrix protein (VSV ΔM51) which enables the virus to replicate only in cells which cannot produce interferon (Stojdl *et al.* 2003). It has been demonstrated that cancer cell lines frequently acquire mutations in the interferon pathway which allows ΔM51 modified

VSV to replicate efficiently. In cells in which the interferon pathway is intact VSV ΔM51 replication is severely impaired.

Similarly, changes to adenoviruses have been introduced that ensure that the virus proteins can only function in the presence of transforming cancer associated mutations. These include the E1B-55K deleted adenoviruses already described but also other viruses containing more specific and defined mutations. Dl922-947 is a serotype five adenovirus in which the CR2 domain of E1A has been deleted (Heise *et al.* 2000). This region of E1A is known to bind to the tumour suppressor protein pRB and this interaction causes cell cycle progression and induces early viral gene transcription (Howe *et al.* 1992). This deletion therefore allows adenovirus to replicate only in cells in which the pRb pathway is dysregulated which includes cells from many forms of cancer (Hickman *et al.* 2002).

These approaches offer promise for oncolytic virotherapy but are often limited by non-specific attenuation or residual levels of inhibitory proteins still present within the cancer cells. For example, E1B-55K deleted adenovirus was designed to replicate only in cells in which the p53 pathway was dysregulated, however, more recent research has shown that the mechanisms of selectivity of this virus is not dependent on p53 but on another aspect of E1B-55K function which involves the efficient export of late viral mRNA from the nucleus into the cytoplasm (Rothmann *et al.* 1998). Similarly, VSV Δ51 shows good replication in cells devoid of interferon but many cancer cell lines and primary tumours still respond to, and produce, substantial quantities of interferon which impairs viral replication and efficacy (Nguyen *et al.* 2008). Given the myriad of mutations in both the interferon response and synthesis pathways that can occur, it is difficult to see how patients could be pre-screened for such mutations. Moreover, these mutations are not

known to be required for cancer development or progression but are instead a side-effect of genetic instability and pathway de-regulation.

The development of oncolytic viruses which are capable of wild-type levels of replication in cancer cells through complementation targeted mechanisms is difficult because the mechanisms on which these changes are based are often not shared by all cancers or even all patients suffering from a particular cancer type. This technique also relies on the sufficient presence or complete absence of particular proteins in heterogenic tumours and it is unlikely that all cancer cells will provide the complementing environment necessary for wild-type levels of virus replication. The design of viruses which are fully active in cancer cells but attenuated in sites where replication is unwanted is therefore highly desirable. It was the objective of this thesis to generate such a virus that would show wild type replication and lysis in all cancer cells with efficient, robust and safe attenuation in non-target tissues.

Limitations to successful virotherapy

Viruses are exquisitely evolved to replicate within the micro-environmental niche in which they normally infect the host. As such, any differences between cancer cells and the tissue cells the virus normally infects may present limiting factors for successful virotherapy.

The search for a virus which demonstrates all the properties required of a virus for successful sustained oncolysis in humans has led to the pre-clinical investigation of viruses from almost all viral families. Given the specificity of most viruses for the tissue

and location in which they normally replicate it is unlikely that any virus isolated from the natural world will be endowed with all the properties required for virotherapy. It is consequently important to identify the key limitations that prevent successful viral therapy so that attempts can be made to modify viruses to circumvent those challenges and enhance therapeutic benefit. Some of the major limitations to successful treatment with oncolytic viruses are described below.

1. *Toxicity*

   Most current cancer treatments induce significant unwanted side-effects in non-target tissues. However, because these treatments do not amplify following administration *in vivo*, their toxicities are scalable and usually decrease if treatment is immediately stopped. Conversely, the replication of viruses in non-cancerous tissues can lead to toxicity and virus induced pathology (Southam *et al.* 1952). Such side-effects are difficult to treat unless specific anti-viral treatments exist. Many viruses have been modified extensively to prevent this from occurring, often at the expense of oncolytic potency.

2. *Delivery*

   The human immune system has developed multiple systems to enable the efficient clearance of viral particles from both the blood stream and tissues and this is a major limitation to the successful delivery and spread of therapeutic viruses systemically. Many groups have circumvented this issue by injecting viruses directly into tumour deposits. However, delivery to distal and deep-seated metastases is still a problem using this technique, and delivery remains a major limitation.

3. *Spread*

Viruses have evolved many strategies to allow their spread from cell to cell and host to host however most infections do not result in the entire infection of an organ and are usually self-limiting. The design of viruses which can spread throughout a disorganised tumour is therefore difficult and many attempts have been made to improve this by encoding matrix degrading proteins or other extracellular mediators in an attempt to allow enhanced intra-tumoral spread of virus particles.

4. *Potency*

A successful virotherapy candidate virus must be capable of killing the cells it infects in order to be of therapeutic benefit. As discussed above, many viruses which show cancer selectivity often do so at the cost of oncolytic potency. Wild type virus strains often show the fastest replication kinetics and represent the most potent viral strains.

5. *Immunity*

The human immune system has co-evolved with viruses and contains multiple mechanisms of removing them from within the circulation and tissues. Antibodies and complement can neutralise and clear virus particles efficiently from within the host and CD8+ T cells are capable killing infected cells before the replication cycle is completed. Each has been shown to increase significantly in patients after virus administration (White *et al.* 2008). These mechanisms prevent both the infection of cells and the production of new progeny virus for further viral spread.

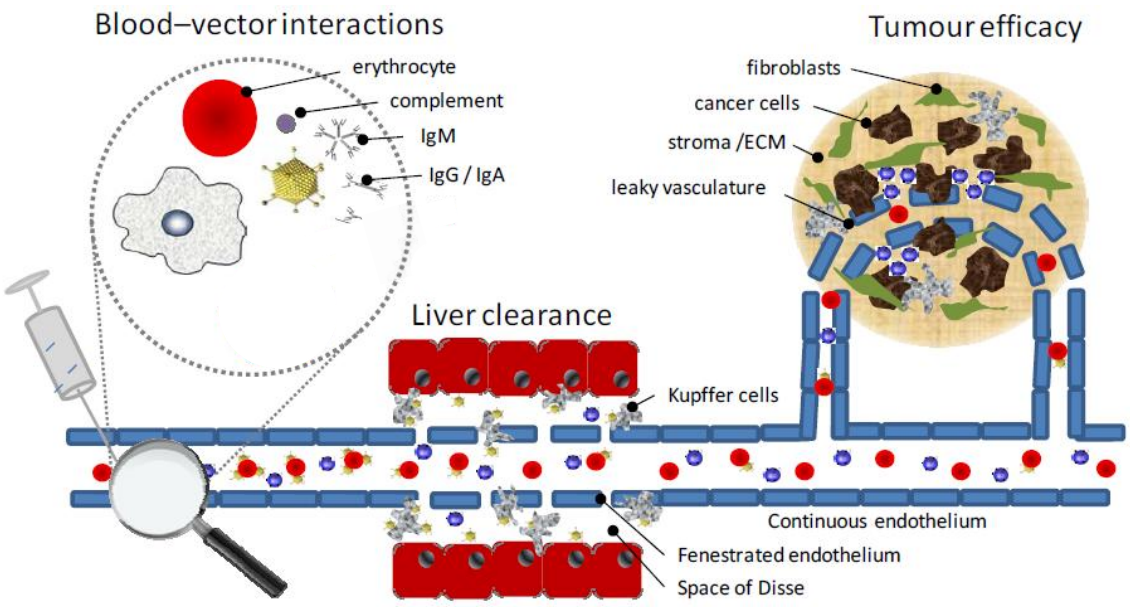


Figure 1: Challenges to systemic delivery of viruses. The systemic delivery of viruses allows access to both the primary tumour and distal metastasis but is limited by multiple clearance mechanisms and physical barriers. The blood stream represents a hostile environment for virus particles which can be opsonised rapidly by antibody and complement. Sequestration into the major filtering organs (primarily the liver but also the spleen) represents a second major hurdle. If virus particles are able to successfully extravasate into the tumour stroma they must still infect, replicate in, and lyse cells before spreading to adjacent cancer cells. The disorganised vasculature and dense stroma of the tumour microenvironment all represent significant challenges to successful therapy. This image was kindly provided by Dr Kerry Fisher.

Adenovirus

Adenovirus biology

Adenovirus is the most commonly investigated and exploited virus in modern scientific research. It has found utility in the fields of vaccination, gene therapy, virotherapy, and also as a complex tool for understanding biological systems (McConnell *et al.* 2004).

The adenoviridae family of viruses are double stranded DNA viruses which were first isolated from the adenoids of infected children in 1953 (Rowe *et al.* 1953). The family consists of many species infecting a broad variety of hosts including humans, birds and amphibians and is divided into four genuses to reflect the wide variety of hosts they infect. The mastadenoviridae consists of 25 unique adenovirus species infecting mammalian hosts. Within this genus there are currently six distinct species of adenovirus that infect humans. They are denoted by a letter as human adenoviruses A-F. Within these species multiple serotypes have been identified that are numbered from 1-51 and are all recognised as independent strains by the International Committee on the Taxonomy of Viruses (Table 2).

Since their discovery, adenoviruses have served as important molecular tools for understanding cell biology and viral replication. Significant findings that have stemmed from adenovirus research include the discovery of mRNA splicing (Chow *et al.* 1977) and the observation that adenovirus serotype 12 can induce tumours in animal models (Trentin *et al.* 1962). This was the first human virus demonstrated to be capable of causing neoplasia although no link to human cancer has been discovered. Today adenoviruses are studied and modified extensively for many purposes and are an important and enabling tool in scientific research.

Species	Serotypes	Known Receptors and Co-receptors
Human Adenovirus A	12,18 & 31	Coxsackie and Adenovirus Receptor (CAR)(12,31)
Human Adenovirus B	3,7,11,14,16,21,34,35 & 50	CD46 (3, 11, 16, 21, 14,35,50), CD80 & CD86 (3)
Human Adenovirus C	1,2,5 & 6	Coxsackie and Adenovirus Receptor (CAR) & Heparin Sulphate, αVβ3 and αVβ5 (2,5), αMβ2 and αLβL2 (2)
Human Adenovirus D	8,9,10,13,15,17,19,20,22,23,24,25,26,27,28,29,30,32,33,36,,37,38,39,42,43,44,45,46,47,48,49,51	Coxsackie and Adenovirus Receptor (CAR)(9,15,19), Sialic Acid (8,19,37), CD46 (37)
Human Adenovirus E	4	Coxsackie and Adenovirus Receptor (CAR)
Human Adenovirus F	40,41	Coxsackie and Adenovirus Receptor (CAR)(41)

Table 2: A List of human adenovirus species and serotypes and their known human cell surface receptors. Adenovirus nomenclature uses letters for the species name and numbers for the serotype. Cell surface receptors are those known for each serotype that is placed in brackets. Many more non-human adenoviruses are known and are now being exploited for scientific research. This table was compiled from a review of adenovirus receptors by Zhang *et al* (Zhang *et al*. 2005).

Adenovirus pathogenesis in humans

Adenoviral infections in immune-competent individuals are usually febrile, self limiting conditions (Rowe *et al.* 1953). They are responsible for approximately 5-10% of respiratory infections in children and are a major cause of respiratory infections in personnel in US military installations (De Witt *et al.* 2000). Although considered primarily a respiratory disease, adenoviruses can also cause epidemic conjunctivitis and infantile gastroenteritis (Uhnoo *et al.* 1984). In immune-compromised patients, including those suffering from acquired immune-deficiency syndrome (AIDS), adenovirus infections can be much more severe and can induce fulminant hepatitis (primarily within transplant patients), pneumonia and encephalitis which can be fatal (Kojaoghlanian *et al.* 2003; McLaughlin *et al.* 2003). This finding strongly suggests that adenovirus is capable of infecting hepatocytes in the human liver which relates significantly to the subject of this thesis.

The self-limiting nature and wide antigenic variance of the adenovirus family has led to the development of very few adenovirus vaccines. Historically US military personnel were administered enteric capsules containing adenovirus serotypes four and seven particles as vaccines. Production of the original vaccine was stopped in 1999 (Hilleman 1958; De Witt *et al.* 2000) although a new product licence has recently been granted to Barr Pharmaceuticals. Immunity to some adenovirus serotypes is widespread in most adult human populations and is acquired early in life with approximately 70%-100% of individuals testing sero-positive for adenovirus serotype five (Ersching *et al.* 2010; Pilankatta *et al.* 2010).

Adenovirus pathogenesis in mice

Human adenoviruses species B and C do not replicate to produce new virus progeny in murine tissue but can undergo abortive replication in some tissues. Following intravenous delivery to mice, adenovirus serotype five can infect the liver and the virus replication cycle is initiated (Duncan *et al.* 1978). This process can cause substantial and often lethal toxicity and this is described in detail in the introduction to chapter 6. Briefly, the adenovirus replication cycle is induced in murine liver by a high level of expression of the E1A protein in hepatocytes. This expression allows the downstream activation of multiple early viral transcription cassettes including E2, E3 and E4 (Figure 2). The trans-activation of early virus gene expression by E1A protein eventually leads to structural gene expression and also the expression of the viral DNA polymerase. The polymerase is able to replicate the adenovirus genome substantially, however, the replication cycle is ultimately abortive and no new virus is produced. This process is toxic to murine hepatocytes and leads to significant alanine and aspartate transaminase release from the liver, which can serve as an accurate measure of this toxicity. Gross morphological changes are also obvious upon visual inspection. Nuclear enlargement, cell lysis and vacuolation are all common histological findings. The maximum tolerated dose of adenovirus serotype five in mice by intravenous injection is $8.8x10^8$ plaque forming units (Duncan *et al.* 1978).

Adenovirus structure

Adenoviruses are small (approximately 90 nm in diameter) non-enveloped viruses with an icosahedral capsid with 12 fibres projecting from the icosahedron vertices (Figure 2). The virus consists of 240 hexon trimers, 12 penton pentamers, 60 IIIa monomers, 12 fiber trimers, 60 VII hexamers, 80 IX trimers as well as two terminal repeat proteins and multiple core proteins. Many of the latter are essential for intracellular trafficking and proteolytic cleavage of endocytosed viral capsids.

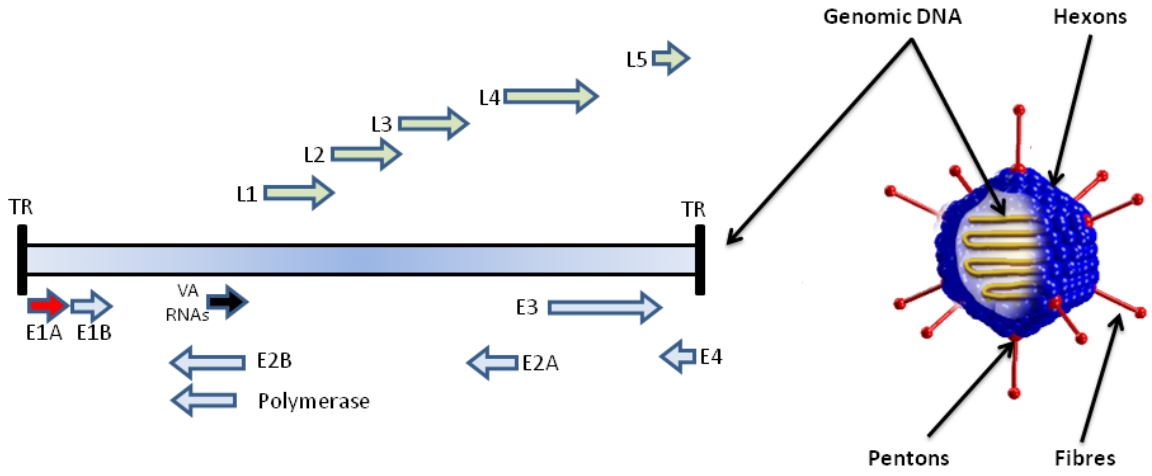


Figure 2: Adenovirus genetics and structure. Diagrammatical representation of the adenovirus serotype five genome (left) and virus particle (right). The genome of adenovirus can be divided into the regions encoding the early and late transcription events. Those regions below the genome denote early transcription events whilst those above the genome are late transcription events. The E1A protein transcription cassette is highlighted in red whilst the region encoding the non-coding VA RNAs are shown in black. TR denotes the terminal repeat regions which are bound by one copy of the terminal repeat protein at each end. The virus particle shows the major proteins comprising the adenovirus virion including the fibre, penton and hexon proteins. Many core proteins exist (including IIIa, VII, IX) but are not shown here.

The DNA genome of adenovirus varies in length dependent on serotype, but is typically 34-36 kilbases in length. The adenovirus serotype five genome which was engineered in this thesis is 35938 base pairs with a 103 base pair terminal repeat at each end and a 58% GC content. The genome is linear within the capsid structure and for the majority of the replication cycle but can form pan-handle circular structures within the nucleus during genome replication.

Cell entry and un-packaging

The process of cell attachment for adenoviruses varies dependent on the serotype. Adenovirus species C (including serotypes two and five) have been shown to attach to cells via their fibre domain binding to the cell surface coxsackie and adenovirus receptor (CAR) (Tomko *et al.* 1997) which is a component of cellular tight junctions (Honda *et al.* 2000). This initial interaction is high affinity and is followed by a lower affinity interaction between the virus penton base and cell surface integrins (αVβ3 & αVβ5) which are abundant on epithelial cells (Wickham *et al.* 1993). Following absorption to the cell surface virions are endocytosed and in a poorly defined step the virus capsid escapes from the endosome into the cytosol. Within the cytosol, adenoviral capsids bind to the microtubule network and are transported via a Dynein dependent mechanism to the nucleus where the DNA is delivered into the nuclear lumen following capsid interaction with the nuclear pore complex (Trotman *et al.* 2001; Kelkar *et al.* 2004).

Adenovirus gene transcription and expression

Our understanding of adenovirus genetics, transcription and translation is primarily derived from virological studies using adenovirus species C serotype five. The sequential process of gene transcription from the adenovirus genome reflects the protein requirements of the virus at each stage of the replication process. The transcription from the adenovirus genome is therefore divided into early and late events dependent on the timing of initiation of transcription from each viral promoter (Figure 2). The first proteins produced from the virus genome are the E1A proteins. The E1A transcription unit produces multiple mRNA molecules through alternative splicing which in turn produce multiple proteins ranging from 6-36 kDa. The E1A proteins have two major roles within an infected cell. Firstly, they induce the cell to enter the S phase of the cell cycle to allow the efficient replication of the viral genome. Secondly, they induce transcription of the other early promoters within the viral genome through transactivation. These promoters control the production of the E1B, E2, E3, E4 and E5 proteins. E1A expression is immediately followed by VA RNA and E1B and E3 protein production. These proteins and RNA molecules help to prevent the development of an anti-viral response. These early events in viral replication help to shape the intracellular environment to allow the replication of the viral genome before packaging. Later transcription events involve the production of structural proteins and proteins essential for cell lysis which are derived primarily from a single promoter (the major late promoter) which transcribes the late regions 1-5. Cell lysis is dependent on the E3-11.6K protein (also termed the adenovirus death protein) which despite its labelling as an early gene is only produced late in infection and from the major late promoter (Tollefson *et al.* 1996).

E1A proteins

E1A proteins are the translation products of the first gene transcription events from the adenovirus genome within the nucleus. This initial transcription is driven by a strong constitutively active enhancer element within the E1A promoter and allows significant quantities of E1A mRNA to be produced. E1A proteins expressed alone in rodent cells are sufficient to allow transformation. They are one of two sets of proteins in the adenovirus genome which are capable of inducing transformation with E1B proteins also able to induce cell cycle progression.

Early in adenovirus replication E1A proteins are produced from two primary alternatively spliced transcripts that are designated as 12S and 13S due to the sedimentation co-efficients of their respective mRNAs. These transcripts encode the large (36kDa) and small (26kDa) E1A proteins which are primarily involved in inducing the cell to enter the cell cycle and initiating viral gene expression. Later in virus replication many smaller E1A transcripts are produced but the functions of the proteins translated from these messages are less well defined. The E1A proteins have been shown to localise equally to the nucleus and the cytoplasm and have a half life of approximately 20-80 minutes (Frisch *et al.* 2002). All E1A mRNAs share the same 5' and 3' un-translated region (UTR) with the difference in transcript sizes resulting from internal splicing of the coding sequence.

The large E1A protein consists of 289 amino acid residues whilst the small E1A protein contains a 46 amino acid deletion (243 amino acids total size) in a central region of the large E1A protein (Figure 3). Both E1A proteins are heavily phosphorylated by the cellular proteins CDK1, 2 and 4 and a MAPK (Mal *et al.* 1996; Whalen *et al.* 1997). E1A proteins have no enzymatic function and do not bind to DNA, however, they are capable

of influencing many cellular processes by binding to important surface and pocket regions of key cytoplasmic and nuclear proteins (Frisch *et al.* 2002). Many of these proteins are pivotal for controlling the cell cycle and include pRB, p21, p300 and p27 (Figure 3). Comparative analysis of the primary protein structure of E1A proteins from multiple adenovirus serotypes has shown that they share distinct modular regions interspaced with less well conserved amino acid sections. These are designated as the N-terminus region and the CR1, CR2, CR3 and CR4 regions (Figure 3).

The large and small E1A proteins are capable of trans-activating many of the early promoters within the viral genome, although the large protein alone can stimulate early viral promoters to 'wild-type' levels (Winberg *et al.* 1984). Studies attempting to identify sequences unique to this effect identified no universal DNA motifs. It is now understood that the trans-activation effect mediated by the large E1A protein is due to the direct binding and trans-activation of many important nuclear transcription factors through its conserved CR3 domain, a region which is absent in the small E1A protein (Frisch *et al.* 2002). Whilst the large E1A protein is the only isoform capable of activating transcription from the majority of the early viral promoters, the small E1A protein is able to activate the E2 promoter region. This effect is mediated by the small E1A protein activating E2F cellular transcription factors and activating transcription from E2F dependent promoters (Phelps *et al.* 1991).

E2F transcription factors are multi-protein complexes which initiate transcription at promoters which are essential for inducing cell cycle progression. Their activity is tightly controlled by the cellular protein pRb and the functionally and structurally related proteins p107 and p130 which act to prevent entry into the cell cycle (Dyson 1998). Both the large

and small E1A proteins have been shown to bind these proteins in the regions that recognise E2F proteins (Frisch *et al.* 2002). This interaction displaces E2F proteins from their regulatory proteins (e.g. pRB) and allows them to trans-activate promoters which encode proteins essential for cell cycle progression. The CR2 region of E1A has been shown to be particularly important for displacing pRb from E2F transcription factors. Interestingly, mutant adenoviruses which express only the large E1A protein can replicate to wild-type levels in rapidly cycling cells but are significantly attenuated in G^0 quiescent cells (Montell *et al.* 1984). This suggests that the role of small E1A is primarily to induce cell cycle progression in order to prime the cell for virus genomic DNA replication in the S phase of the cell cycle.

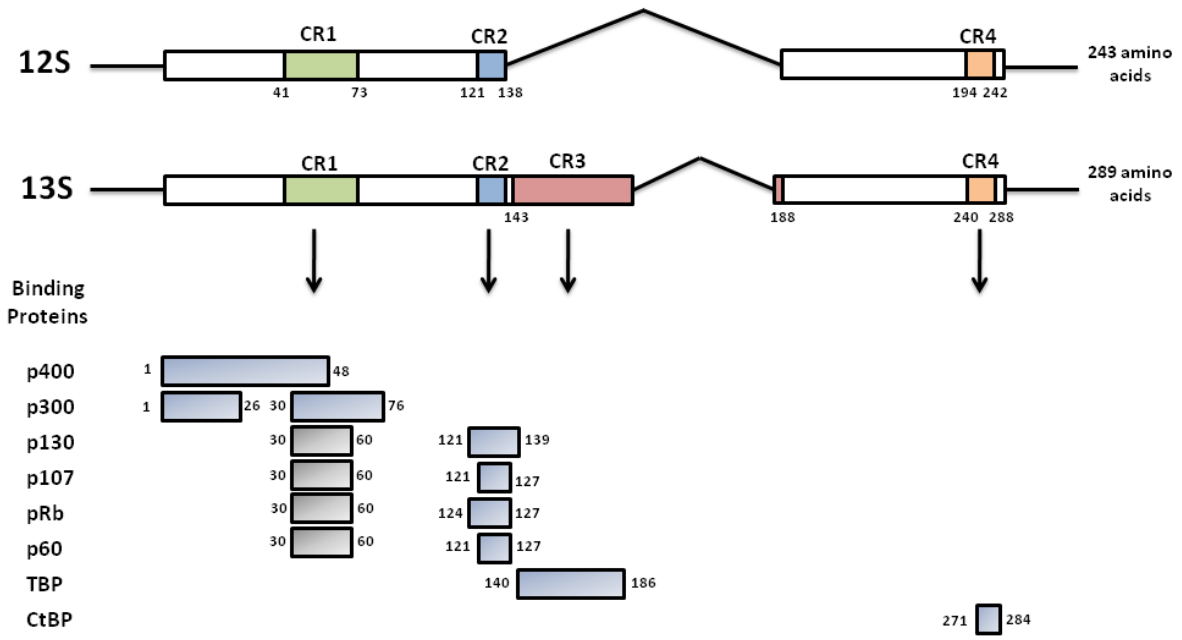


Figure 3: E1A major spice variants and protein interactions. E1A proteins are produced as two major isoforms encoding the large E1A protein produced from the 13S transcript and the small E1A protein produced from the 12S transcript. This diagram shows the major domains of both the large and small E1A proteins labelled as CR1-CR4. Some of the important proteins which interact with E1A proteins are shown and the regions of interaction are designated with either grey or blue bars. Blue bars represent the primary interacting regions with grey bars showing regions of secondary, less important contact. The small E1A protein lacks the CR3 region which interacts with TATA-binding protein (TBP). The presence of CR3 in the large E1A protein allows it to enhance transcription from the viral genome more than the small E1A protein. Numbers denote amino acid residues and the numbers highlighting interacting regions are based on the residues in the large E1A protein. Smaller E1A transcripts exist including the 11S, 10S and 6S transcript but these are not shown here.

MicroRNAs

The discovery of microRNAs and the microRNA network

In 1993 Lee *et al* reported on the observation that the Lin-4 gene in *Caenorhabditis elegans* encoded a small RNA molecule which was capable of regulating the transcript level of the Lin-14 gene through anti-sense recognition (Lee *et al.* 1993). Lin-4 was the founding member of the vast array of small non-coding RNA molecules which regulate gene expression which is now called the microRNA network. MicroRNAs are small non-coding RNA molecules which range in size from 16-30 bases although typically they are 21-23 nucleotides in length (Nilsen 2007). There are currently 1048 known microRNAs in the human genome and 672 have been discovered in mice (Griffiths-Jones *et al.* 2008). Many of these microRNAs are conserved in both sequence and function across many species which allows the effects of some microRNAs to be accurately tested in animal models.

The transcription of microRNA genes is mediated by RNA polymerase two which is unique among non-coding RNA molecules which are typically transcribed by RNA polymerase one and three (Lee *et al.* 2004). They are produced as primary microRNA transcripts (Pri-miRNA) which are 5' 7-methylguanosine capped and poly-adenylated and contain a hair-pin region containing the microRNA. These primary transcripts are then cleaved in the nucleus by the nuclease Drosha to become Pre-miRNAs before they are exported out through the nuclear pore complex by the Exportin-5 protein pathway into the cytosol (Lee *et al.* 2003; Yi *et al.* 2003). In the cytosol, the Pre-miRNAs are processed by the nuclease protein DICER before loading of the mature single stranded microRNA molecule into the RNA induced silencing complex (RISC) (Figure 4) (Hutvagner *et al.*

2001). MicroRNAs can also be encoded within introns of other genes so that their expression is closely allied with the expression of a specific gene. Such microRNAs are primarily processed by the nuclease Drosha, however, a subset can undergo Drosha independent Spliceosome dependent maturation within the nucleus and are referred to as Mirtrons (Kim *et al.* 2007; Ruby *et al.* 2007).

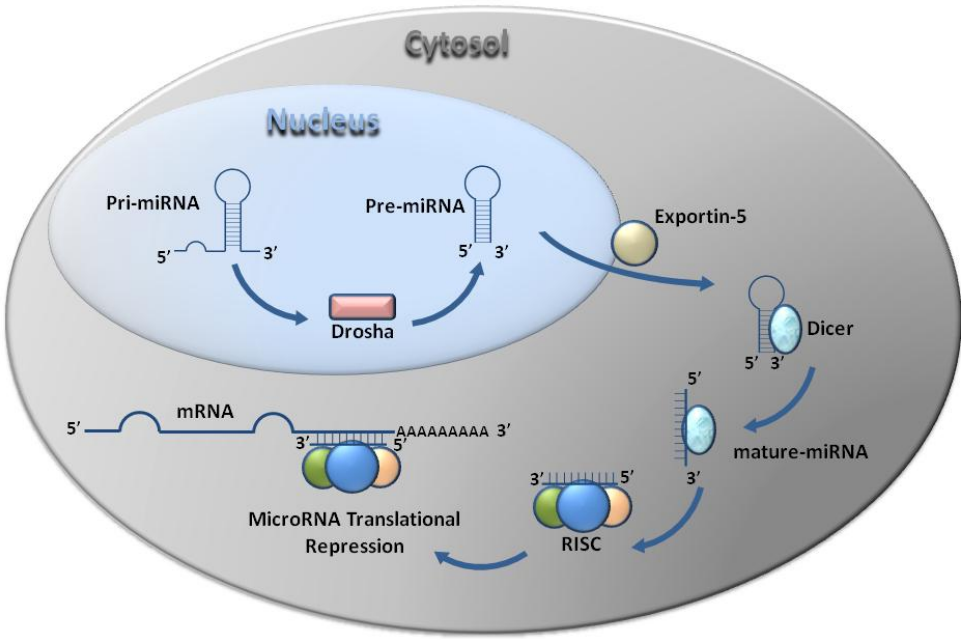


Figure 4: MicroRNA processing. MicroRNAs are transcribed as primary-miRNA transcripts by RNA polymerase two in the nucleus and then cleaved by Drosha into pre-miRNAs. These RNAs are then exported by Exportin-5 into the cytosol where they are further cleaved by Dicer and incorporated into the RISC complex. Binding of the microRNA to mRNA mediates translation inhibition through a variety of mechanisms.

MicroRNAs act by negatively regulating cellular gene expression post-transcriptionally through a number of mechanisms that all involve binding of the microRNA to complementary regions within a messenger RNA (mRNA), leading to decreased protein production (Nilsen 2007). Each microRNA is estimated to recognise on average 200 distinct mRNA species and it is estimated that in excess of 30% of all mRNAs produced from human genes are regulated to some extent by microRNA mediated silencing (Nilsen 2007).

The consequences of a microRNA binding to a mRNA can differ substantially and depend largely on the homology between the microRNA and the target binding site within the mRNA molecule (Nilsen 2007). The region bound by microRNAs is often referred to as the seed region and is typically 6-8 nucleotides in length (Lewis *et al.* 2003; Stark *et al.* 2005). In general terms, as the homology between the microRNA and the mRNA binding site increases, the ability of the microRNA to repress mRNA translation also increases. If sequences are perfectly complementary then the microRNA is capable of inducing cleavage of the mRNA molecule in an Argonaute-2 nuclease dependent reaction between the 10^{th} and 11^{th} base pairs within the binding site (Liu *et al.* 2004). The varying influences of microRNAs on mRNA transcripts can be divided into either direct or indirect effects whereby the indirect effects result in mRNA degradation or sequestration and the direct effects do not alter mRNA stability or quantity but directly influence protein translation (Nilsen 2007).

Indirect effects:

De-adenylation: MicroRNA binding can induce the poly-adenylation region of an mRNA to become degraded (Behm-Ansmant *et al.* 2006). This indirectly prevents translation and leads to increased degradation of the remaining mRNA through normal degradatory pathways.

Degradation: Binding of microRNAs to mRNA with high affinity can cause the mRNA to become directly cleaved by the RISC complex in an Argonaute-2 nuclease dependent reaction which leads to further degradation and prevents translation (Lim *et al.* 2005).

Sequestration to P bodies: Processing bodies are general sites of mRNA storage and degradation where mRNAs are not translated (Eulalio *et al.* 2007). Binding of microRNAs has been shown to induce sequestration to P bodies thereby reducing protein production (Liu *et al.* 2005).

Direct effects:

Inhibition of Initiation: MicroRNA binding has been shown to prevent ribosomal engagement onto some mRNA molecules and so prevents initiation of protein translation directly (Humphreys *et al.* 2005).

Pre-mature Ribosome drop-off: Some microRNAs can influence protein production after a ribosome has engaged the Kozak sequence of an mRNA by causing the ribosome to prematurely terminate translation (Petersen *et al.* 2006).

Slowed or Stalled Elongation: A decrease in protein production without a change in mRNA stability or ribosomal engagement has been observed and called

ribosomal stalling. It is currently unclear how important this poorly defined mechanism is for preventing protein translation (Nottrott *et al.* 2006).

Co-translational degradation: Rather than directly inhibiting or slowing translation or degrading the mRNA it has been demonstrated that the binding of microRNAs to mRNA can cause an increase in the proteolytic cleavage of the translated protein (Nilsen 2007).

MicroRNAs are unlikely to be limited to using one mechanism of protein translation inhibition and a single microRNA may use multiple mechanisms to prevent protein production. Indeed, the method of suppression has been shown to differ for different mRNAs after binding of the same microRNA (Behm-Ansmant *et al.* 2006), which suggests that the effect the microRNA has on any particular mRNA species is largely dependent on the seed region or secondary structure within the mRNA, not the microRNA itself.

Tissue specific microRNA expression

Whilst the expression of most microRNA molecules is ubiquitous throughout most human tissues, some have also been shown to demonstrate tissue-selective expression (Figure 5 and Table 2) (Landgraf *et al.* 2007). Some of these microRNAs have been shown to be essential for tissue identity and regulate key developmental lineages and pathways. MicroRNAs are involved in the differentiation of cells of the haematopoietic lineage and neural lineage as well as influencing cardiogenesis, brain morphogenesis, insulin secretion

and cell growth and apoptosis (Cao *et al.* 2006; Plasterk 2006; Shivdasani 2006). Interestingly, when the neural microRNA mir124 was transfected into HeLa cells the expression of over 170 genes was down-regulated, which were known to be reduced in brain tissue compared to other tissues. In other words, the expression of mir124 induced a phenotypic transition of HeLa cells towards that of the brain suggesting a role for mir124 in neuronal identity (Lim *et al.* 2005). Similarly, transfection of the skeletal and cardiac specific microRNA mir1 into HeLa cells also reduced the expression of many genes which were known to be down-regulated in those tissues (Lim *et al.* 2005). These results suggest that microRNAs have important regulatory functions that help to define the lineage and differentiation status of cells and tissues.

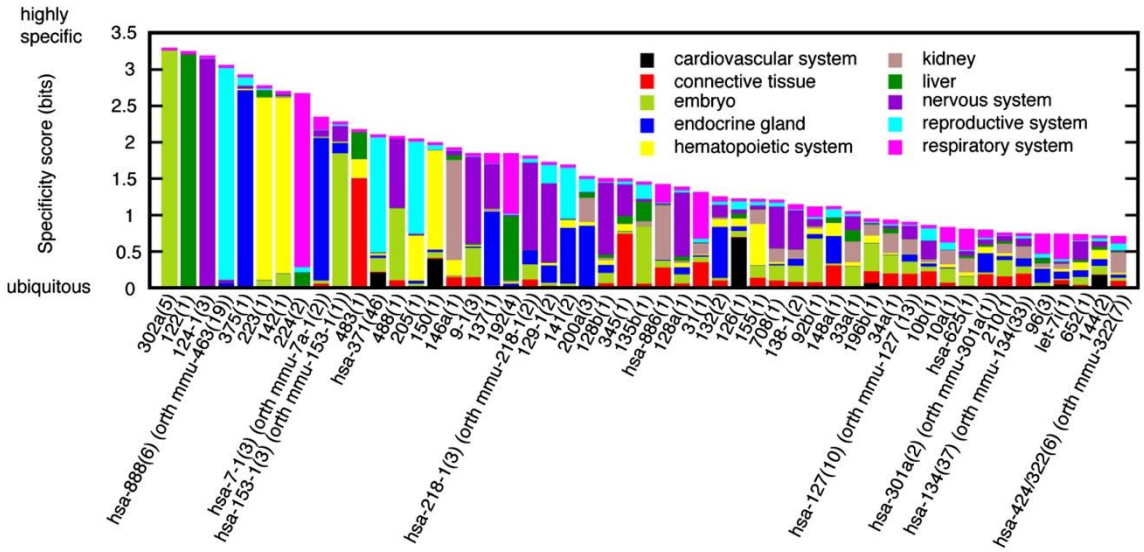


Figure 5: MicroRNA tissue specificity. The selective expression of microRNAs can be plotted by quantifying a specificity score. Of note is the microRNA mir122 which is shown as the microRNA with the second highest specificity score. This diagram is included with permission from the authors and Cell Press and was published in 2007 after profiling of human tissues. A description of the technique used to calculate this score is available within the primary manuscript (Landgraf *et al.* 2007).

Tissue/Cell Lineage	MicroRNA
Liver	mir122, mir192, mir194, mir135, mir224
Brain	mir124a&b, mir125, mir128, mir132, mir134, mir135, mir138, mir153, mir9
Colon	mir143, mir194
Heart	mir1, mir126, mir133, mir206, mir208
Heamatopoetic	mir142, mir144, mir155, mir181, mir195, mir205, mir221, mir222, mir425, mir223 (myeloid only), mir150 (B and T cells only)
Kidney	mir192, mir194, mir204, mir215, mir30
Lung	mir130, mir24, mir32
Ovary	mir189
Pancreas	mir7, mir216, mir375
Skeletal Muscle	mir1, mir133, mir206
Spleen	mir127, mir150, mir151, mir212
Stomach	mir148
Testis	mir204
Epithelium	mir29, mir194
Endothelium	mir126, mir130

Table 2: Tissue selective microRNAs. This list has been compiled through the analysis of genome wide microRNA profiles published elsewhere (Liu *et al*. 2004; Landgraf *et al*. 2007). It shows tissue specific or tissue selective microRNA expression profiles for individual tissues.

MicroRNAs in cancer and other diseases

The role of microRNAs in regulating such diverse pathways can lead to disease when they become dysregulated. The alteration of microRNA regulation pathways has been shown to be involved in spinal muscular atrophy (Haramati *et al.* 2010), fragile X syndrome (Cheever *et al.* 2010), DiGeorge syndrome (Landthaler *et al.* 2004) and important roles for microRNAs in many other diseases is rapidly emerging.

Changes in microRNA profiles have also been linked with many different forms of neoplasia. This finding has been linked in part to the presence of clusters of microRNA genes in regions of known genomic instability. Approximately 52.5% (Calin *et al.* 2004) of microRNA genes are located in such genetic regions in human cancers. The microRNA cluster mir15a/16-1 is located in a region which is frequently deleted in chronic B cell lymphoma and known to contain a putative tumour suppressor gene which is now believed to encode a microRNA (Calin *et al.* 2002). Similarly, deletions of the microRNA cluster encoding genes let7g/mir135-1 is also frequently deleted in many human neoplasias (Calin *et al.* 2004). It has also been shown that the genomic locus encoding the microRNA cluster mir17-92 is frequently amplified in lymphoma and this same region is often translocated in T cell acute lymphoblastic leuakemia (T-ALL) (Tagawa *et al.* 2005). Mir26a is also amplified in many glioblastomas (Huse *et al.* 2009).

The observation that some microRNAs are down or up-regulated in cancer has led to their respective labelling as either tumour-suppressor or oncogenic microRNAs. Many studies have now confirmed the critical role some microRNAs can play in either preventing or promoting tumour growth. For example, over-expression of the tumour-suppressor microRNA let7g is capable of reducing tumour burden in a murine k-ras lung cancer

model (Kumar *et al.* 2008) whilst the over expression of the oncogenic microRNA cluster mir17-92 leads to lympho-proliferative conditions (Xiao *et al.* 2008). The under and over-expression of microRNAs can also influence important phenotypic behaviours of some cancers and microRNAs which are associated with tumour aggressiveness (mir210) (Foekens *et al.* 2008) and metastasis (mir10b, mir9, mir31 and mir335) (Hurst *et al.* 2009) have been identified.

The activity of microRNA promoters has also been linked with cancer progression and oncogenesis. The promoter regions of some tissue specific microRNAs are potent initiators of transcription including both the haematopoietic specific microRNA mir142 and the hepatic specific microRNA mir122. In both B cell leukemia and hepatocellular carcinoma is has been found that the C-MYC oncogene can be translocated to become under the control of these microRNA promoters respectively (Etiemble *et al.* 1989; Gauwerky *et al.* 1989). This leads to over expression of C-MYC and induces cell cycle progression and oncogenesis.

MicroRNA and virus interactions

The ability of a single microRNA to control the quantity of many proteins makes them powerful tools for regulating multiple cellular pathways. Therefore it is not surprising to find that viruses have evolved to exploit this pathway in order to control cellular behavior and phenotype. Nine viruses which infect humans have been shown to encode microRNAs including Epstein Barr virus (EBV), Human Adenovirus, Human Immuno-deficiency virus (HIV) and Herpes Simplex viruses 1 and 2 (HSV 1 & 2) (Griffiths-Jones *et al.* 2008). Epstein Barr virus alone expresses 25 unique microRNAs. Many of these

microRNAs have been shown to influence pathways which are important for the development of an antiviral response.

Although DNA viruses can encode microRNAs to subvert cellular processes, RNA viruses are less suited to this task because it would require the cleavage of the genome itself, nevertheless, this does not mean that RNA viruses have not adapted to exploit the microRNA network in order to enhance viral replication. Hepatitis C virus (HCV) is an RNA virus of the flaviviridae family which has been shown to be dependent on the expression of microRNA mir122 in hepatocytes for replication (Jopling *et al.* 2005). Interestingly, two binding sites for mir122 have been found in the 5' UTR of the HCV genome and without these binding sites the viral genome is not replicated and protein translation is reduced (Jangra *et al.* 2010). This is the converse of normal microRNA-mediated protein inhibition and appears to operate by enhancing IRES dependent translation from mir122 bound mRNAs through an as yet unidentified mechanism (Jangra *et al.* 2010). Mir122 is now considered an important therapeutic target for the treatment of chronic HCV with many small molecule inhibitors and anti-sense targeting approaches are being attempted clinically to down regulate it (Tyczewska *et al.* 2010).

MicroRNA control of viral gene therapy vectors

The observation that microRNA molecules could potently down-regulate mRNA translation quickly led to the development of novel therapeutics which exploited their utility for therapeutic purposes. One such development was the design of non-replicating gene therapy vectors in which the transgene was regulated by tissue specific microRNAs

to prevent unwanted gene expression. This principle was originally developed by Brown *et al*, who showed that the inclusion of binding sites for the microRNA mir-142-3p within the 3'UTR of retrovirally-encoded transgenes prevented their expression in antigen presenting cells (Brown *et al.* 2006). This modification prevented stimulation of an immune response against the transgene protein product and allowed long term transgene expression in non-hematopoietic cells without immune rejection. This initial finding was followed by the modification of a non-replicating adenoviral serotype five vector encoding the Herpes Simplex 1 thymidine kinase (HSV-TK) suicide gene containing binding sites for the liver specific microRNA mir122. This vector performed well and demonstrated anti-cancer efficacy and reduced hepatic expression of HSV-TK by up to 1500-fold (Suzuki *et al.* 2008).

MicroRNA regulation of virus vaccine strains

The control of single mRNA transcripts produced from non-replicating gene therapy vectors described above showed that microRNA mediated silencing of mRNA molecules that were produced from a strong viral promoter was possible. However, the control of a replication competent virus, many of which are capable of systematically shutting down host RNA and DNA synthesis, may not have been possible using this mechanism because even small amounts of replication may shut down microRNA and protein synthesis. The sequential staging of viral replication and the redundant nature of many viral proteins must also be considered when engineering microRNA responsiveness into replication competent viruses because, if the incorrect protein is targeted, the system may fail to completely prevent virus replication and toxicity. Perhaps because of this complexity the

first virus in which this strategy was attempted was genetically one of the simplest. The picornavirus polio virus (PV) is a positive sense single-stranded RNA virus responsible for the debilitating condition poliomyelitis due to its infection of, and replication within, neurons. Barnes *et al* hypothesised that the inclusion of four binding sites for either the ubiquitously expressed microRNA let7a or the neuronal specific microRNA mir124 may prevent this toxicity and allow the development of a potent, immunogenic, yet safe virus vaccine strain (Barnes *et al.* 2008). Following the intramuscular injection of microRNA-regulated PV into poliovirus receptor transgenic mice, it was demonstrated that the inclusion of either let7a or mir124 binding sites prevented virus replication in neurons and enhanced survival. This work demonstrated that microRNA control of virus replication was possible and that immunogenic replication competent vaccines could be produced using tissue specific microRNA control.

This approach has also been adapted to generate conditionally attenuated Influenza A virus in a species specific manner. Perez *et al* searched for microRNAs that were ubiquitous in human and murine tissues but absent from chicken tissue. This would allow the virus to be grown to high titre in chicken eggs whilst preventing viral replication in murine or human tissue. By including microRNA binding sites for the microRNA mir93 into genetically stable coding regions of the influenza A genome it was possible to show species specific attenuation in murine animal models (Perez *et al.* 2009). This virus showed reduced toxicity but equal immunogenicity when compared to the wild type Influenza A strain.

MicroRNA control of oncolytic viruses

The use of viruses in the treatment of cancer requires that such viruses do not cause unacceptable toxicity in sites where the virus normally replicates. The use of microRNAs to control virus replication should allow the creation of replication competent viral vectors which are both safe and efficacious. The small size of the required insertion (typically only 21-24 base pairs per binding site) provides considerable flexibility in the design of capacity-restricted therapeutic viruses. Equally, the negative regulatory principle of using microRNA regulation to inhibit viral replication could allow significant therapeutic potency by avoiding attenuation in target sites. Although at the beginning of this project this technique had not been applied to any replicating virus, it has since been used to generate many microRNA controlled conditionally-replicating viruses by other groups. The first virus to which this technique was applied was VSV (Edge *et al.* 2008; Kelly *et al.* 2010) into which binding sites for the microRNA let7a were inserted. Let7a is often down-regulated in cancers and it was hypothesised that this modification might allow attenuation of the neural toxicity of VSV without attenuation in cancer cells which have down-regulated let7a. More recently, a VSV variant has been produced which was attenuated by exploiting the neuron specific microRNA mir124 (Kelly *et al.* 2010). Both viruses showed increased cancer selective replication and reduced neural toxicity, however, the use of let7a to control virus replication also attenuated viral replication in cancer cells.

In other studies, Coxsackie virus strain 21 (CAV21) was modified by the insertion of tissue-specific microRNA binding sites (Kelly *et al.* 2008). CAV21 causes myelitis in small animal models which is due to infection and replication within skeletal muscle

tissue. The insertion of four binding sites for the muscle specific microRNAs mir206 and mir133 (two binding sites of for each) significantly reduced virus replication mediated toxicity in mice and allowed systemic efficacy with CAV21 to be achieved. Unfortunately, in some mice the loss of microRNA binding sites was shown to allow muscle infection and replication, which caused fatal toxicity.

MicroRNA regulation of Herpes Simplex virus has also been demonstrated (Schaefer *et al.* 2009). Lee *et al* inserted either four or five binding sites for the microRNAs mir143 and mir145 into the 3' UTR of an essential viral gene, ICP4. Mir143 and mir145 are expressed in most human tissues but have been shown to be down regulated in prostate cancer cells. This modification was therefore designed to exploit cancer specific changes in microRNA levels rather than tissue specific microRNA expression.

Replication competent retroviruses have also been produced which are regulated by microRNAs but results have yet to be published (Prof N Kasahara, personal communication). Finally, adenoviruses have been modified by the insertion of binding sites for liver specific microRNA mir122 into the 3' UTR of the E1A transcription cassette. Leja *et al* published the toxicity profile and efficacy of a serotype five adenovirus in which E1A was placed under the chromogranin-A promoter and was then further modified by the inclusion of microRNA binding sites for the liver specific microRNA mir122 (Leja *et al.* 2010). Ylosmaki *et al* similarly produced an adenovirus containing three binding sites for microRNA mir122 in the 3' UTR of E1A although this virus was never tested *in vivo* and was constructed from the dl922-947 modified virus strain (described above) (Ylosmaki *et al.* 2008).

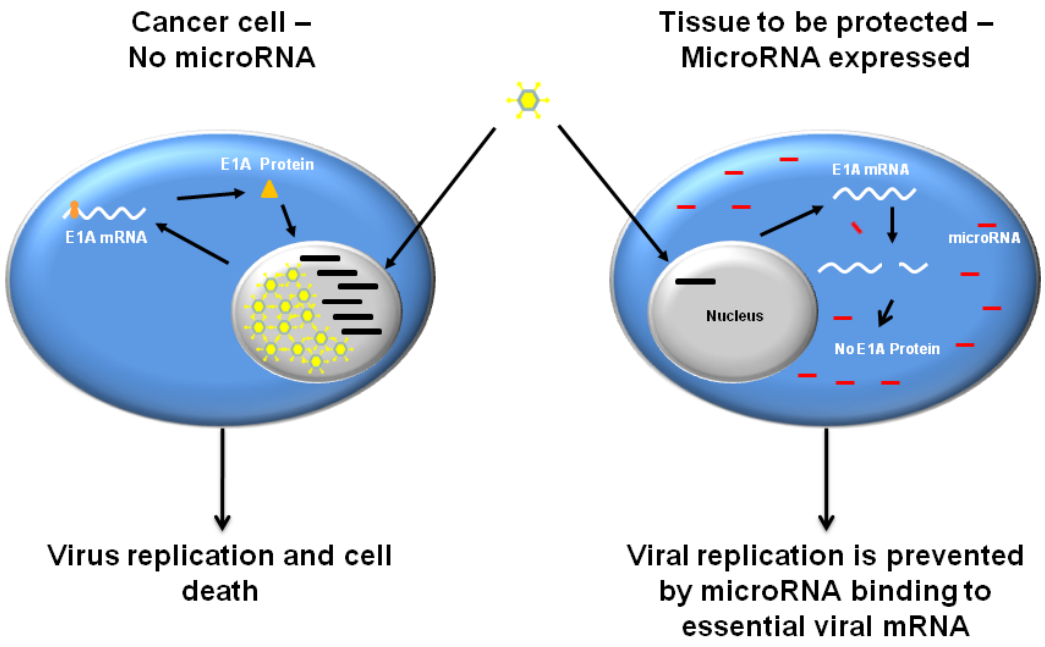


Figure 6: The principle of exploiting microRNAs to regulate virus replication. The differential expression of microRNAs in tissues can be exploited to control protein translation from essential mRNAs. Shown here is a cancer cell (left) in which a particular microRNA is not expressed and a cell from a tissue which is normally infected by the virus (right) in which the microRNA is expressed. When the virus enters the cancer cells the absence of the microRNA allows the virus to replicate to wild-type levels because no proteins are limiting. In the tissue cell on the right the presence of the microRNA allows the selective destabilisation of an essential mRNA which prevents the translation of a key viral protein. This limits viral replication and reduces or prevents toxicity. The diagram shows the replication cycle of an adenovirus as an example.

Mir122

The ability of microRNAs to regulate multiple mRNA targets involved in diverse biochemical pathways is particularly well exemplified by the liver specific microRNA mir122 (Lagos-Quintana *et al.* 2002; Girard *et al.* 2008). The discovery of mir122 and its liver specificity pre-dates the discovery of the microRNA network as we know it today. In 1989 Moroy *et al* discovered a mutation in a non-coding gene called *hcr* in Woodchuck liver tumours which was associated with *c-myc* transforming mutations (Moroy *et al.* 1989). This gene encoded an RNA molecule which was 85 base pairs in length that localised to the nucleus and was subjected to nuclease cleavage, however, no role was attributed to its expression. It is now understood that the *hcr* gene encodes mir122 and that the transcript discovered by Moroy *et al* was the primary mir122 transcript which is cleaved to a 66 nucleotide pre-microRNA in the nucleus. This transcript has a hairpin structure and base pairs along 79% of its nucleotides. In humans the *hcr*, or mir122 gene, is located on chromosome 18 between base pairs 56118306 and 56118390 and encodes an un-spliced RNA molecule (Accession NR029667, MirBase MI0000442). In mice the mir122 gene is also found on chromosome 18 and lies between base pairs 65408515-65408580 (Accession NC000084, Mirbase MI0000256).

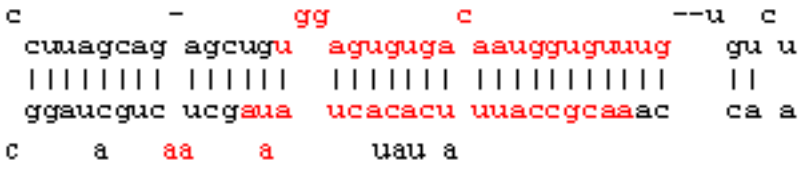


Figure 7: Pre-mir122 hairpin. This diagram shows the structure and base pairing relationship of the primary mir122 transcript. The final DICER cleavage product is highlighted in red with the mature mir122 sequence shown in red on the top strand 5' to 3'.

Mir122 is the most abundant tissue specific microRNA in both humans and mice in the adult liver. It accounts for up to 70 % of all the microRNA content in primary human and murine hepatocytes (Chang *et al.* 2004). In fetal liver this is not the case with mir93a and mir483 demonstrating higher levels of expression than mir122, perhaps suggesting developmental roles for these liver specific microRNAs (Girard *et al.* 2008). Mir122 expression is observed in human, mouse and woodchuck liver as well as in primary human hepatocytes and hepatic derived cell lines including the mouse Hep1-6 cell lines and the human cell line Huh7 (Chang *et al.* 2003; Jopling *et al.* 2005).

The sequence of the mature mir122 RNA (5' uggagugugacaaugguguuug 3') is identical between mice and humans although the complete gene sequence varies between mice and humans (Figure 8). The human gene contains an additional nine nucleotides at the 5' end and an additional ten nucleotides at the 3' end which are absent in the murine gene. The remaining sequence shares 97% homology across 66 base pairs with only two mismatches.

```
Identities/Homology = 64/66 (97%), Base Pair Gaps = 0/66 (0%)

Human 10 AGCTGTGGAGTGTGACAATGGTGTTTGTGTCTAAACTATCAAACGCCATTATCACACTAA
69
         ||||||||||||||||||||||||||||| |||| ||||||||||||||||||||||
Mouse 1  AGCTGTGGAGTGTGACAATGGTGTTTGTGTCCAAACCATCAAACGCCATTATCACACTAA
60

Human 70 ATAGCT  75
         ||||||
Mouse 61 ATAGCT  66
```

Figure 8. Sequence alignment of murine and human mir122 genes. Results show that the region encoding mature mir122 is identical (highlighted in red). Two mismatches within the gene sequences are visible and aligning numbers show that the human gene is longer than the murine gene.

Mir122 regulation of target genes

The small size of seed region binding sites in mRNA molecules makes predicting microRNA binding sites difficult (Lewis *et al.* 2003). This problem is also enhanced by the observation that most microRNAs control many hundreds of different mRNAs and usually only by a limited amount (<5-fold) (Lim *et al.* 2005). This makes determining genuine microRNA mediated signals difficult to discern from background experimental noise. Chang *et al* exploited the Lewis prediction algorithm (Lewis *et al.* 2003) to search for mRNA molecules that might contain mir122 binding sites. One site was detected in the 3' UTR of the mRNA encoding the cationic amino acid transporter one (CAT-1) protein. Knockdown of mir122 in human hepatoma Huh7 cells proved that mir122 inhibited the CAT-1 expression by demonstrating that CAT-1 protein levels increased when mir122 was inhibited (Chang *et al.* 2004). It was also demonstrated that reducing the concentration of essential amino acids in the culture medium prevented mir122 mediated repression of CAT-1 suggesting a dynamic role for mir122 in reacting to cellular conditions.

Other proteins which have been demonstrated to be regulated by mir122 include Aldolase A protein and Lag-1 longevity assurance homolog 6 (LASS6) (Esau *et al.* 2006). LASS6 is believed to be involved in sphinogolipid synthesis and it is interesting to find that many similar genes involved in lipid synthesis are also regulated by mir122. Inhibition of mir122 in mice has uniformly shown that the level of circulating cholesterol decreases suggesting that mir122 has an important role in regulating lipid metabolism (Moore *et al.* 2010). Mir122 has since been proposed as a potential therapeutic target for reducing circulating cholesterol.

MicroRNA mir122 is believed to prevent the expression of many genes which are 'non-liver' and thereby help to define phenotypic hepatocyte identity (Coulouarn *et al.* 2009). It has also been shown that the level of mir122 expressed in primary hepatoma is inversely correlated with the aggressiveness of the tumour (Coulouarn *et al.* 2009). This observation is perhaps unsurprising given that tumour aggressiveness correlates with a lack of tissue differentiation. More importantly, it has been shown that the expression of the oncogene *N-Myc* and the cell cycle inducing protein *Cyclin G1* are repressed by mir122 (Gramantieri *et al.* 2007). Levels of *Cyclin G1* have also been shown to correlate with the loss of mir122 in tissue culture cell lines suggesting a role for the loss of mir122 expression in cell cycle control and enhancing tumour development.

Reporter viruses and monitoring microRNA regulation

"*Count what is countable, measure what is measurable, and what is not measurable, make measurable*" (Galileo Galilei)

Reporter viruses are an important and essential tool in modern biology and virology. They allow the direct quantification of virus replication and gene expression and can be used to monitor the effects of host cells on the virus life cycle. Within this thesis the ability to accurately quantify even small levels of microRNA mediated regulation of adenovirus translation was essential. Traditional reporter adenoviruses exist which express luciferase (Mittal *et al.* 1993), green fluorescent protein (deMartin *et al.* 1997) and β-galactosidase (Wilkinson *et al.* 1992) under the control of either strong viral promoters (e.g. SV40 and CMV) or tissue specific promoters (Quantin *et al.* 1992) however these vectors are replication deficient and do not measure activity from an endogenous adenovirus promoter and are therefore at best an indirect measurement of viral gene expression. No wild type based replication competent serotype five adenoviruses expressing luciferase have previously been reported and for this reason within this thesis an attempt was made to develop such a virus. It was hypothesized that the role of adenovirus E1A protein as a labile binding protein with no enzymatic function might allow the fusion of reporter proteins which would allow direct and accurate quantitation of E1A production. This method might also allow indirect measurement of viral replication because E1A transcription is active from replicated viral genomes and therefore increases throughout the viral life cycle. The fusion of luciferase to E1A protein might also allow sensitive *in*

vivo imaging of virus replication which is not possible using non-reporter viruses without euthanizing animals at multiple time points. This is because luciferase emits light with the maximum emission peak at 520 nm which is able to transmit though tissue depths which are not possible using other reporter proteins. However, it was unclear at the start of this thesis if the addition of luciferase to the E1A protein would allow the generation of viable replication competent virus or if this modification would abrogate E1A activity and interfere with virus progeny production.

Thesis objective

In this thesis the use of microRNA regulation to control a wild type serotype five adenovirus, a DNA virus, was explored. By inserting binding sites for the hepatic specific microRNA mir122 within the 3' UTR of the E1A transcription cassette it was hypothesised that E1A protein would be down regulated specifically in hepatocytes whilst virus replication would demonstrate wild-type kinetics in cells not expressing microRNA mir122.

Mir122 is highly and selectively expressed in both human and murine hepatocytes (Lagos-Quintana *et al.* 2002; Chang *et al.* 2004), and this modification should prevent expression of E1A within the liver *in vivo*, thereby reducing adenovirus replication and hepatotoxicity whilst maintaining its therapeutic replication within tumour cells. It was unclear; however, if a microRNA would be sufficiently potent to prevent mRNA translation from a DNA virus to the level required to prevent further viral transcription, translation and replication.

This thesis also developed two novel approaches to assess the activity and toxicity associated with the microRNA regulated virus. Firstly, an attempt was made to generate the first replication competent luciferase reporter virus based on an adenovirus serotype five backbone. Secondly, the toxicity of the viruses produced in this thesis was tested in a novel pharmacodynamics led dose escalation study in order to determine both the optimal therapeutic dose and the maximum tolerated dose of microRNA regulated adenovirus compared to the wild type strain.

Thesis aims:

1. Determine whether the use of microRNA mir122 to regulate E1A mRNA is capable of abrogating adenoviral replication selectively in hepatocytes.

2. Demonstrate that the use of microRNA mir122 regulation can reduce virus replication induced hepatic toxicity in a murine model.

3. Assess the side effects of exploiting microRNA regulation to control adenovirus replication in the murine liver to determine if this method of control affects normal physiological liver function.

4. Determine if microRNA regulated adenovirus demonstrates enhanced anti-cancer efficacy or improved therapeutic index compared to Ad5 WT in a microRNA mir122 negative xenografted model of human cancer.

Chapter Two: Materials and Methods

2.1: Materials and methods

Plasmid amplification in prokaryotic systems

Transformation of *Escherichia coli* (*E.coli*) allows amplification of plasmids and subsequent screening for desired clones. DNA transformation was performed by thawing *E. coli* cells on ice, followed by the addition of 5-100 ng of DNA per transformation. Cells were incubated in ice for 20 minutes followed by 3 minutes heat-shock at 37 ºC. LB medium (0.9 mL per sample) was added to each tube and incubated for a further 15 minutes at 37 ºC. Samples were either poured into nutrient agar plates containing selection antibiotic or 100 µL of each sample was streaked out to allow individual colony isolation.

Successfully transformed clones were amplified and selected using the appropriate antibiotic in growth media. Bacterial culture broth and plates containing either Kanamycin (50 µg/mL, Kanamycin Sulfate, Invitrogen, UK) or Ampicillin (50 µg/mL, Ampicillin Sodium salt, Sigma Aldrich) were prepared using Lennox LB Broth Base (20 g/L) and Lennox LB Agar (32 g/L) (Invitrogen, UK), respectively. Single bacterial colonies obtained on culture plates after transformation were picked and grown overnight in broth (5 mL mini-prep or 250 mL maxi-prep) at 37 ºC with agitation (200 rpm) using a Sanyo orbital incubator (SANYO Electric Biomedical Co. Ltd, USA).

Plasmids were purified by mini-prep using the QIAprep Spin Miniprep Kit (Qiagen, UK) or maxi-prep using the Qiagen High Speed Maxi-prep kit (Qiagen, UK). Plasmid clones were selected and confirmed by diagnostic restriction digestion and DNA sequencing.

Bacterial cells

For the majority of small (<10 Kb) DNA constructs DH5α *E.coli* bacteria (Invitrogen) were used to amplify plasmids. For larger (>10 Kb) constructs XL10 Gold Ultracompetent *E.coli* bacteria (Strategene) were used to amplify DNA. These cells have been engineered in order to allow the efficient delivery of large DNA plasmids via heat-shock treatment and reduce bias towards selecting for small plasmids libraries. For growing DNA which was free of DAM and DMC methylation at key restriction sites JM110 *E.coli* (Stratagene) were used. These cells are deficient in methyl transferase enzymes and allow high purity methyl deficient DNA to be amplified. For recombination of DNA molecules BJ5183 *E.coli* cells were used. These cells allow the recombination between two DNA molecules due to both the mutation of Endonuclease I and the expression of a RecET homologue which allows the efficient repair of double strand breaks at homologous regions. All strains were grown in Lennox LB media or on LB agar plates at 37 ºC.

DNA restriction digests (preparative and qualitative)

Restriction endonucleases were used to generate diagnostic digestion patterns or to produce DNA molecules with sticky (non-blunt) ends or blunt ends, allowing subsequent ligation of DNA fragments with other DNA molecules with either blunt or compatible overhangs. All digestions were performed according to conditions specified by the manufacturer (enzymes purchased from New England Biolabs, UK or Promega, UK). Where digestions were performed for molecular engineering purposes, DNA fragments of

correct sizes were visualised by gel electrophoresis and purified either from excised gel bands using the QIAEX II Gel Extraction Kit for large fragments (>8 Kb) (Qiagen, UK) and the MinElute Gel Extraction Kit for small fragments (Qiagen, UK), or, where it was unnecessary to resolve fragments of different sizes, purified directly from the digestion reaction (QIAquick PCR Purification Kit). HyperLadder™ I (Bioline Ltd, UK) was used as a molecular weight marker for fragments ranging from 1-10 Kb. For larger DNA fragments a Lambda Hind3 digest ladder was used (New England Biolabs, UK). For fragments less than 1 Kb a 100 base pair ladder was used (New England Biolabs, UK).

DNA ligation

Donor (insert) and recipient (vector) DNA were digested with single or double (for directional cloning) restriction enzyme(s) generating compatible overhangs for complementary base-pairing. Digested insert was gel-purified or cleaned up, while the vector was de-phosphorylated by treatment with 2 µL of calf intestinal alkaline phosphatase (37 ºC, 1 h) (Invitrogen, UK) to reduce background contamination resulting from vector re-ligation. Vectors were then purified using the MinElute Gel Extraction Kit (Qiagen, UK) or the QIAquick PCR Purification Kit (Qiagen, UK) using the manufacturers protocols. All purified DNA was eluted in nuclease-free water. Ligation reactions were prepared using T4 DNA Ligase (New England Biolabs, UK) according to the manufacturer's protocol.

Heat shock competent cells

Heat Shock DNA competent *E. coli* cells were produced by inoculating a single colony of *E. coli* into 5 mL 2xYT broth media (16 g/L Bacto Tryptone, 10g/L Yeast Extract, 5g/L NaCl, pH 7.0 with 5M NaOH) which was shaken at 37 ^{o}C for 4 hours. This was transferred to 200 mL pre-warmed 2xYT media. When the culture reached OD 480 the cells were pelleted at 3000 rpm in a swing out centrifuge at 4 ^{o}C for 10 minutes. Cells were re-suspended in a total of 80 mL cold TFBI1 (30 mM $KC_2H_3O_2$, 100 mM RbCl, 10 mM $CaCl_2 \cdot 2H_2O$, 50 mM $MnCl_2 \cdot 4H_2O$, 15% v/v glycerol, adjusted to pH 5.8 using 0.2 M CH_3COOH, filter sterile). Cells were spun as above and re-suspended in 8mL of TFBI2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2 \cdot 2H_2O$, 15% v/v glycerol, adjusted to pH 6.6 using 5 M KOH, filter sterile). 100 µl of cells were pipette into pre-chilled 1.5 mL microfuge tubes and frozen on dry ice. Tubes were then transferred to -80 ^{o}C for long term storage.

Polymerase chain reaction (cloning)

Polymerase chain reactions (PCR) were performed using oligonucleotide primers designed according to the requirements for molecular manipulation, sequencing or screening procedures (purchased from Sigma-Genosys, UK). Primer sequences employed for vector engineering and molecular characterisation purposes are described in the appropriate text.

For sub-cloning of DNA fragments generated by PCR amplification from DNA templates, high fidelity AccuPrime™ Pfx proofreading DNA polymerase (Invitrogen, UK) was used.

For routine screening for the presence of specific DNA sequences by PCR, PCR SuperMix (Invitrogen, UK) was used. Primers were dissolved in de-ionised, nuclease-free water at 10 µM; 0.8-1 µL of each of the forward and reverse primers were used per PCR reaction. Reactions were carried out according to recommended protocols provided by the suppliers of enzymes and reagents. Where necessary, primer concentrations were varied and a gradient of annealing temperatures was run to determine optimal reaction conditions. PCRs were performed using a PTC-225 Peltier Thermal Cycler DNA Engine Tetrad (MJ Research, USA).

Agarose gel electrophoresis

Size fractionation of DNA on 1% (w/v) agarose (Invitrogen, UK) gels containing ethidium bromide (Sigma-Aldrich, UK) (0.5 µg/mL) in 0.5 x TAE buffer (4.84 g Tris-base, 1.09 g glacial acetic acid, 0.29 g EDTA in 1 L) enabled visualisation of DNA and size estimation, by relative comparison to commercially available molecular weight markers described above. DNA molecules from purified plasmids or fragments from PCR reactions or restriction digestions were subjected to gel electrophoresis (10 volts per cm gel length, 30-120 minutes) in horizontal DNA electrophoresis gel tanks (Bio-Rad Laboratories Ltd, UK). DNA bands were visualised using a gel and fluorescent imaging system (AlphaImager, Alpha Innotech Corporation, USA) under ultraviolet light.

DNA precipitation and purification

In order to obtain high purity, contaminant free, high yield DNA for both transfection or hydrodynamic injection it was necessary to pellet DNA using either ethanol or

isopropanol. This protocol was used for direct purification of DNA from large restriction digestion volumes or from eluted DNA after plasmid maxi-prep. A volume of isopropanol or ethanol equivalent to or exceeding 1.5 volumes of the DNA solution was added to the DNA, mixed thoroughly and centrifuged at >15,000 x g for 30 minutes at 4 ºC. The supernatant was discarded using a fine glass pipette and the DNA pellet was air-tried thoroughly and re-suspended in either nuclease free water or TE buffer (10 mM Tris, pH7.5, 1 mM EDTA).

Double stranded deoxyribonucleic acid quantitation

DNA was quantified using a Nanodrop (Thermo Scientific, UK) spectrophotometer. Prior to analysis equipment was blanked using distilled water and then background readings were performed using the solution in which the DNA was dissolved (TE, EB or H_2O). Samples were tested in triplicate and data was interpreted using the ND-1000v 3.1.0 software according to the manufacturer's instructions. The final quantity of DNA was expressed as nanograms/microlitre.

Luciferase plasmid expression assays in vitro

Cells were seeded in triplicate in 12 well plates. After 24 h plasmid DNA (0.5 µg) was added to 50 µL of HBS buffer and mixed with 2.5 µL DOTAP reagent (Roche) also in 50 µL sterile HBS. The complex was incubated at room temperature for 30 min. 100 µL of transfection mixture was added to each well and incubated at 37 °C for 4 h. Cells were washed with PBS and incubated with DMEM containing 2 % fetal calf serum (FCS)

(PAA Laboratories, Yeovil, UK). 24 h following transfection media was removed and 150 µL of reporter cell lysis buffer (Promega) was added to the cells. Cells were then frozen at -80°C for 1 h before thawing. Luciferin (25 µL) (Promega, Southampton, UK) was added to 25 µL aliquots of cell lysate and relative luminescence was measured by luminometry (Lumat LB9507, Berthold Technologies, Redbourn, UK).

Assessment of hepatic luciferase expression in mice by hydrodynamic injection

Plasmids or cells were administered by hydrodynamic injection in a volume of PBS equivalent in volume to 10% of the body weight of each mouse (typically 2 ml) over 5–10 seconds with a 27 gauge needle into the superficial tail veins of mice under isofluorane anesthetic. Non-invasive measurement of luminescence was performed at multiple time points using an IVIS 100 system (Xenogen, MA) under isofluorane anaesthetic.

In vivo animal imaging

In vivo virus activity was assessed by non-invasive luciferase imaging using an IVIS 100 system (Xenogen, MA). D-Luciferin (potassium salt) (Gold Biotechnology Inc) was prepared in PBS at 15.8 mg/mL. 100 µl Luciferin was administered via intra-peritoneal injection and mice were imaged 4 minutes later. Flux levels were analyzed with Living Image Software (Xenogen, MA).

Calcium phosphate transfection for adenovirus recovery

SwaI linearised adenoviral plasmids containing either recombinant adenoviral genomic DNA or the wild-type adenovirus genome were transfected into A549 lung carcinoma cells using calcium phosphate transfection. A549 cells were seeded in wells of a 6-well plate 24 h prior to transfection so that they were 70-80% confluent at the time of transfection. Calcium Chloride (0.5 M, 250 µL) was mixed with DNA solution (20 µg in 250 µL sterile water) before being added drop-wise to 500 µL of 2 x HEPES buffered saline (HBS) (sigma, UK) while vortexing the tube containing HBS. The resulting transfection mixture (sufficient for the transfection of four 6-well plates) was allowed to stand for 1 minute before it was added drop-wise onto A549 monolayers which were growing in DMEM with 10% FBS (PAA Laboratories, Yeovil, UK) including penicillin (25 U/mL) and streptomycin (10 µg/mL) (Sigma Aldrich, UK).

The culture media containing the calcium phosphate transfection complex was removed at 24h post-transfection, transfected A549 cells were washed gently with PBS and collected by gentle pipetting – at this stage the transfected cells were not highly adherent and were amenable to removal without trypsinisation. Fresh DMEM media containing 2% FCS was added to each well seven days after plating; cytopathic effects (CPE) were observed in wells containing successfully transfected cells between 12 and 15 days post-transfection. Virus stocks were serially 10-fold diluted into 96 well plates. Single clones were picked and amplified in 10 cm dishes from which infectious supernatants were collected and stored as seed stock for further amplification and virus production.

Virus production and purification by banding on caesium chloride gradients

Single virus clones were amplified in A549 cells cultured in DMEM media containing 5% FCS, using approximately 15-25 confluent 175 cm^2 monolayers for each purification. A549 monolayers containing virus-packed cells were harvested (72 hours post-infection, when CPE was observable but infected cells were not lysed) by gentle agitation and pelleted cells were re-suspended in infectious supernatant (16 mL, volume accommodated in three banding columns), then lysed by three freeze-thaw cycles to release virus particles. The mixture containing lysed cells and free virus particles was centrifuged at 245 g (10 min, 4 ºC). The mixture was incubated on ice for 60 min, followed by centrifugation at 800 g (10 min, 4 ºC). The pellet was discarded and the supernatant (containing virus particles) was loaded onto centrifuge tubes (Ultra-clear™ Beckman Centrifuge tubes, Beckman Coulter UK Ltd) containing the Caesium Chloride (CsCl) gradient as illustrated in Figure 2.1. The gradients were centrifuged (25,000 rpm, 10 ºC, 120 min without deceleration using a Beckman L8-70M Ultracentrifuge with rotor type SW40 TI) (Beckman Coulter, Inc, USA). Two discrete bands were obtained after centrifugation – a faint band higher in the column containing 'empty' viral particles, and a thicker, opaque lower band containing intact infectious viral particles (Figure 2.2). The virus was harvested by puncturing the tube below the level of the virus band with an 18-gauge needle and extracting the desired band into a syringe.

CsCl was subsequently removed by consecutive dialyses in buffers (500 mL, 4 ºC) containing 50 mM HEPES, 1x PBS, 0.1 g/L $CaCl_2$, 0.2 g/L (initial dialysis) or 0.1 g/L (final dialysis) $MgCl_2$ and 10% glycerol at pH 7.8, using a 3-15 mL dialysis cassette for the initial dialysis (Pierce Slide-A-Lyzer® Dialysis Cassette, Pierce Biotechnology, Inc.,

USA) after which the virus was recovered and treated with Benzonase DNA nuclease (6 µL/mL, Novagen, UK) for 30 minutes at room temperature. The virus was subsequently re-banded by CsCl gradient centrifugation and dialysed overnight in the final dialysis buffer using a 0.5-3 mL cassette (Pierce Slide-A-Lyzer® Dialysis Cassette, Pierce Biotechnology, Inc., USA). For each dialysis the buffer was changed and replaced with fresh buffer after 1 hour and 2 hours, followed by an overnight dialysis. Virus obtained after the final dialysis was aliquoted and stored immediately at -80 ºC.

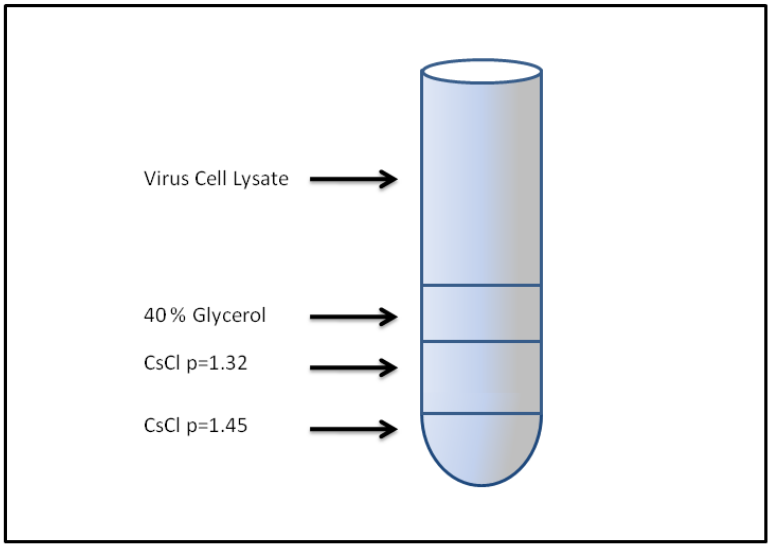


Figure 2.1: Caesium chloride gradient column for banding and purification of adenovirus. The gradient consists of 3 mL of CsCl ρ = 1.32 (32.0g CsCl, 6.8 mL of 0.5 M Tris-HCl at pH 7.9, 61.2 mL dH2O), underlayed with 2 mL of CsCl ρ = 1.45 (20.5g CsCl, 2.9 mL 0.5 M Tris-HCl at pH 7.2, 28.5 mL dH2O). Finally 2 mL of 40 % glycerol (40 g glycerol, 2 mL of 0.5 M Tris-HCl at pH 7.9, 0.5 mL of 0.2 M EDTA at pH 8.0, made up to 100 mL using dH2O) was placed on top of the 1.32 CsCl layer.

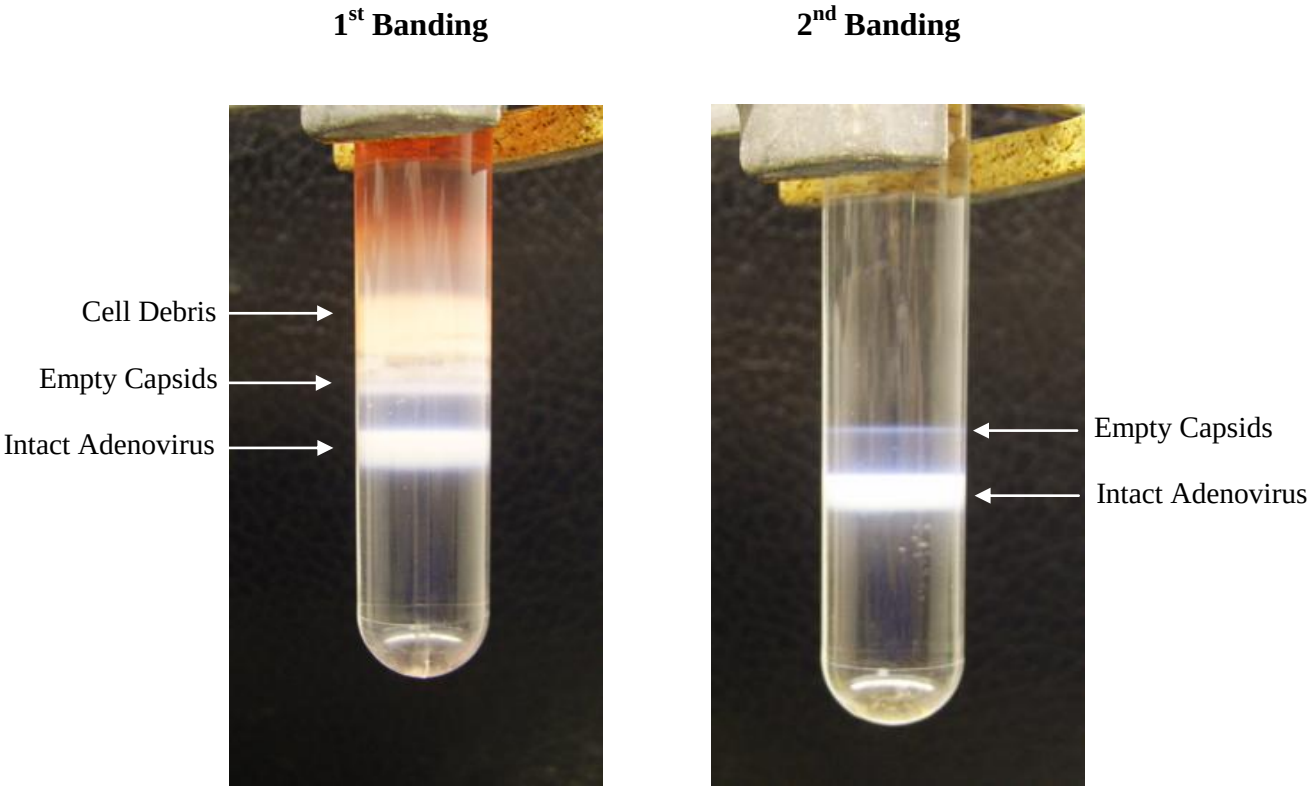


Figure 2.2: CsCl banding of virus preparations. The left image shows a tube following the initial banding of a crude adenovirus preparation. Unwanted cell protein and membranes are clearly visible above the two bands of virus as a flocculated diffuse band. The lower, larger, band corresponds to intact virus particles containing DNA. The middle band correlates with empty viral capsids. The right image shows the same sample after dialysis and re-banding. The faint upper band shows empty capsids whist the larger, lower, band shows intact virus particles. Intact virus bands are extracted using an 18 gauge needle from below to minimize empty capsid contamination. Pictures kindly provided by Sam Illingworth (Psioxus Therapeutics, Oxford).

Double-stranded DNA measurement for adenovirus quantitation

Adenoviral DNA concentrations were determined using the PicoGreen assay (Quant-iT™ PicoGreen ® dsDNA Reagent, Molecular Probes, Invitrogen). The assay contains a fluorescent nucleic acid probe for double-stranded DNA (dsDNA), allowing quantification of adenoviral genome content. Appropriate dilutions of virus stock solutions (10 to 100-fold) were made and were transferred (15 µL) to a solution containing 1 x TE buffer (255 µL) and 0.5 % Sodium Dodecyl Sulphate (SDS) in 1 x TE (30 µL). Samples were incubated at 56 ºC (30 min) to disrupt virus particles. Six four-fold serial dilutions of known concentrations of the bacteriophage lambda DNA provided in the Quant-iT™ PicoGreen® Kit were carried out (highest concentration at 1 µg/mL), allowing the construction of a standard curve (last standard blank) from which the DNA content in unknown samples were calculated. The PicoGreen reagent was diluted 200-fold in 1 x TE buffer. 100 µL of the diluted reagent was placed in wells of a black 96-well plate (Corning, UK) for each standard or unknown sample. 50 µL of appropriately diluted standards and samples were added to the wells in duplicate. The plate was read in a Wallac 1420 Victor^2 multi-label counter, using the 'Fluorescein 485/535 nm, 1 second' program for analysis. The number of viral particles present in each sample was calculated on the basis that 1 µg of DNA is approximately equal to 2.7 x 10^{10} adenoviral particles.

Calculation of plaque forming units of adenoviral preparations using the tissue culture infectious dose 50 (TCID50) method

The tissue culture infectious dose 50 (TCID50) method (Karber 1931) was used to estimate the number of infectious virus particles, or plaque forming units (pfu), and is

based on the development of cytopathetic effect (CPE) in A549 cells after infection with 10-fold serially diluted samples of virus preparation. This method is described in full in the manual for the AdEasy non-replicating virus platform available from QBiogene (France).

Adenovirus preparations

All adenoviruses were grown in A549 cells, purified by double banding in CsCl gradients as described above. Viral particle (vp) number was determined by measuring DNA content using a modified version of the PicoGreen assay (Invitrogen, Paisley, UK) (Mittereder *et al.* 1996). Infectivity was calculated using the $TCID_{50}$ system with the KÄRBER statistical method (Karber 1931) and was used to estimate the adenovirus titer ($TCID_{50}$ units/mL) and corrected to determine plaque forming units/mL (pfu/mL). Adenovirus preparation characteristics are as follows: Ad5 WT: 1.13×10^{12} vp/mL, 1.98×10^{11} pfu/mL and particle:infectivity (P:I) ratio of 5.6; Ad5-mir122: 1.29×10^{12} vp/mL, 2.01×10^{11} pfu/mL and particle:infectivity (P:I) ratio of 6.4.

Maintenance of cell lines

HT29-Luc and Lovo-Luc colorectal cell lines were obtained from Caliper Life Sciences (Runcorn, UK). Human hepatocellular carcinoma Huh7, HepG2 and Hep3b cell lines, A549 lung carcinoma cells, OVCAR-3 ovarian cancer cells and HEK293 human embryonic kidney cells were obtained from the European Collection of Cell Cultures (Porton Down, UK), and maintained in DMEM media with 10 % FCS (PAA Laboratories,

Yeovil, UK) including penicillin (25 U/mL) and streptomycin (10 mg/mL) at 37 ºC in 5 % CO2 in a humidified incubator.

Virus luciferase in vitro assays

Cells were seeded in triplicate in 48 well plates at $2.5x10^4$ cells/well. Virus infections were performed at 10 vp/cell. 24 h following infection the media was removed and 150 µl reporter cell lysis buffer (1x) (Promega) was added. Cells were frozen at -80 ºC for 1 hr before thawing. Luciferin (25 µl) (Promega, Southampton, UK) was added to 25 µl of cell lysate and relative luminescence was measured by luminometry (Lumat LB 9507, Berthold Technologies, Redbourn, UK).

MTS assays

Cell viability was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). This is a colourmetric assay that measures cytoxicity by quantifying the number of viable living cells in a sample. The reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling agent (phenazine ethosulphate; PES). The MTS tetrazolium compound is reduced in living cells to a formazan coloured compound that is soluble in tissue culture media.

Cells were seeded at $1x10^4$ /well in 96 well plates in 100 µl media. After 24 hours, viruses were added at 10 and 100 vp/cell in 100 µl media. Cells were left for either 3 or 6 days. At each time point media was removed and 20 µl MTS reagent in 180 µl media was added

and cells were incubated for 30 minutes. Plates were read at 490 nm for 0.1 seconds. Blank wells containing MTS reagent and media but no cells served as background and non-infected cells represent 100 % survival in each cell type. N=5 for each cell type.

Pre-mir 122 transfection

A549 cells were seeded at $5x10^4$ cells per well and incubated overnight. Pre-mir122 (Ambion) and pre-mir negative control (Ambion) were re-suspended to 50 µM and then further diluted 10-fold. 3 µL per well of this dilution of each pre-mir was added to 22 µL Opti-MEM medium (Invitrogen). 2 µL per well of NeoFx transfection reagent (Ambion) was added to 23 µL Opti-MEM solution. Pre-mir/Opti-MEM was mixed with the NeoFx/Opti-MEM and allowed to complex for 10 minutes. A549 cells were washed with PBS and the transfection mixture added to cells at a total volume of 50 µL. Total amount of pre-mir is 15 pmol/well. Immediately following transfection Ad5-mir122-Luc was added at 10 vp/cell in 450 µL DMEM media (10% FCS). 18 h later, 30 pmol/well of pre-cursor mir122 and negative control pre-cursor microRNAs were added to each well in addition to the 500 µL already added. Luciferase levels were determined at 24 h post-initial transfection.

Primary hepatocyte culture

Frozen Human primary hepatocytes (NHeps, CC-2591)), Hepatocyte Basal medium (HBM™, CC-3199), supplements and growth factors HCM™ SingleQuots (CC-4182) were purchased from Lonza (Basel, Switzerland). Cells were seeded and manipulated

according to Lonza's protocol. A 96 well plate was coated with rat tail collagen type 1 (Sigma, C3867) at 60 µg/cm^2. Cells were thawed at 37ºC in a water bath, centrifuged at 4 ºC for 3 minutes at 50 g and then washed in cold culture medium. Cells were re-suspended and the number and viability was confirmed using the Trypan blue dye exclusion method. The viability obtained was 60 % at seeding. Cells were seeded at 5x10^4 /well with 120 µl medium each well. The medium was changed at 3 hours after seeding. Virus infections were performed 24 h after seeding in 100 µl fresh medium at 1 vp/cell, another 100 µl medium was added at 2 h post-infection. 100 µl of media was exchanged each day and until experiment completion (72 h). The supernatant was removed by aspiration and cells were lysed in 100 µl Promega reporter lysis buffer (E397A) and frozen at -80 ºC before luciferase quantitation.

Endotoxin characterisation

The endotoxin content of adenovirus preparations was determined using a QCL-1000 chromogenic end point assay (Lonza, UK). All assays were conducted based on the manufacturer's instructions. Briefly, serial dilutions of virus stock were added to a 96 well plate in conjunction with an endotoxin standard curve with a maximum reading of 1.8 endotoxin units/ml. 50 µL reagent was added to each well and the colour was allowed to develop for 15 minutes. Plates were read at 405 nm in a spectrophotometer and the endotoxin content of individual adenovirus preparations was calculated based on standard curve values.

Evaluation of the activity of adenoviruses containing microRNA binding sites in vivo

Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. It was encapsulated in liposomes as described by Van Rooijen *et al* (Vanrooijen *et al.* 1994). Viruses were administered intravenously (unless otherwise indicated) and all animals were pre-treated with bisphosphonate liposomes (100 µL/mouse, obtained from Dr Nico Van Rooijen) 24 h before.

Human and viral genomic DNA extraction

Where determination of adenoviral genome numbers were necessary, viral DNA was extracted from culture supernatants or cell lysates prepared in either lysis solution C (Sigma, UK) or lysis solution from the luciferase assay system (Promega, UK). Samples were treated with proteinase K (1 mg/mL) and incubated at 56 ºC for 20 minutes to degrade viral capsid proteins, followed by a 20 minute incubation at 70 ºC. Total cellular DNA extraction was then performed using the Genelute mammalian DNA extraction kit (Sigma-Aldrich, UK) according to the manufacturer's instructions.

Real time (quantitative) PCR (Q–PCR) for Ad5

The Q-PCR methodology for measurement of adenoviral particles has been previously described (Green *et al.* 2004). Briefly, viral DNA from infected cells or tissue samples was extracted using a mammalian genomic Genelute DNA extraction kit (Sigma). Reactions were performed using Applied Biosystems master mix following the manufacturer's protocol. The cycles were as follows: 94 °C 10 min then 40 times at 94 °C

30 s, 60 °C 1 min. Primers sequences for targeting Ad5 fiber are: Forward primer – 5' TGG CTG TTA AAG GCA GTT TGG 3' (Ad5 32350–32370 nucleotides) and reverse primer - 5' GCA CTC CAT TTT CGT CAA ATC TT 3' (Ad5 32433–32411 nt) and the TaqMan probe- 5' TCC AAT ATC TGG AAC AGT TCA AAG TGC TCA TCT 3' (Ad5 32372–32404 nt), dual labeled at the 5' end with 6-carboxyfluorescein and the 3' end with 6-carboxytetramethylrhodamine. The results were analyzed with the Sequence Detection System software (Applied Biosystems). Standard curves for tissues and cells were prepared by spiking samples of cell lysate or tissue homogenate with serial dilutions of known concentrations of virus particles followed by extraction and analysis of each sample separately by Q-PCR as described above.

Measurement of serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) levels

50 µl of blood was taken from mice by tail bleed and allowed to clot (15 min, room temperature) and spun at 1200 g for 10 min. Serum was isolated and immediately frozen at -20 °C. Samples of thawed serum (5 µl) were added to ALT reagent (995 µl, Microgenics) in a 1 mL quartz cuvette, incubated at 37 °C and the change in absorbance (340 nm) per minute was measured. Units of ALT per litre were calculated according to the manufacturer's instructions using the following equation: Activity in Units/Litre = Δ Absorbance change per minute x factor (factor = total reaction volume x 1000 / 6.3 x sample volume x path length).

Liver histology

The left liver lobe from each mouse was immersed in 10% buffered formalin overnight at room temperature, embedded in wax and sectioned using a vibratome. Sections were stained with haematoxylin and eosin and analysed by light microscopy at X40 magnification.

Western blots

Protein was extracted from murine liver by homogenisation in Promega lysis solution at 80 mg/mL wet weight. Samples were spun at 2000 rpm for 5 minutes at 4 °C to remove cell debris. 30 µg of liver protein was loaded onto a 10% polyacrylamide gel after quantitation using a QuantiPro BCA assay (Sigma Aldrich, cat: QPBCA-1KT). The gel was run at 160 V for 1 h and then protein was blotted to a nitrocellulose membrane overnight at 4 ºC at 30V. Nitrocellulose membrane was stained with Ponceau solution (described below) to confirm equal loading and transfer and then blocked using 5 % milk powder (Fluka, Sigma Aldrich) for 2 h (E1A) or overnight (Aldolase A). Membrane was washed twice with PBS 0.1 % Tween 20 and then once with PBS. For the E1A western blot, primary antibody recognising E1A (AbCam, Cambridge, UK, cat number: Ab33183) was added at 1:500 dilution in 2.5 % milk powder in PBS for 1 hr. Membrane was washed as above. Secondary anti-rabbit HRP labelled antibody was added at 1:1000 dilution in 2.5 % milk powder for 1 h. For the Aldolase A western blot, primary antibody recognising Aldolase A (Sigma-Aldrich, cat number: AV48130) was added at 1:1000 dilution in 2.5 % milk powder in PBS for 1 h. Membrane was washed as above. Secondary antibody was

the same as used for Adenovirus E1A western blot but used at a 1:4000 dilution for 1 h. The membranes were washed as above and then bathed in ECL western blotting detection reagent (Amersham, GE Healthcare) at 0.125 mL/cm^2 for 1 minute. Blot was visualised in an Alpha Innotech gel documentation system for 1, 5 and 10 minutes using chemi-luminescence detection. Molecular weights were calculated against a dual colour molecular weight ladder (Bio-Rad).

Ponceau staining

To confirm successful transfer of protein from poly-acrylamide gels onto nitrocellulose membranes and to ensure equal loading and transfer, membranes were stained for 2 minutes with Ponceau S solution (0.1 % w/v Ponceau S in 5 % v/v Acetic Acid made up with de-ionised distilled H_2O) prior to blocking. The stain was poured off and membranes were washed briefly in PBS twice before imaging. Membranes were then washed twice with PBS 0.1 % Tween 20 and then once with PBS to remove all residual stain prior to blocking.

RNA extraction and reverse-transcription quantitative PCR for E1A mRNA

Total RNA from murine livers was extracted using mirVana miRNA isolation kit (Ambion) without the miRNA enrichment step. Reactions were performed using TaqMan RNA-to-C_T 1-step kit (Applied Biosystems), following the manufacturers protocol. PCR cycles were as follows: 95 ^oC 10 minutes, then 40 cycles at 95 ^oC 15 seconds, 60 ^oC 1 minute. Primer sequences for targeting E1A 13S were: Forward primer – 5’ ATG TTT

GTC TAC AGT CCT GTG TCT GAA 3', Reverse primer – 5' GAT AGC AGG CGC CAT TTT AGG 3' and probe – 5' CCA GAA CCG GAG CCT GCA AGA CCT AC 3' (Sigma-Aldrich), dual labeled at the 5' end with 6-carboxyfluorescein and the 3' end with 6-carboxytetramethylrhodamine. Results were analysed with StepOne Plus Real-Time PCR Systems software (Applied Biosystems). Standard curves were made by serial dilutions of known quantities of E1A DNA using an Ad5 E1A encoding plasmid and assumes 100 % extraction efficiency.

MicroRNA quantitation

RNA was extracted from murine liver as described above and was diluted to 1 ng/µl in nuclease free water. Mature mir122 levels were quantified using a TaqMan MicroRNA Assay (Applied Biosystems) specific for mir122 (Part number: 4427975 Assay ID: 002130). Reverse transcription (RT) reactions (15µl) were set up according to manufacturer's guidelines using Multiscribe Reverse Transcriptase (50U/reaction), dNTPs (1mM final concentration), 0.188 µl RNase inhibitor, 1.5µl 10X RT buffer, 3 µl 5X mir122 TaqMan MicroRNA RT primer and 5 µl RNA sample (1 ng/µl). Reaction conditions were 30 minutes 16 ºC, 30 minutes 42 ºC, 5 minutes 4 ºC. cDNA (1.33 µl of RT reaction) was added to 1 µl 20 x mir122 Taqman MicroRNA assay, 10 µl TaqMan 2X Universal PCR master mix and made up to 20 µl using nuclease free water. Real-time PCR conditions were 10 minutes at 95 ºC, followed by 40 cycles of 15 seconds at 95 ºC then 1 minute at 60 ºC. Samples were run on a Step One Plus real-time PCR system (Applied Biosystems). The microRNA let7a was used as an internal standard using the conditions described above and using the TaqMan MicroRNA Assay (Applied

Biosystems) specific for mature let7a RNA (Part number: 4427975 Assay ID: 000377). Relative standard curves were produced by ten-fold serial dilutions of liver RNA extracted from a mouse receiving PBS.

Reaction properties were as follows; mir122 assay: slope 3.28, 101.78 %, efficiency 2.02 and Let 7a assay slope 3.27, 102.29 %, efficiency 2.01. Relative expressions were calculated using the method published by Pfaffl, M (Pfaffl 2001). Probes were labeled at the 5' end with 6-carboxyfluorescein and at the 3' end with a non-fluorescent quencher coupled to a minor groove binder (MGB).

mRNA microarray analysis

RNA was extracted from murine liver as described above. Each group contains three mice and independent chips were used for each sample. Whole-genome gene expression analysis was performed using Illumina Single Colour Mouse WG-6_V2_0_R0_11278593_A BeadChip with direct hybridization assay (Illumina, Essex, UK) according to the manufacturer's directions starting with 300 ng total RNA from each sample. Biotinylated cRNA was prepared using the Illumina TotalPrep-96 RNA Amplification Kit (# 4393543 Ambion, Inc., Austin, TX). This briefly includes a first- and second-strand reverse transcription step, followed by a single *in vitro* transcription (IVT) amplification that incorporates biotin-labelled nucleotides. Subsequent steps include array hybridization, washing, blocking, and streptavidin-Cy3 staining. Fluorescence emissions by Cy3 were quantitatively detected by BeadArray Scanner for downstream analysis. GenomeStudio Data Analysis Software was used to visualize and analyze data generated. This software provides results in standard file formats that can be readily processed with

most commercial expression-analysis software programs. Data was imported to GeneSpring GX 11.0.2 (Agilent Technologies, Inc, Santa Clara, CA) normalized with Shift to 75 percentile and baseline transformed to median of all samples for detailed analysis to identify significantly differentially expressed genes. The data generated is MIAME compliant and has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and is accessible through GEO Series accession number GSE23854 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE23854)

Advanced protein assay

Samples obtained from tissue culture lysate or primary tissue homogenate were standardised against protein content to ensure equal loading of samples in poly-acrylamide gels and to correct luciferase readings for direct comparison. Typically, 1-10 µL of lysate was added to a 96 well plate followed by the addition of 250 µL of Advanced BCA solution (Sigma Aldrich, UK) which was produced following the manufacturer's instructions. Standard curves were constructed using 2-fold dilutions of bovine serum albumin (BSA). The highest standard was 2 mg/ml which is within the linear range of the assay. Background levels were determined using Advanced BCA reagent alone in independent wells. Colour was allowed to develop for at least 5 minutes but not exceeding 60 minutes and samples were read at 595 nm in a spectrophotometer. Readings were then correlated against the standard curve and corrected against the original quantity loaded to calculate protein content as µg/ml.

Animal experimentation ethics statement

All animal experimentation was performed in accordance with the terms of the UK Home Office guidelines and the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia. The home office project license number under which these experiments were conducted is PPL 30/2333. All experiments were conducted with approval of the Medical Sciences Animal Ethics Committee, University of Oxford.

Statistical analyses

In vitro and *in vivo* data are expressed as the mean of three, four or five replicates $\pm$ standard deviation unless otherwise stated. Significance was evaluated using a student's two-tailed t-test for comparative analysis of experiments producing two datasets. For analysis of data in which more than two means are to be compared an analysis of variance (ANOVA) method was used to limit the chances of observing a type one statistical error. Significances are denoted on the graphs as * = $P \leq 0.05$ ** = $P \leq 0.005$ or *** = $P \leq 0.0005$.

Chapter Three: Molecular engineering of mir122 controlled plasmids and preliminary characterisation of mir122 activity

3.1. Introduction:

MicroRNA regulation

The degree to which a microRNA can influence the stability of an mRNA depends on multiple factors. These include the level of homology between the mature microRNA and the microRNA binding site, the position of the binding site within the mRNA strand, the number of microRNA binding sites and the abundance and intrinsic repression capability of the microRNA itself. To establish the effectiveness of mir122 at controlling gene expression a series of plasmids was engineered to assess the number of binding sites required to mediate a significant reduction in protein production. These vectors also served to determine if mir122 was functionally active against artificially introduced microRNA regulated mRNA targets under the control of strong viral promoters.

Plasmids were developed which contained either zero, four or eight perfectly complementary binding sites for microRNA mir122 in the 3' un-translated region of luciferase under the control of the strong CMV immediate early promoter. A vector was also generated containing four anti-sense microRNA binding sites to control for the influence of the DNA insertion into the 3' UTR. Further plasmids were developed which exploited the use of the less transcriptionally active E1A viral promoter to drive luciferase expression where luciferase was fused to the luciferase coding sequence. This approach had not previously been attempted and it was undetermined whether this technique would yield either functional luciferase and/or E1A. All of these vectors were assessed using both *in vitro* and *in vivo* assays and models to determine if mir122 was capable of reducing luciferase expression in the presence of substantial mRNA target.

Primary experimental aims:

1. Engineer plasmid DNA vectors to quantify the capacity of mir122 to knockdown mRNA translation.

2. Determine if the number of microRNA binding sites inserted into the 3' UTR influences the level of microRNA mediated translation inhibition.

3. Assess the capacity of microRNA mir122 to suppress mRNA translation *in vitro* by plasmid transfection and *in vivo* by hydrodynamic injection of plasmid DNA.

3.2. Results and discussion:

Engineering of microRNA regulated luciferase reporter plasmids

Luciferase reporter plasmids sensitive to mir122 were engineered by introducing concatamers of binding sites for mir122 (four or eight sense or four antisense binding sites) into the 3'UTR of the luciferase transcription cassette. A CMV-driven, pGL3 based, luciferase-expressing plasmid vector pCIKLux (a kind gift from Dr Deborah Gill and referred to as pCMV-Luc) was cleaved with NotI. Oligonucleotides encoding perfectly complementary binding sites for microRNA mir122 were annealed at 95 °C for 5 minutes in 1X restriction buffer 3 (New England Biolabs) and then cooled and ligated into the dephosphorylated vector. An Xba1 site was included within the oligonucleotides for directional screening with Xba1 and BamH1 restriction enzymes.

The insertion of mir122 binding sites also re-constituted the Not1 sites at either end of the insert. Screening of pCMV-Luc recombinants with Not1 and Xba1/BamH1 following ligation produced plasmids containing four and eight mir122 binding sites (Figure 3.1). This produced vectors pCMV-Luc-mir (shown in Figure 3.2) and pCMV-Luc-mir122X8. pCMV-Luc-mir was cleaved with Not1 to excise the four microRNA binding sites and re-ligated to its own insert to produce a vector with four anti-sense microRNA binding sites. This vector was termed pCMV-Luc-mir122-anti. The insertion of microRNA mir122 binding sites was confirmed by DNA sequencing using the forward primer 5'AAG AAA AAT CAG AGA GAT CCT CAT AAA G 3' and the reverse primer 5'AAA TAA AGC ATT TTT TTC ACT GCA TTC T 3' which bind to regions flanking the microRNA insertion site in pCMV-Luc and all derived plasmids.

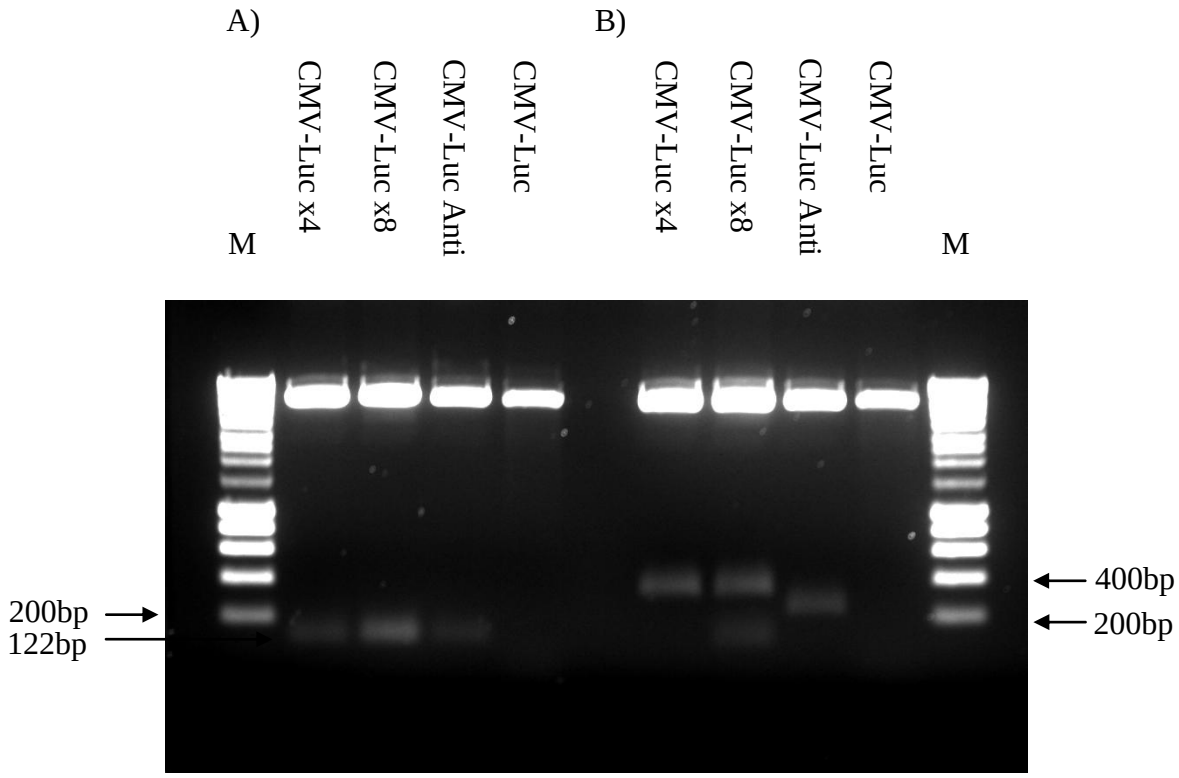


Figure 3.1: Screening of CMV driven luciferase encoding plasmids into which mir122 binding sites have been inserted. A) Plasmids encoding 4, 8, 4 anti-sense and 0 mir122 binding sites were screened with Not1 restriction enzyme. This screen cuts out all binding sites inserted and the presence of a 122 base pair fragment shows insertion. All lanes except the parental vector show this fragment with the plasmid containing eight binding sites producing a band of increased intensity showing multiple inserts. B) Screening of the same plasmids digested in A) with Xba1 and BamH1 restriction enzymes. Mir122 sites inserted in the correct orientation produces a fragment of 355 base pairs. The same fragment inserted in the incorrect orientation produces a fragment of 245 base pairs. No Xba1 site is found in the parental plasmid and so no fragment is produced. The presence of a 122 base pair fragment from CMV-LucX8 shows multiple insertions. All insertions were confirmed by DNA sequencing.

Engineering of plasmids encoding a fusion protein between E1A and luciferase under the E1A promoter to allow luminescent monitoring of microRNA regulation

In order to produce vectors in which the E1A coding region was fused to the luciferase coding region under the E1A promoter, the C-terminal half of E1A was PCR amplified using Accuprime PFX (Invitrogen) and primers (forward ATT ATA AGA TCT GGA TAG CTG TGA CTC CGG TCC TTC, reverse TAT TCC ATG GAT GGC CTG GGG CGT TTAC) using a plasmid containing the wild-type Ad5 genome as a template. PCR conditions were as follows: 95 ºC for 15 s, 68 ºC for 1 min, 30 cycles (Figure 3.3). These primers introduced unique BglII and Nco1 restriction sites to the 5' and 3' termini respectively. The purified PCR product was cleaved with BglII and Nco1 and cloned into pCMV-Luc and pCMV-Luc-mir described above, using the same enzymes, producing a fusion between the C-terminal half of E1A and luciferase, including zero or four microRNA sites in the 3' UTR (Figures 3.3 and 3.4). These products were sub-cloned using PshA1 and Hpa1 into a plasmid pAd5-Kpn1 (produced by restriction digestion of the wild-type Ad5 genome with Kpn1, described in detail in Chapter Four) to produce plasmids (pE1A-Luc and pE1A-Luc-mir, figure 3.2) in which the penultimate codon of E1A was fused to the start codon of the luciferase coding sequence.

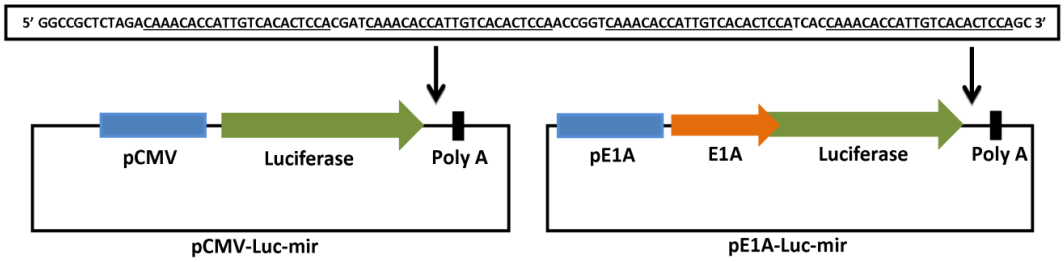


Figure 3.2: MicroRNA regulated plasmid construction. pCIK-Lux (referred to as pCMV-Luc) was cleaved with Not1 and concatamers of mir122 binding sites (four or eight sense, or four antisense; the sequence of the four sense insert is shown at the top of the figure) were inserted into the luciferase 3'UTR. Both pCMV-Luc and the version containing four microRNA sites (pCMV-Luc-mir) were modified to contain the C-terminal half of the E1A expression cassette, isolated from pAd5WT (Ad5 wild type) by PCR. Both resulting constructs were then cloned into pAd5Kpn1, which contains the E1A promoter and remaining coding sequence, to produce E1A promoter regulated E1A-luciferase fusion constructs termed pE1A-Luc and pE1A-Luc-mir.

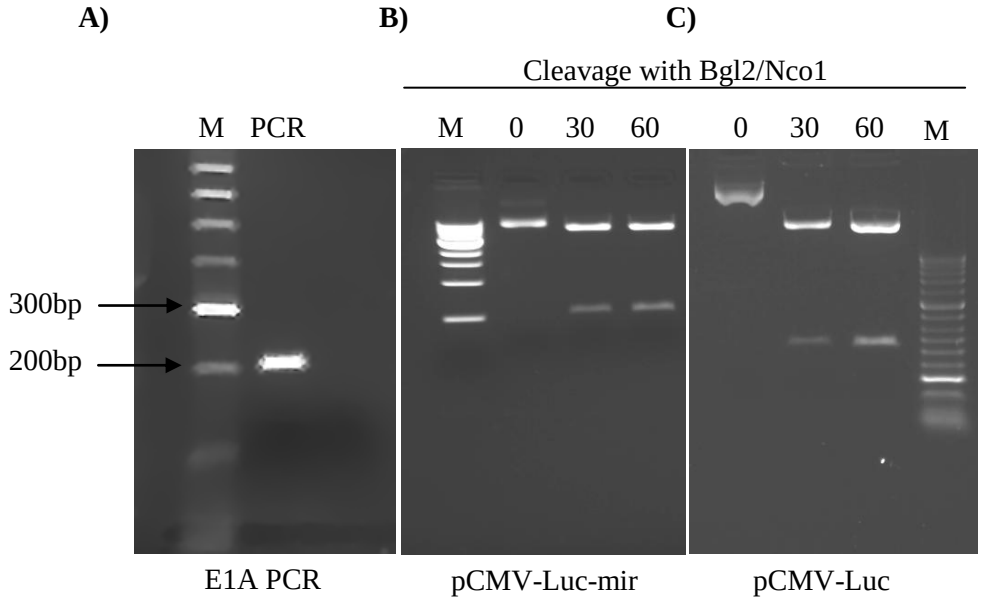


Figure 3.3: Fusion of the coding sequence of E1A and luciferase. A) The final 190 base pairs of the E1A coding region was PCR amplified to add compatible cohesive ends. The PCR product was run on a gel (shown here), purified and cleaved with Bgl2 and Nco1 restriction enzymes. The PCR product before cleavage was 204 base pairs in size and the primer sequences are described within the text above. B & C) Cleavage of pCMV-Luc-mir and pCMV-Luc with Bgl2 and Nco1 restriction enzymes. Restriction digest length in minutes is expressed above each image (0, 30, 60). Both vectors are successfully cut by both enzymes to release 500 base pair fragments. M= DNA ladder/marker.

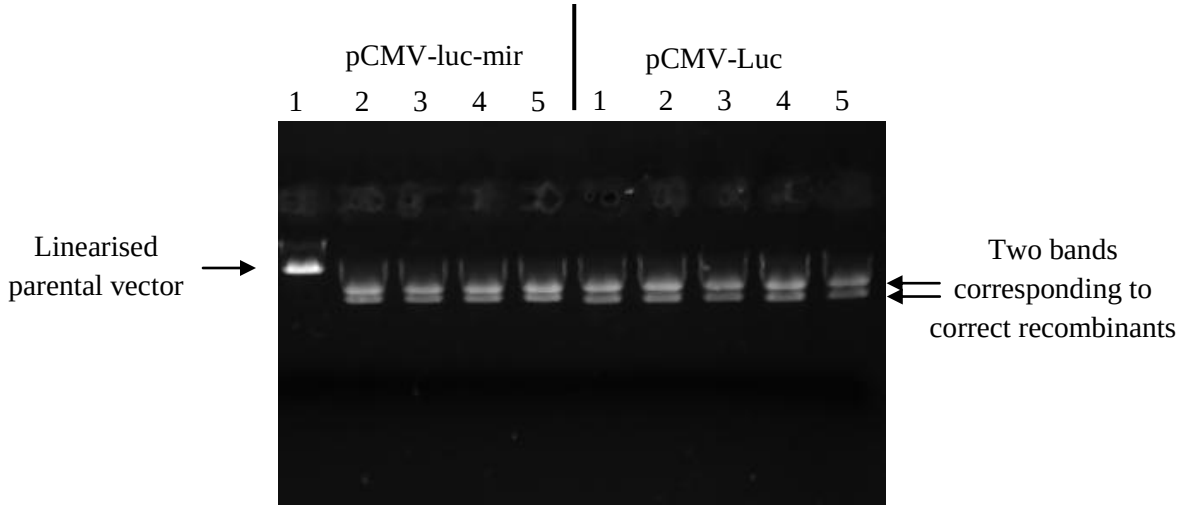


Figure 3.4: Screening for E1A luciferase fusion plasmids. Recombinant plasmids derived from the ligation of DNA shown in figure 3.3 were screened using Hpa1 and PshA1. The parental vectors contain one Hpa1 site and no PshA1 sites. Successful recombinants should show the addition of a PshA1 site within the inserted PCR product. All recombinants are correct except for lane one (pCMV-luc-mir) which demonstrates the DNA pattern expected from the parental vector (linearization). Correct plasmids should show two bands of roughly equal size (1993 and 2762 base pairs). The ladder, cropped from the image, showed these band sizes were correct although the presence of two bands also demonstrates correct PCR product insertion. All insertions were confirmed by DNA sequencing.

Evaluation of the potency of microRNA mir122 regulation in vitro by plasmid transfection

To assess the repression capabilities of microRNA mir122, CMV promoter-driven luciferase plasmids containing zero, four and eight sense or four anti-sense microRNA binding sites were transfected into HEK-293, OVCAR-3 and Huh7 cell lines using DOTAP (Roche) and luciferase activity was measured by luminometry after 24 h. The presence of the microRNA binding sites had no effect on luciferase levels detected in the microRNA mir122 negative cell lines HEK-293 and OVCAR-3 (Figure 3.5). In contrast, in microRNA mir122 positive Huh7 cells, luminescence produced from the plasmid encoding four anti-sense microRNA binding sites (control vector) was $7.9x10^5$ RLU/µg whilst the plasmid containing four mir122 binding sites produced a reading $9.9x10^4$ RLU/µg, an eight-fold lower reading. The transfection of a plasmid containing eight mir122 binding sites produced a reading of $3.4x10^4$ RLU/µg (23-fold lower than control). The inclusion of four anti-sense microRNA binding sites did not affect luciferase activity compared to the un-modified control plasmid in any cell type.

Whilst the inclusion of eight microRNA binding sites did show improved repression in comparison to four binding sites, there was concern that the use of eight repetitive sequences within a replication competent virus might be unstable and encourage the loss of microRNA control through deletion and recombination. Therefore, for further work only four binding sites were investigated. However, if at any point four binding sites proved ineffective at controlling gene expression, eight binding sites would be re-considered.

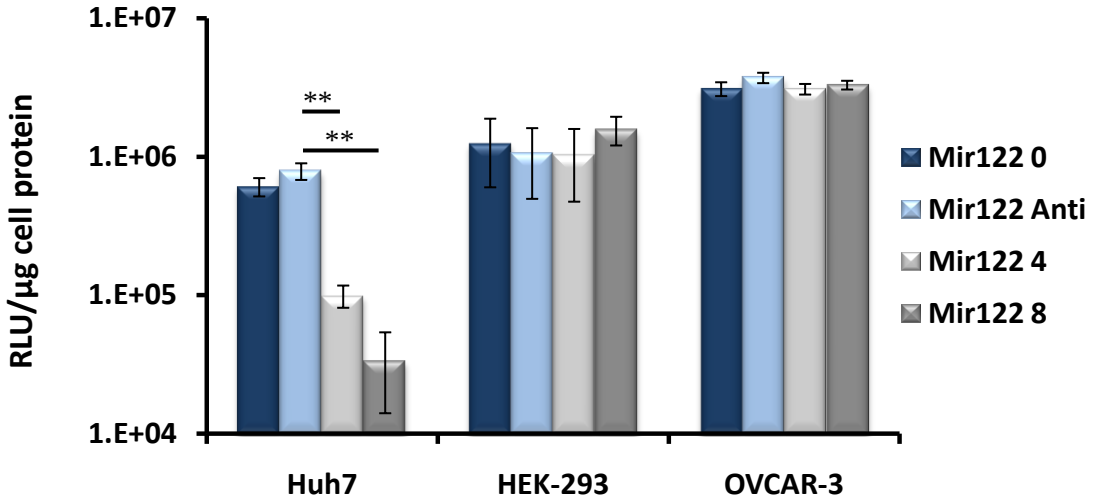
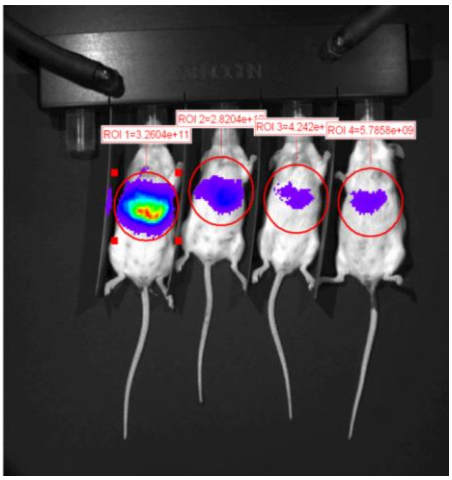


Figure 3.5: Effects of microRNA binding sites on expression of CMV promoter driven luciferase plasmids *in vitro*. Cells were seeded in triplicate in 12 well plates. After 24 h 0.5µg of plasmid DNA (containing zero (dark blue), four (light grey) or eight (dark grey) sense microRNA mir122 binding sites, or four antisense binding sites (light blue)) was mixed with 2.5 µl DOTAP (Roche) reagent. 24 h following transfection cells were lysed and relative luminescence was measured using 25 µl cell lysate. N=3, Error bars +/- standard deviation and data is shown as RLU/µg cell protein, determined by BCA protein assay. (** P<0.005 by ANOVA).

Preliminary evaluation of microRNA mir122 activity in vivo by hydrodynamic injection of plasmid DNA

A pilot study was undertaken to assess the number of animals required to observe a significant microRNA effect on luciferase expression *in vivo*. CMV driven luciferase plasmids containing either zero or four microRNA mir122 binding sites were injected into the superficial veins of Balb/C mice by hydrodynamic delivery (20µg DNA / mouse in 2 ml PBS). Luminescence readings were calculated after eight hours by measuring the emission of light from identically sized regions of interest surrounding the murine liver (Figure 3.6A). These regions are shown in figure 3.6A as red circles in each image. Results showed that a control mouse which was injected with a luciferase plasmid containing no microRNA binding sites produced $3.18x10^{11}$ RLU at eight hours post-injection whilst the average of three mice injected with a CMV driven plasmid containing four microRNA binding sites for mir122 produced a reading of $1.29x10^{10}$ RLU, a 25-fold lower reading (Figure 3.6B).

A)



B)

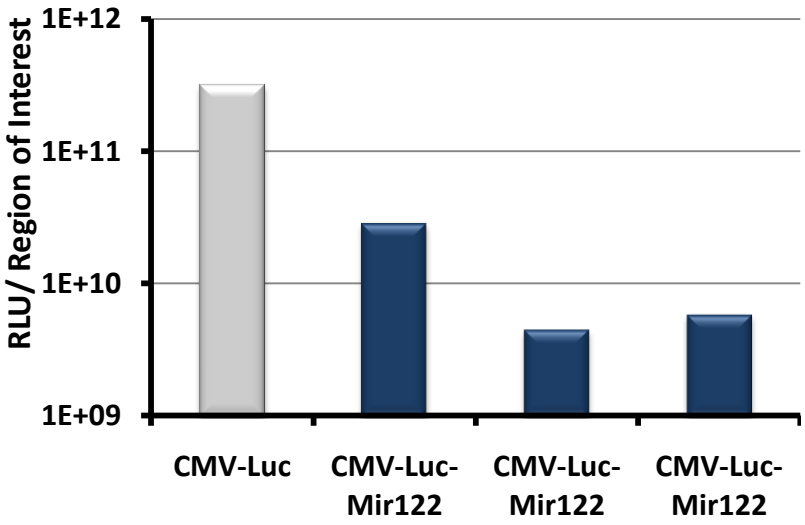


Figure 3.6: Pilot study to assess microRNA control of luciferase expression following hydrodynamic injection of plasmid DNA. Balb/C mice were injected with 20µg of DNA by hydrodynamic injection in 2 ml of PBS per mouse and imaged after eight hours. Four minutes prior to imaging mice were administered luciferin (100 µL, 15.8 mg/ml) via intra-peritoneal injection. A) IVIS luminescent imaging of mice 8 hours-post DNA delivery. The mouse on the left received an un-regulated CMV driven luciferase plasmid whilst the three mice in the right of the image received a CMV driven plasmid containing four mir122 binding sites in the luciferase 3' UTR. Regions of interest are shown as red circles and were used to calculate flux levels. B) Luminescence quantitation of mice shown in A). RLU = relative light units.

Evaluation of the potency of microRNA mir122 regulation in vivo

The 25-fold decrease in luciferase expression observed in the pilot study described above (Figure 3.6) suggested that microRNA regulation was capable of significantly reducing mRNA translation. To quantify this effect in more detail, Balb/C mice (N = four per group) were injected with either microRNA regulated or unregulated plasmid vectors in equimolar amounts by hydrodynamic delivery. IVIS luminescence imaging was performed at 8, 24 and 48 h post injection.

Mice receiving an un-regulated control CMV promoter-driven plasmid gave high levels of transgene expression after 8 h (2.7×10^{11} RLU) while the inclusion of four microRNA binding sites in the same plasmid decreased expression to 5.7×10^{9} RLU, a 47-fold decrease in expression (Figure 3.7). Total levels of luciferase expression fell substantially over the next 40 h, although the differential expression increased up to 129-fold ($p = 0.0064$) 48h post injection.

Plasmids containing the E1A promoter and E1A coding sequence fused to the luciferase coding sequence (described above) were tested to determine if the insertion of four mir122 binding sites into the 3' UTR would reduce protein translation. Following hydrodynamic delivery of equimolar amounts of both vectors to mice, expression from the plasmid producing the E1A-luciferase fusion protein with no microRNA mir122 binding sites was 16-fold lower than from the equivalent un-regulated CMV vector, probably reflecting the weaker activity of the E1A promoter in murine cells. The inclusion of four microRNA sites within this plasmid again mediated a significant decrease in expression (86-fold after 8 h, $p=0.01$) (figures 3.8 and 3.9).

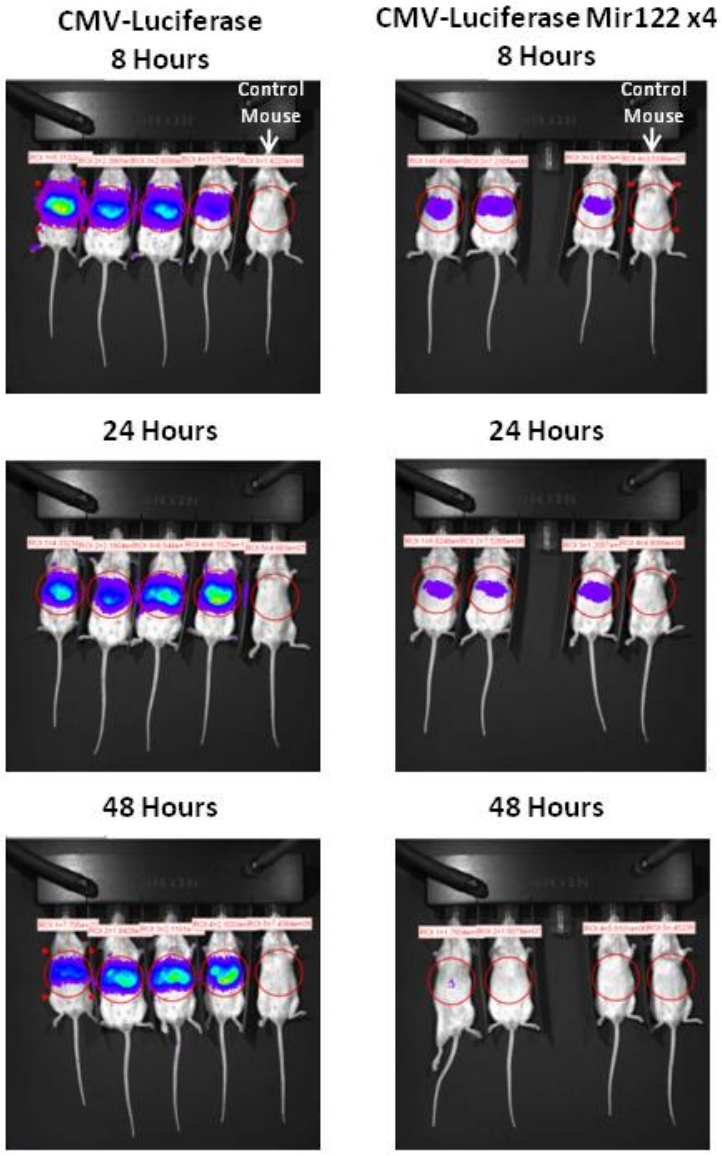


Figure 3.7: Effects of including binding sites for microRNA mir122 on the expression of CMV promoter driven plasmids *in vivo* following hydrodynamic delivery to mice. Imaging luminescence of mice administered CMV driven plasmids containing either no microRNA binding sites (left panels) or four microRNA mir122 binding sites (right panel). The animal on the right side of all images is a control treated with PBS, but mock injected with luciferin, used to provide a background reading. The two images at each time point are directly comparable; however, scaling varies between time points. Red circles define regions of interest from which flux levels were calculated.

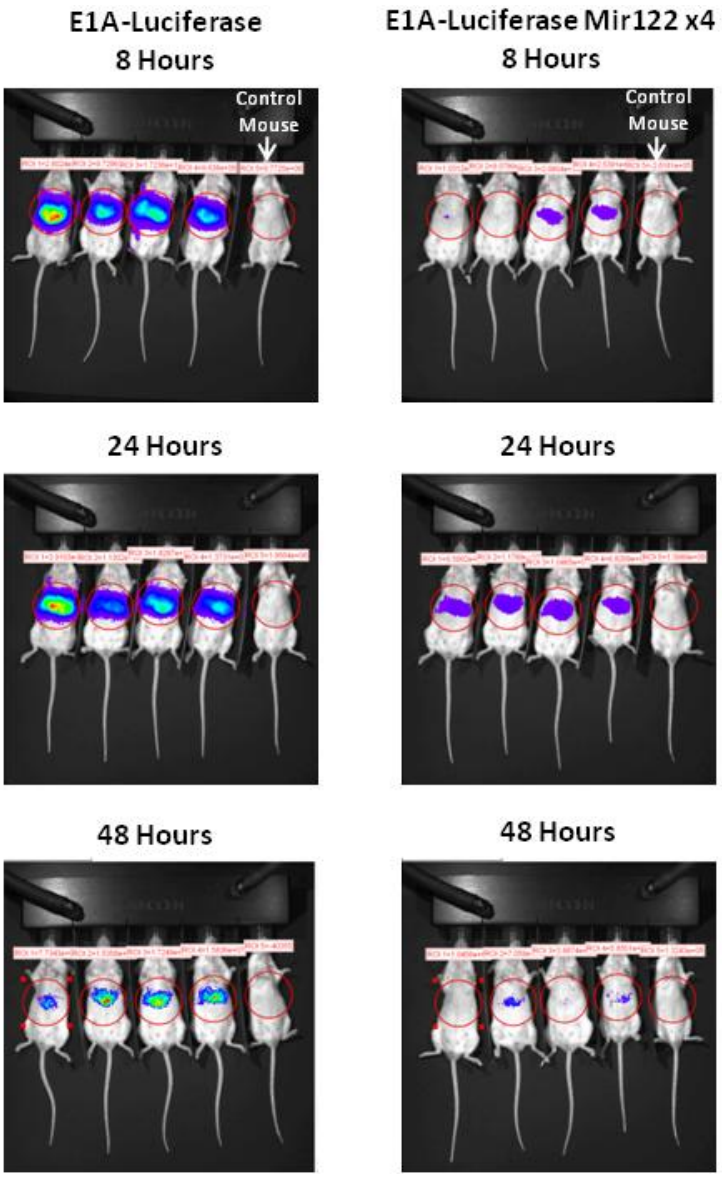


Figure 3.8: Effects of including binding sites for microRNA mir122 on the expression of E1A promoter driven plasmids *in vivo* following hydrodynamic delivery to mice. Imaging luminescence of mice administered plasmids containing the E1A promoter and coding region fused to luciferase containing either no microRNA binding sites (left panels) or four microRNA mir122 binding sites (right panel). The animal on the right side of all images is a control treated with PBS, but mock injected with luciferin, used to provide a background reading. The two images at each time point are directly comparable; however, scaling varies between time points. Red circles define regions of interest from which flux levels were calculated.

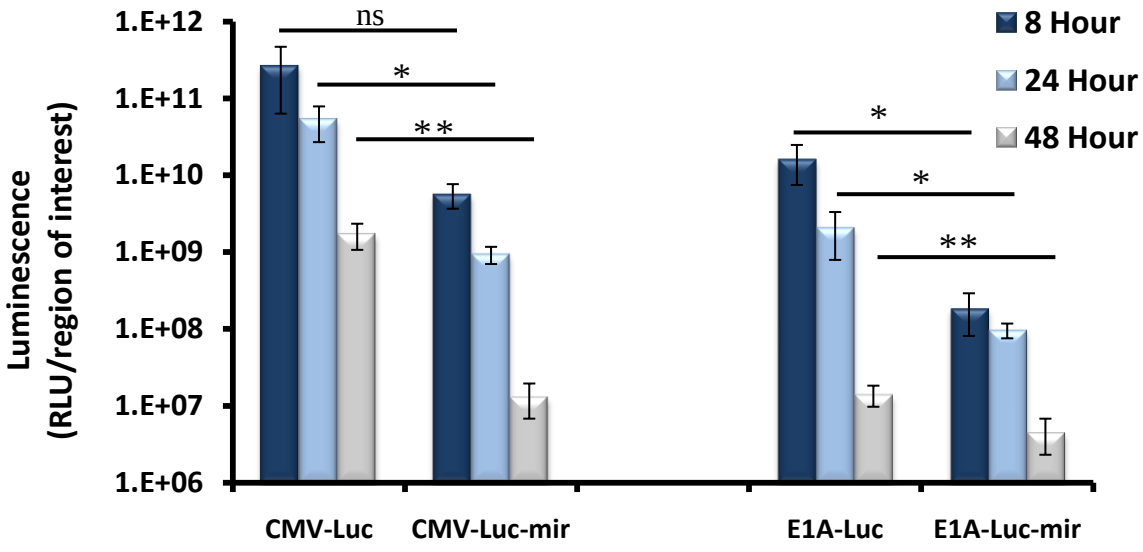


Figure 3.9: Quantitation of luminescence over time from mice administered CMV and E1A promoter driven plasmids. The levels of luminescence produced from each plasmid within the regions of interest defined in figures 3.7 and 3.8 from all mice in study are shown. N = 4 in all groups (except pCMV-mir122 where n=3) and error bars are shown as standard deviation. Time points include 8 h (dark blue), 24 h (light blue) and 48 h (grey). Statistical analysis was performed using a two-way ANOVA with a Bonferroni post-test.

3.3. Concluding discussion:

The ability of microRNA mir122 to reduce mRNA protein translation from an artificially introduced plasmid was undetermined and it was, therefore, necessary to confirm that this method of control was effective at inhibiting protein production. A better understanding of the regulatory capacity of mir122 was also essential for future virus design, production and testing. To monitor and quantify the effects of mir122, plasmids were engineered to contain four, eight or four anti-sense perfectly complementary binding sites for mir122 within the 3' UTR of luciferase transcription cassettes. Perfectly complementary binding sites were inserted to maximise Argonaute-2 mediated mRNA cleavage which should provide robust and efficient translation inhibition.

Two luciferase plasmid systems were genetically engineered in which transcription was driven by either the strong, highly transcriptionally active, CMV promoter or the weaker adenoviral E1A promoter. These vectors allowed the capacity of mir122 to inhibit mRNA translation to be measured from viral promoters of varying strengths and testing of the hypothesis that mRNA produced from the adenoviral E1A transcription unit was capable of being successfully repressed by microRNA mir122. The results of this would be crucial for virus design and engineering.

Initial *in vitro* analysis of CMV driven plasmids showed that the inclusion of four microRNA binding sites for mir122 did not affect the level of luciferase translated in cells that did not express mir122. However, in the hepatocellular carcinoma cell line Huh7 (which expresses approximately 12% of the mir122 found in primary human hepatocytes) luciferase translation was reduced 8-fold when compared to either a plasmid containing

no mir122 binding sites or a plasmid with four anti-sense mir122 binding sites. This showed that mir122 was capable of mediating significant translational knockdown in microRNA mir122 positive cells. These data also showed that eight microRNA binding sites were more effective at controlling gene expression than four binding sites. However, for virus design is was predicted that eight identical copies of a DNA sequence might encourage recombination within the virus genome which could produce a revertant with Ad5-WT properties and toxicity. Eight microRNA binding sites were therefore not investigated further but would be re-considered if four binding sites were unable to control virus gene expression.

In vivo analysis of the ability of mir122 to knock down mRNA translation was demonstrated by delivering plasmid DNA by hydrodynamic injection into the liver of Balb/C mice. The injection of CMV driven plasmids showed that mir122 was able to reduce the level of protein translation by up to 129-fold for up to 48 hours. Importantly, expression from the CMV driven plasmid containing no microRNA binding sites was higher at eight hours than any signal which has previously been obtained in our laboratory using virus vectors and yet microRNA mir122 was still capable of reducing luciferase production by 47-fold. This suggested that the regulatory capacity of mir122 had not been quenched even when the level of mRNA produced is very high.

The hydrodynamic injection of E1A promoter driven plasmids encoding a fusion protein between E1A and luciferase produced strong luminescence readings from the murine liver. This was the first demonstration that luciferase was functionally active even when it was fused to the E1A protein. This was an encouraging finding that spurred the design of reporter viruses carrying the same genetic alteration, described in Chapter Four in detail.

The luciferase expression level from the E1A-Luciferase plasmid containing no microRNA binding sites was significantly lower than that produced from the CMV promoter driven plasmids but the level of repression mediated by mir122 was similar (87-fold at 8 h).

The use of reporter plasmids to directly monitor the effect of mir122 on luciferase translation allowed non-invasive temporal analysis of microRNA regulation *in vivo*. This ability made it possible to determine that the regulatory activity of mir122 is durable up to at least 48 hours and is able to down-regulate protein translation from substantial quantities of mRNA transcribed from two independent strong viral promoters.

Chapter Four: Cloning and *in vitro* characterisation of microRNA controlled adenovirus

4.1. Introduction:

Genetic modification of adenovirus serotype five

The adenovirus serotype five genome consists of a 35,938 base pair linear double stranded DNA molecule that can be genetically modified to allow the production of stable recombinant viruses endowed with new phenotypes and properties. Such genetic modifications have allowed adenoviruses to be produced for vaccination, gene therapy and oncolytic virotherapy purposes as well as being used as analytical tools to increase our understanding of a wide variety of biological pathways and mechanisms. The majority of genetic changes that have been made to the adenovirus genome have involved the deletion of significant portions (up to 35 kilobases of the parental genome) followed by the insertion of large extraneous DNA fragments to reconstitute the genome to its normal size (Morsy *et al.* 1999; Steinwaerder *et al.* 1999). This approach is used primarily in the development of helper-dependent, replication defective adenoviruses and involves the deletion of essential virus genes.

It has also been demonstrated that the packaging capacity of wild-type adenovirus serotype five is flexible and the successful packaging of genomes that are 105 % of the parental size can be achieved (Bett *et al.* 1993). This would increase the genome size from 35.9 kilobases to a total of 37.7 kilobases. The ability of adenovirus to allow the insertion of up to 1.8 kilobases of exogenous DNA without the deletion of endogenous genetic material allows the generation of replication competent viruses with new phenotypes and properties which do not include the deletion of important viral genes. Such modified

viruses have been shown to be stable over multiple passages and demonstrate little to no decrease in growth kinetics (Bett et al. 1993).

By exploiting the capacity of Ad5 to accommodate exogenous DNA insertion, whilst maintaining the Ad5-WT gene complement, a recombinant 'wild type' adenovirus was designed and engineered that contained a 122 base pair insertion in the 3'UTR region of the E1A transcription cassette. This insertion was positioned within the 66 base pairs interspacing the terminal codon of the E1A coding region and the E1A poly-adenylation signal (Adenovirus genomic base pairs 1545 and 1611 respectively). This virus modified genome, termed Ad5-mir122 and the Ad5 WT genome were then further modified to include the coding sequence of luciferase which was contiguous with the E1A coding region by being inserted between the final (stop) and penultimate codon of E1A. These viral genomes are 37.7 and 37.8 kilo bases or 105.1% and 105.9% compared to the wild type genome respectively.

Mir122 in vitro models

MicroRNA mir122 is expressed exclusively within hepatocytes and is one the most abundant microRNAs in any human tissue. *In vitro* models for quantifying the influence of mir122 of mRNA translation are therefore limited to cells of hepatocyte origin. These include the use of primary human hepatocytes in tissue culture as well as hepatoma cell lines. Quantitation of mir122 in multiple hepatoma cell lines has revealed a propensity to lose the expression of mir122 (Coulouarn *et al.* 2009). This loss has been positively correlated with an increase in tumour aggressiveness *in vivo* and suggests that the loss of

hepatocyte phenotype, or at least the loss of microRNAs that help to define hepatocytes, may be an important component of hepatoma progression. Although mir122 expression is completely abolished in most hepatoma cell lines, including Hep3b and HepG2, partial expression is maintained in the cell line Huh7. Whilst the number of mir122 molecules per cell in primary human hepatocytes is approximately 135,000, the quantity found in Huh7 cells is approximately 16 000, representing a 88% decrease from endogenous normal levels(Chang *et al.* 2004).

The experimental work conducted within this chapter utilises both of the models described above to determine the effect of mir122 on E1A expression using both wild-type and microRNA controlled adenovirus in conjunction with their luciferase encoding counterparts. The assays were used to determine if the insertion of four microRNA binding sites into the 3'UTR of E1A would influence virus replication and E1A expression, however, the reduced level of mir122 in Huh7 cells make this a poor model compared to primary human hepatocytes *in vivo*. The use of primary hepatoyctes within this chapter was an attempt to improve this model, however, the viability of primary human hepatocytes at seeding is typically <60% and they do not survive beyond 3-5 days in culture. As such neither model is an accurate reflection of *in vivo* hepatocyte activity or function but both models allowed the concept of microRNA regulation within adenovirus to be tested.

Luciferase Reporter Viruses

The ability to monitor microRNA mediated knockdown indirectly by quantifying luciferase produced from an E1A-luciferase fusion protein was useful in determining the regulatory capacity of mir122 in Chapter Three. However, these studies utilised plasmid DNA and it was undetermined if the E1A protein could function correctly in the context of a virus carrying the same fusion gene. The cloning of adenoviruses in which the coding region of luciferase was inserted downstream of the E1A coding region was therefore performed. Importantly, this insertion should allow direct visualisation and measurement of all E1A proteins because all coding sequences are co-terminal at the 3' end. Therefore, no E1A transcripts will be produced which do not also encode luciferase. This method of monitoring E1A levels is unique and had not previously been attempted or proven possible.

Primary Scientific Objectives:

1. Design and engineer ‘wild type’-based adenoviruses containing microRNA binding sites and/or luciferase to allow quantitation of translation inhibition.

2. Determine if adenoviruses containing E1A protein fused to luciferase are viable, stable and express functional and quantifiable levels of luciferase.

3. Determine if adenovirus containing four microRNA mir122 binding sites in the 3’ UTR of E1A maintains Ad5 WT potency in mir122 negative cells.

4. Quantify mir122 mediated knockdown in mir122 positive cells including Huh7 hepatoma cells and primary human hepatocytes.

4.2. Results:

Cloning of mir122 regulated Ad5 WT

A plasmid containing the wild-type genome of Ad5 and kanamycin resistance (a kind gift from Dr Reuben Hernandez) was cleaved with Dra1 after multiple passages to ensure the vector was full size and stable (Figure 4.1). Results showed that the vector was full size after repeated passage and all expected bands were visible. This vector was then cleaved with BstZ17I and re-circularised by blunt ended ligation. This vector, termed pAd5-BstZ17I, was then further cleaved and re-ligated using Kpn1 to increase the number of unique restriction sites available for further cloning (Figure 4.2). This vector is referred to as pAd5-Kpn1. The four microRNA binding sites for mir122 within pCMV-Luc-mir (described in Chapter Three) were PCR amplified to introduce Dra1 sites on to each end. The purified PCR product was cleaved with Dra1 and blunt end ligated into pAd5-Kpn1 which was cleaved with Hpa1 in the E1A 3' UTR to produce pAd5-Kpn1-mir containing four mir122 binding sites. Insertion of microRNA binding sites downstream of E1A was confirmed by DNA sequencing in both directions using the primers 5' CGA CAT CAC CTG TGT CTA GA 3' and 5' AAC CAA GAT TAG CCC ACG 3'. Ad5-Kpn1-mir was reconstituted to pAd5-BstZ17I by linearisation with Kpn1 and ligation to the Kpn1 gel-extracted fragment from pAd5-BstZ17I to produce pAd5-BstZ17I-mir (Figure 4.3). Screening of recombinants with Hpa1 produced multiple correct clones (Figure 4.4).

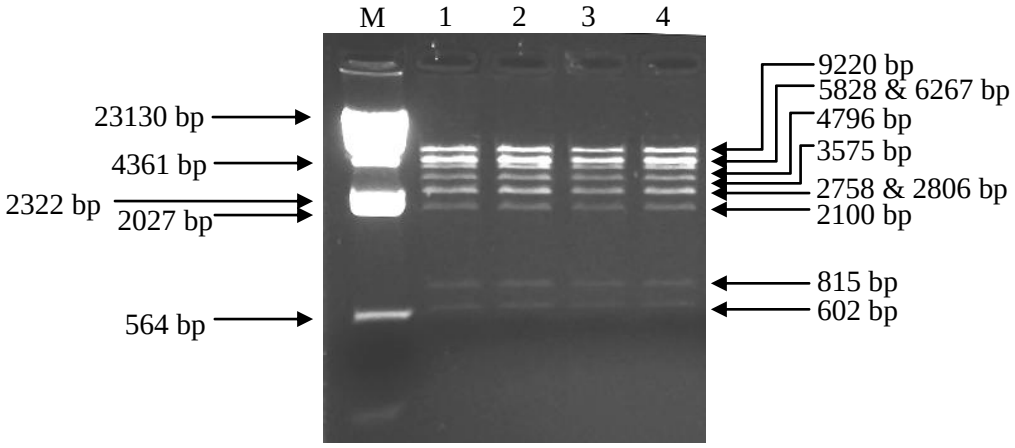


Figure 4.1: The Ad5-WT genome was pre-screened with Dra1 prior to beginning cloning to ensure that the vector was intact, full size and stable over sequential passage. Each lane represents the growth of the plasmid in the previous lane in *E.coli*. Lane one is passage one from the parental stock. The vector is stable and full-size throughout testing. M= DNA ladder/marker.

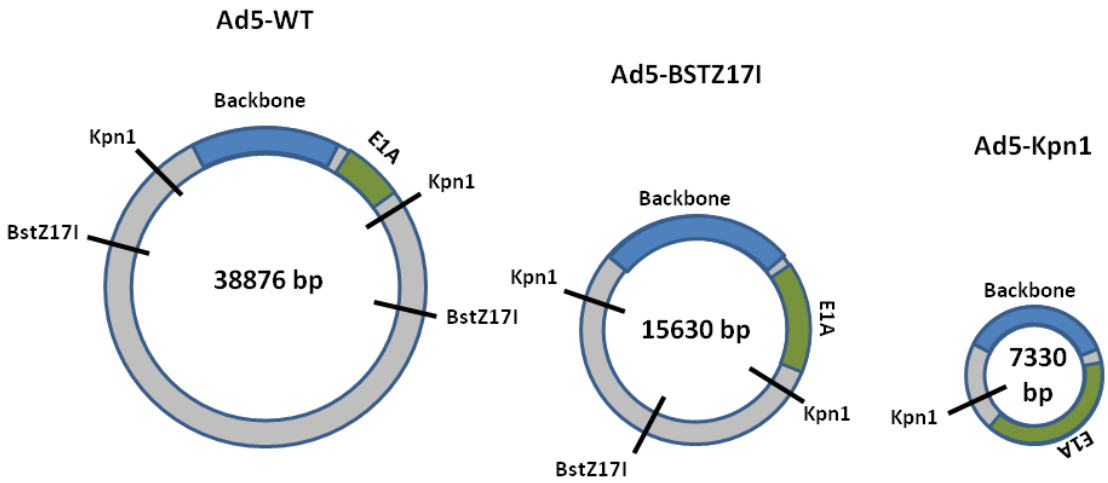


Figure 4.2: Serial cleavage of Ad5-WT to produce unique restriction sites. In order to produce a plasmid with restriction sites that would allow the insertion of both E1A-luciferase fusion cassettes and microRNA binding sites it was necessary to decrease the size of the adenovirus genome by sequential restriction digestion. These schematics show the approximate positions of the relevant restriction enzymes and the key restriction sites used for both minimising and reconstituting the virus genomes. Plasmid sizes are shown in the centre of each vector in base pairs (bp) and represent the sizes of the unmodified parental vectors.

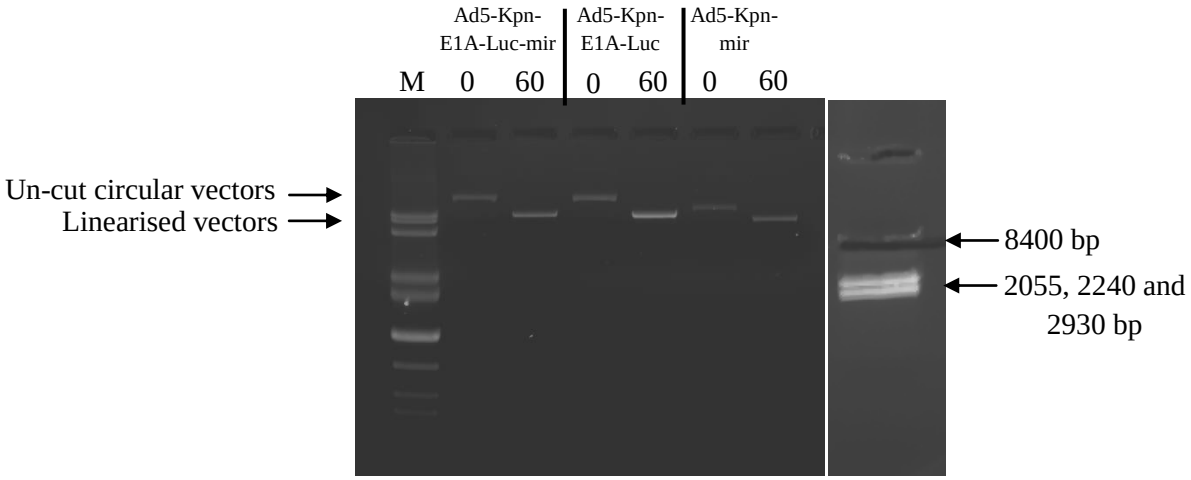


Figure 4.3: Reconstitution of modified vectors (Ad5-Kpn1 derivatives) to become 'half sized' Ad5-BstZ17I adenovirus genomes. Ad5-Kpn1 (Figure 4.2) based vectors encoding E1A-luciferase fusion cassettes and/or mir122 microRNA binding sites were linearised with Kpn1 before re-constitution to become Ad5-BstZ17I (Figure 4.2) based vectors. The left panel shows the successful cleavage of each vector with Kpn1. Vector names and the length of time in restriction digestion are shown above each lane. The right panel shows the gel excision of the Kpn1 fragment from Ad5-BstZ17I cut with Kpn1 and Pac1 (Pac1 cuts the vector backbone to eliminate contamination in downstream ligations). M = DNA ladder/marker.

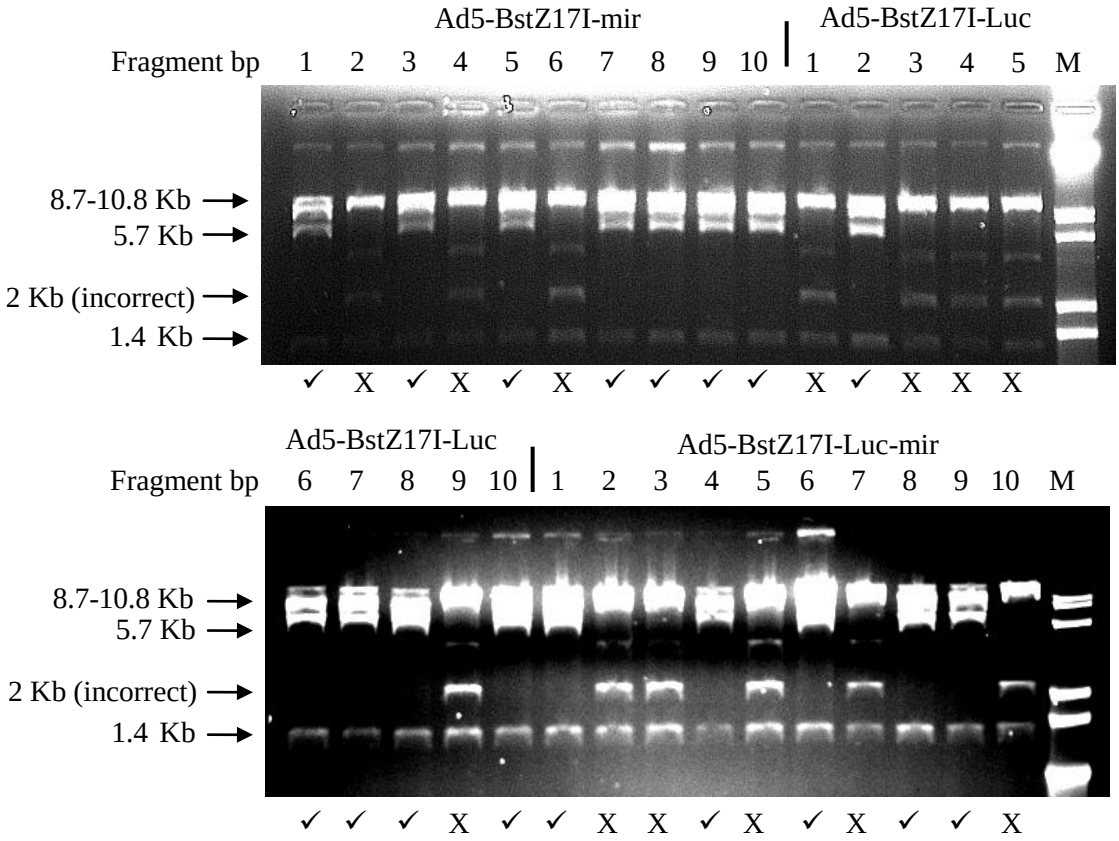


Figure 4.4: Screening for 'half-size' Ad5-BstZ17I recombinant vectors containing luciferase and/or mir122 binding sites. Recombinant plasmids generated from the ligation of DNA products produced in figure 4.3 were screened with Hpa1. All lanes show the insertion of the Kpn1 fragment, determined by the presence of a 1.4 Kb band, although many are inserted in the incorrect orientation. A fragment of 2 Kb is present in the incorrect colonies. The correct colonies show three bands equating to the conserved 1.4 Kb and 5.7 Kb bands and a band of higher molecular weight which varies from 8.7 Kb to 10.8 Kb depending on the vector. The faint bands at the top of some lanes show the digestion was not complete in some samples although the presence and absence of the correct bands is clear. Tick marks (✓) denote correct recombinants whilst X marks denote incorrect recombinants beneath each lane in the gel images. M = DNA ladder/marker.

To generate full size adenovirus genomes Ad5-BstZ17I-mir was cleaved with BstZ17I, de-phosphorylated and subject to homologous recombination in BJ5183 *E. coli* with full size wild-type Ad5 ampicillin resistant vector (a kind gift from Dr Peter Searle) and selected on kanamycin (Figure 4.5). Insertion of microRNA binding sites was confirmed by DNA sequence analysis using the primers 5' CGA CAT CAC CTG TGT CTA GA 3' and 5' AAC CAA GAT TAG CCC ACG 3'. The regions predicted to contain homologous recombination sites were also sequenced in both directions to ensure no deletions had been produced. Primers used to sequence the 5'recombination site (E1A side, figure 1.2 in Chapter One) in both directions were 5' AGA CTT TTG AGG GCG TAG 3' and 5' CTG ACA ATC TTA GCG CAG 3'. Primers used to sequence the 3' recombination site (E4 side, figure 1.2 in Chapter One) in both directions were 5' GCT AAT GAG TGC ACC ACT 3' and 5' GCA GTT AGT GGT GAC ATT AGC 3'. Restriction digestion with multiple enzymes confirmed that the resulting vectors contained full size adenovirus genomes (Figure 4.6).

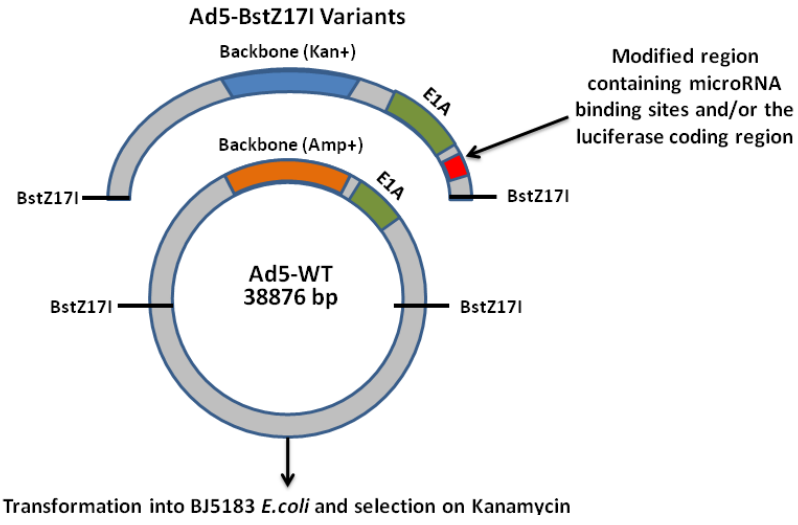


Figure 4.5: Schematic diagram of the homologous recombination strategy used to reconstitute full size adenovirus genomes.

Cloning of microRNA regulated luciferase reporter based on Ad5 WT

pE1A-Luc-mir and pE1A-Luc (which are modified forms of Ad5-Kpn1 described in Chapter Three) were reconstituted to Ad5-BstZ17I using the Kpn1 gel-extracted fragments from Ad5-BstZ17I (Figure 4.3). To generate full size adenovirus genome Ad5-BstZ17I-Luc-mir and Ad5-BstZ17I-Luc were cleaved with BstZ17I, dephosphorylated and subject to homologous recombination with full size wild-type Ad5 vector (a kind gift from Dr Peter Searle) and selected on kanamycin (Figure 4.5). Insertion of microRNA binding sites and luciferase was confirmed by sequence analysis using primers 5' CGA CAT CAC CTG TGT CTA GA 3' and 5' AAC CAA GAT TAG CCC ACG 3'. Restriction digests of the resulting vectors confirmed full size adenoviruses had been recovered (Figure 4.6). Genomic structures and sizes of the viruses are shown in Figure 4.7.

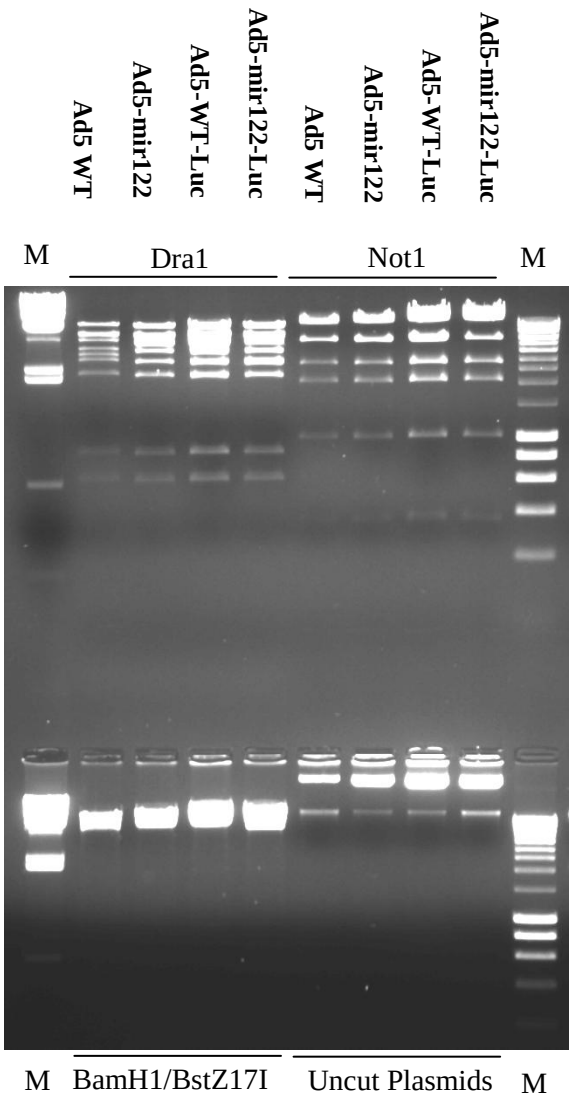


Figure 4.6: Restriction digestion of completed viruses to confirm full sized plasmids. The full size adenoviral vectors produced by recombination (figure 4.5) were cleaved with either Dra1, Not1 or double digested with BamH1 and BstZ17I to ensure full size plasmids were obtained prior to virus rescue. All digests produced the correct band patterns. M = DNA ladder/marker. The left marker is a HindIII digest of Lambda virus DNA whilst the right ladder is Hyperladder 1 (Bioline).

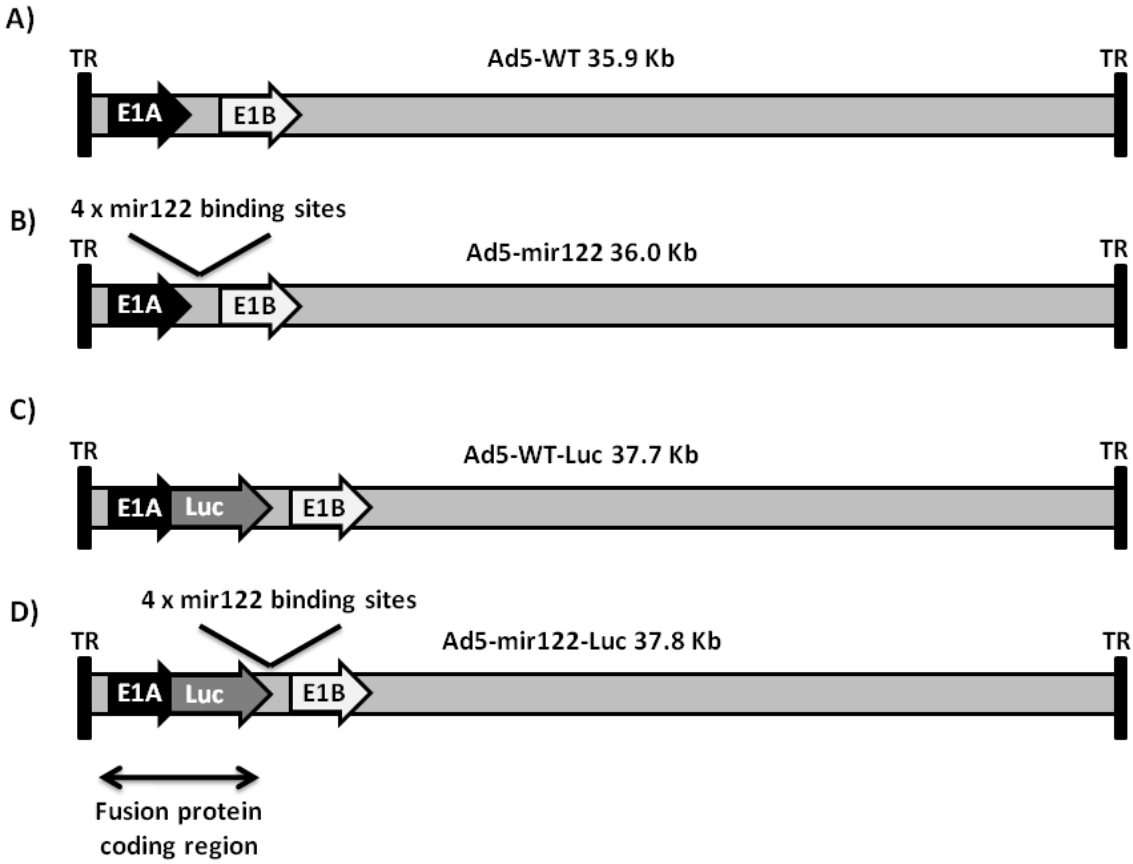


Figure 4.7: Viruses produced by genetic engineering to contain microRNA mir122 binding sites and/or luciferase. (A) Ad5 WT genome with E1A and E1B coding regions highlighted. (B) Four tandem repeats of binding sites for mir122 were inserted into the 3' UTR of E1A. (C) The luciferase coding sequence was inserted into Ad5 WT genome to generate a fusion with the E1A coding sequence which will encode a fusion protein between E1A and luciferase. (D) Four microRNA binding sites for mir122 were inserted into the 3' UTR of luciferase encoded within the virus described and shown in C). Virus names and sizes in kilobases are shown above each construct.

Recovery and growth of E1A-luciferase reporter viruses

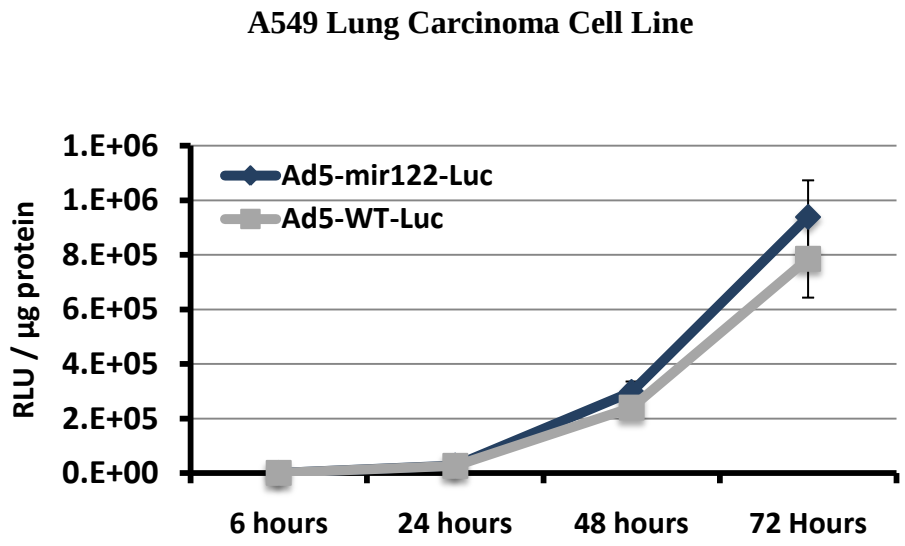
Adenovirus genomes, with and without mir122 binding sites, had been engineered in which the luciferase coding sequence had been inserted 3' to the E1A coding sequence. Previous analysis using plasmid transfection had shown that this fusion cassette produced functional luciferase, however, in order for adenovirus to replicate the E1A portion of this fusion protein would also need to remain active. Therefore, plasmid DNA (20 µg/ 10cm dish) encoding the wild-type Ad5 genome with E1A fused to luciferase and the same plasmid containing four mir122 binding sites were linearised and transfected using the calcium phosphate method into A549 lung carcinoma cells. Adenoviruses are commonly recovered in HEK-293 cells but these cells were avoided because they trans-complement E1A which would make it impossible to determine if the E1A-luciferase proteins were functionally active. Both viruses grew successfully in A549 cells which was visible by the presence of advanced CPE in some wells after 12-15 days. This result confirmed that viable virus could be produced despite the fusion of E1A to luciferase. The quantity of luciferase produced by independent batches of virus was both high and consistent suggesting the viruses were stable. Bulk preparation confirmed this finding with both viruses amplifying from less than $1x10^5$ viral particles to $3.22x10^{12}$ vp/mL of Ad5-WT-Luc and $3.10x10^{12}$ vp/mL of Ad5-mir122-Luc after three sequential passages and double banding. The wild type strain of adenovirus serotype five was also recovered alongside the same virus modified to contain four microRNA mir122 binding sites within the 3'UTR of E1A (Ad5-mir122). Both viruses grew with equal kinetics and after three sequential passages and double banding titers of $1.29x10^{12}$ vp/mL (Ad5 WT) and

$1.11x10^{12}$ vp/mL (Ad5-mir122) were obtained.

Mir122 binding sites do not affect adenovirus activity in mir122–negative cells in vitro and in vivo

Replication competent adenoviruses encoding E1A-luciferase fusion proteins were used to infect mir122-negative A549 and OVCAR-3 cell lines *in vitro* at an MOI of 10 vp / cell. Luciferase activity from both Ad5-WT-Luc (containing no mir-122 binding sites) and Ad5-mir122-Luc (containing four mir122 binding sites) increased slowly between 8 h and 24 h and then showed a more rapid rise that was sustained up to at least 72 h (Figure 4.8 A and B). This profile of luminescence may reflect initial transcription from the input viral genomes that increases rapidly following viral genomic replication. The microRNA insertion into the 3′ UTR did not affect the profile of luciferase expression in these cells, suggesting the modification did not influence the stability of mRNA encoding the E1A-luciferase fusion protein, nor did it inhibit virus replication in these mir122-negative cells.

A)



B)

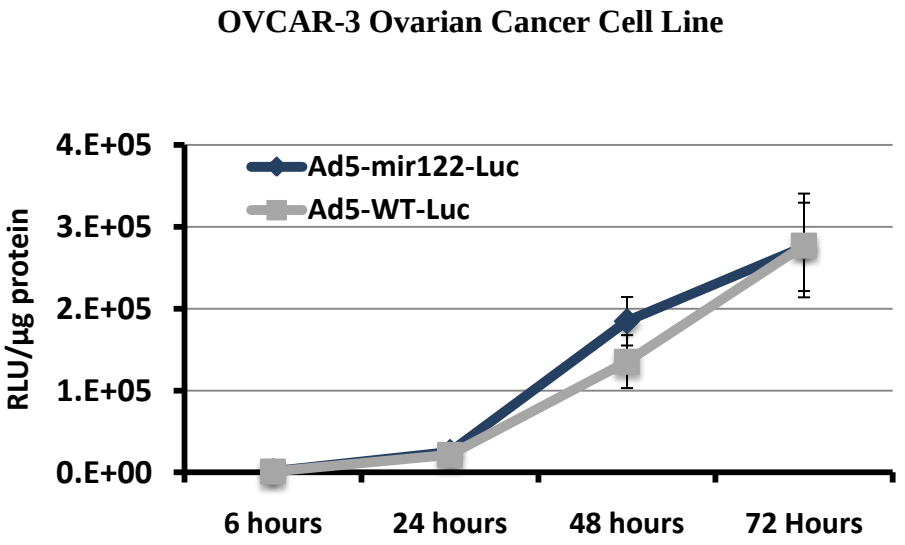


Figure 4.8: MicroRNA binding sites do not affect E1A expression in mir122 negative cells. A) A549 cells were seeded at $5x10^3$ cells per well and incubated with either Ad5-WT-Luc (grey line) or Ad5-mir122-Luc (blue line) at an MOI of 10 vp/cell. Luciferase expression was determined at 6, 24, 48 and 72 h. B) OVCAR-3 cells were seeded at $5x10^3$ cells per well and incubated with either Ad5-WT-Luc (grey line) or Ad5-mir122-Luc (blue line) at an MOI of 10 vp/cell. Luciferase expression was determined at 6, 24, 48 and 72 h. Error bars at each time point are shown as standard deviation. No statistically significant differences were found between the viruses at any time point throughout study.

Ad5-mir122 kills mir122 negative cells with Ad5 WT potency

The ability of Ad5-mir122 to kill tumour cells not expressing mir122 was compared to Ad5 WT to determine if the insertion of four mir122 binding sites affected the ability of Ad5-mir122 to kill cancer cells *in vitro*. Cell lines tested included the lung carcinoma cell line A549, two hepatoma cell lines (Hep3B and HepG2) which are reported to no longer express mir122 and two colorectal cell lines (LoVo and HT-29). Cells were seeded at $1x10^4$ / well in 96 well plates. After 24 h at 37 °C cells were incubated at an MOI of either 10 or 100 vp/cell. Cell viability was determined after 3 or 6 days using an MTS assay to quantify viable cells.

Equal cell killing was observed with both viruses at both MOIs by day 3 although no cell line showed a level of cell kill above 60% (data not shown). Almost complete cell killing in some cell lines was observed at the higher MOI by day 6 (Figure 4.9). The cell line HT-29 seemed less susceptible to killing with Ad5 WT than Ad5-mir122, however, future experiments determined that this result was not consistently significant. Interestingly, the cell line most susceptible to killing by both viruses was HepG2 human hepatocellular carcinoma, which is reported to be mir122 negative (Chang *et al.* 2004). Given that decreases in mir122 levels in primary hepatomas is an indicator of poor prognosis, HepG2 cells represented a promising cell line for future testing as an *in vivo* tumour model.

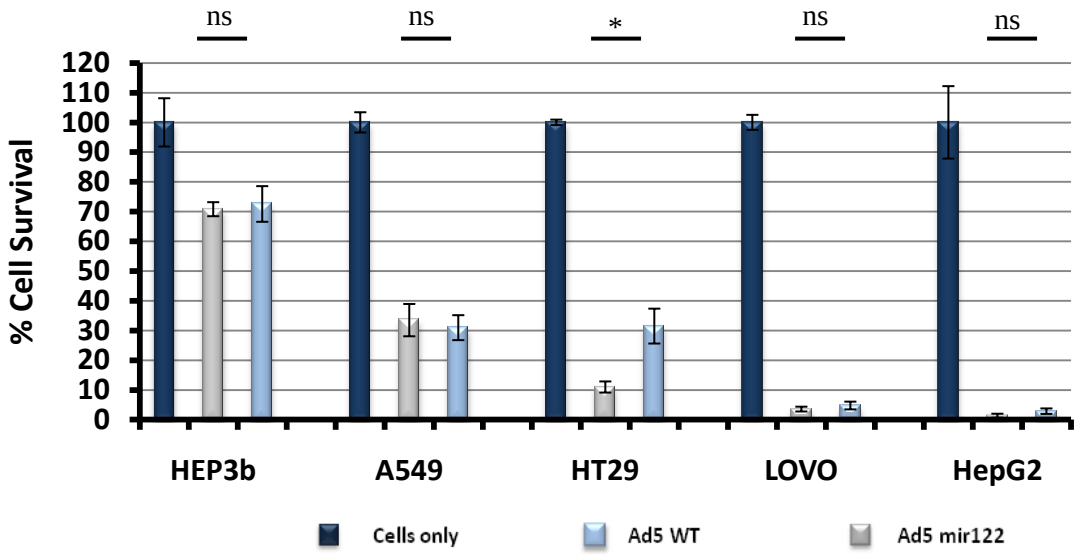


Figure 4.9: Ad5-mir122 kills mir122 negative cells with Ad5 WT potency. Comparison of Ad5-mir122 and Ad5 WT in cancer cell lines incubated at a multiplicity of infection of 100 viral particles per cell. The percentage cell survival is shown 6 days post infection (N=5) using an MTS cell survival assay. Statistical analysis was performed using one way ANOVA (*=$p<0.05$, NS = not significant)

MicroRNA binding sites decrease activity of Ad5-mir122-Luc virus in mir122–positive cells in vitro

In order to determine if microRNA mir122 could control virus replication by reducing E1A expression (as measured by luciferase) the mir122 positive hepatoma cell line Huh7 was incubated with adenoviruses encoding the E1A-luciferase protein with and without four microRNA binding sites. E1A-luciferase expression was monitored by luminometry from 6 h to 72 h post-infection (Figure 4.10). Luciferase expression from Ad5-WT-Luc (containing no mir122 binding sites) showed a small but significant rise between 0 and 24 h (reaching 1.1×10^5 RLU/µg protein) and then increased rapidly, rising to 1.7×10^6 RLU/µg protein by 72 h. These findings suggest that E1A transcription and DNA replication proceeded similarly to the situation in A549 and OVCAR cell lines presented in figure 4.8. In contrast, Ad5-mir122-Luc showed significantly less luciferase expression at all time points, reaching only 6.3×10^4 RLU/µg after 72 h (P = 0.0001 for both 48 and 72 h). The differential in luciferase expression between the viruses with and without microRNA binding sites increased over time, suggesting decreased genome replication of Ad5-mir122-Luc compared to Ad5-WT-Luc.

In order to determine if the effect observed in Huh7 cells was mir122 specific and not an artifact of hepatoma cell lines Ad5-mir122-Luc and Ad5-WT-Luc were incubated with the mir122 negative HepG2 cell and Huh7 cells at an MOI of 1 vp/cell. Results showed that whilst Ad5-mir122-Luc produced significantly lower luciferase when compared to Ad5-WT-Luc in the mir122 positive Huh7 cells, the level of luciferase produced by both viruses in HepG2 was equal (Figure 4.11). Interestingly, the luciferase expression from Ad5-WT-Luc was higher in Huh7 than HepG2. This result was found to be consistent and

may relate to virus receptor expression or differences in the intracellular protein complement between cell lines.

Knockdown of E1A expression in mir122 negative cells using pre-mir122 mimics

The hepatoma cell line Huh7 is the only human cell line which expresses mir122 and the reduction in E1A-luciferase observed with Ad5-mir122-Luc when compared to Ad5-WT-Luc may not have been mir122 specific. In order to confirm that this expression differential was due to mir122 knockdown of E1A, a precursor RNA mimic of mir-122 (Ambion) was introduced by transfection into A549 cells to simulate hepatocyte mir122 expression. Ad5-mir122-Luc and either the mir122 pre-cursor, or negative control pre-mir (Ambion) were added to cells and luciferase readings performed after 24 h. Results showed that the introduction of the pre-mir122 reduced luciferase, and therefore E1A, expression from 9.2×10^4 RLU (negative control pre-mir) to 3.4×10^3 RLU, a 27-fold differential ($P = 0.0001$, Figure 4.12).

Ad5 mir122 shows decreased E1A expression in primary human hepatocytes in vitro

Results had previously shown that the inclusion of microRNA mir122 binding sites within the 3'UTR of adenovirus E1A could reduce the expression of an E1A-luciferase fusion product in both Huh7 cells and by artificial mir122 transfection. However, Huh7 cells express only 12% of the mir122 levels of primary human hepatocytes (Chang *et al.* 2004) and transient transfection of mir122 into a non-hepatic cancer cell line is a poor model for primary human liver. It was therefore desirable to measure the level of control of viral

E1A-luciferase transgene expression that could be achieved in primary human hepatocytes as a more accurate surrogate for human liver.

Primary human hepatocytes seeded at $1x10^4$ / 96 well and were incubated overnight at 37 ^oC. Cells were then incubated with either Ad5-WT-Luc or Ad5-mir122-Luc at an MOI of 10 vp/cell. After 72 hours, cells were lysed and the luciferase levels produced from each virus were calculated. Data showed that primary human hepatocytes infected with Ad5-WT-Luc produced >30-fold more luciferase than those infected with Ad5-mir122-Luc ($2.7x10^4$ RLU/mg protein compared to $9.5x10^2$ RLU/mg protein respectively) (Figure 4.13). This is the first demonstration of control of a microRNA regulated virus in primary human cells and highlights the potential clinical utility of exploiting microRNA regulation in therapeutic viruses.

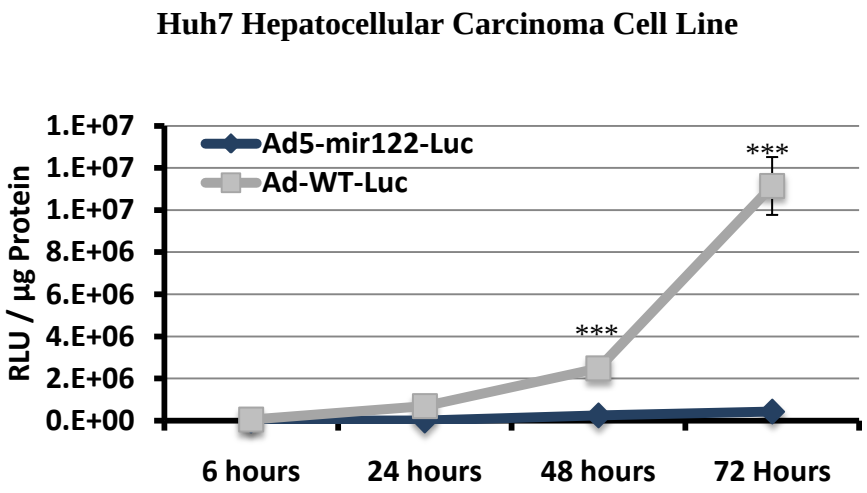


Figure 4.10: MicroRNA binding sites reduce E1A expression in mir122 positive cells. Mir122 positive human hepatoma Huh7 cells were seeded at $5x10^3$ cells per well and incubated with either Ad5-WT-Luc (grey line) or Ad5-mir122-Luc (blue line) at 10 vp/cell. Luciferase expression was determined at 6, 24, 48 and 72 h. Error bars represent standard deviation (*** P<0.0005).

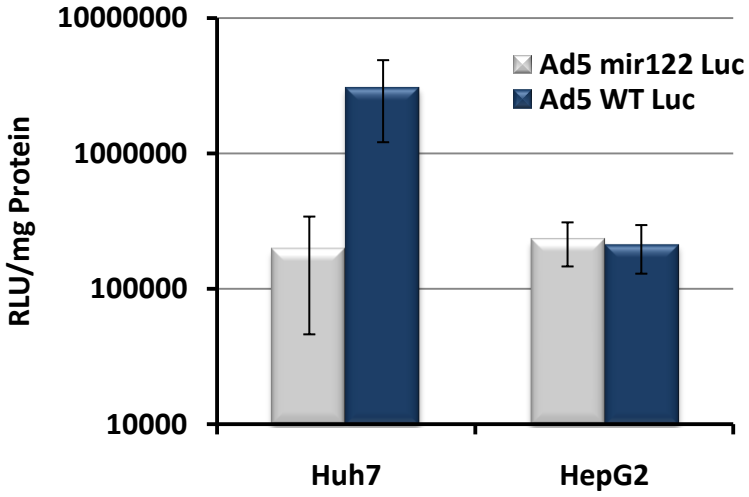


Figure 4.11: E1A-Luciferase expression from Ad5-mir122-Luc is unaffected in mir122 negative hepatoma cells. Huh7 cells and HepG2 cells were incubated with either Ad5-WT-Luc or Ad5-mir122-Luc at 10 vp/cell for 24 h followed by luciferase quantitation (N=3). Similar to the results shown in figure 4.10, Huh7 cells show knockdown in luciferase expression from Ad5-mir122-Luc compared to Ad5-WT-Luc whilst no difference is observed in the mir122 negative cell line HepG2.

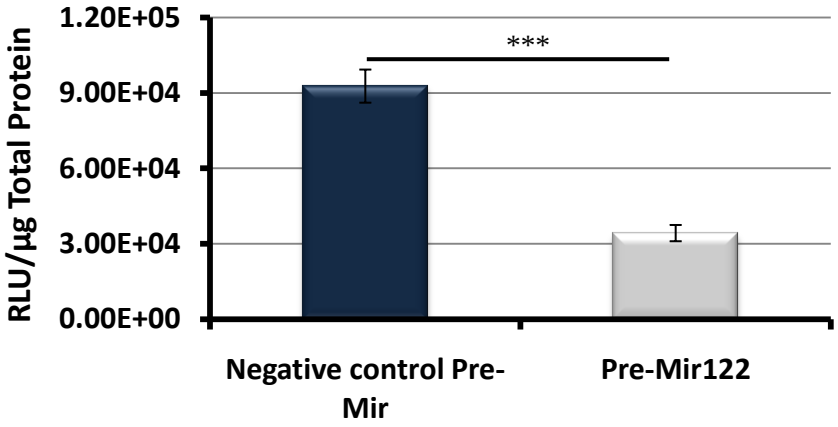


Figure 4.12: Mir122 specific knockdown of E1A-luciferase from Ad5-mir122-Luc. A549 cells were seeded at $5x10^4$ cells per well and transfected with pre-mir122 (Ambion) or pre-mir negative control (Ambion). Immediately following transfection Ad5-mir122-Luc was added at 10 vp/cell. 18 h later, 30 pmol/well of pre-cursor mir122 and negative control precursor microRNAs were added to each well. Error bars represent standard deviation (*** P<0.0005).

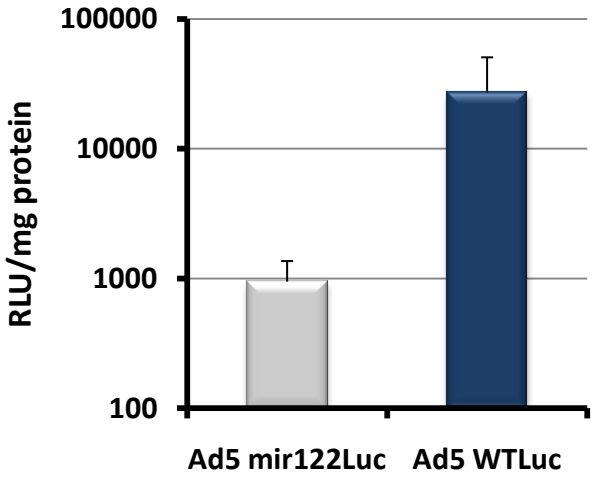


Figure 4.13: Ad5-mir122-Luc shows reduced E1A-luciferase activity in primary human hepatocytes. Primary human hepatocytes were incubated with either Ad5-mir122-Luc or Ad5-WT-Luc at 10 vp/cell. Luciferase levels are shown 3 days post infection (n=3) as relative light units per mg total protein. Error bars represent standard deviation.

4.3. Concluding Discussion:

Adenoviruses encoding E1A-luciferase fusion proteins are stable and functionally active

The ability to accurately quantify microRNA mir122 mediated knockdown of viral translation was essential. Therefore, luciferase reporter viruses were designed and engineered that increased the genome size of Ad5 WT to 105% of its normal size. Whilst the addition of similar sized DNA inserts has previously been shown to be possible and stable (Bett *et al*. 1993), the composition and position of the inserted DNA within the adenoviral genome could cause instability and prevent viral replication. The luciferase coding region that was inserted was fused to the E1A coding region and it was also uncertain if this addition would prevent E1A from functioning. It was hypothesised that the labile nature of E1A as a non-enzymatic protein binding protein would make it an ideal candidate for modification.

Attempts to rescue the adenovirus encoding an E1A-luciferase fusion protein proved successful. An identical virus containing four microRNA mir122 binding sites was also successfully rescued. Both viruses were stable in culture and showed no loss of luciferase expression over multiple passages. These modifications were therefore well tolerated and importantly both luciferase and E1A were both functional. These are the first examples of reporter adenoviruses encoding and expressing all of the genes that are normally expressed in the wild type strain of Ad5. These modified viruses are important tools for quantifying the effects of microRNA mediated knockdown both *in vitro* and *in vivo*. They are also now being tested for other purposes within the laboratory as they are the only replication competent adenovirus serotype five reporter viruses currently available.

MicroRNA binding site insertion does not reduce Ad5 WT potency in mir122 negative cells

The use of microRNA binding sites to control gene expression from a DNA virus had not previously been demonstrated. These data show that the inclusion of four microRNA binding sites for microRNA mir122 into the 3' UTR or adenovirus E1A mRNA does not reduce virus replication or cell cytotoxicity in cells not expressing microRNA mir122. These data also demonstrate that the quantity of E1A translated (measured indirectly by monitoring luciferase levels) is significantly reduced throughout infection in cells expressing mir122 up to 72 hours.

The use of cancer cell lines or murine models to demonstrate the proof of concept of targeting modalities that may eventually be used in humans is not always ideal. As such, the use of primary human hepatocytes was adopted herein as an alternative approach to attempt to confirm that any knockdown observed using either murine or cancer cell line models was applicable to human tissue. The use of primary human hepatocytes showed that the level of microRNA repression observed is equal to or exceeds that observed in the mir122 positive Huh7 cells and similar to the level of knockdown obtained following the hydrodynamic injection of microRNA controlled plasmid DNA into mice. It can therefore be concluded that the knockdown observed in all these assays is reproducible in human primary non-transformed cells.

MicroRNA mir122 can reduce viral replication and E1A production in vitro

The primary purpose of this research was to demonstrate that the hepatic specific microRNA mir122 was able to control the quantity of E1A produced from a modified adenovirus serotype five virus. The data produced here shows that mir122 is capable of knocking down expression of E1A in a mir122 dependent manner in multiple settings. Firstly, it was shown that in the mir122 positive cell line Huh7 the inclusion of four mir122 binding sites could reduce E1A expression by up to 29-fold. Similar results were observed following infection of primary human hepatocytes. This effect was confirmed to be mir122 specific by the transient transfection of a mir122 mimic into the lung carcinoma cell line A549 which significantly knockdown E1A expression when compared to A549 cells transfected with a control microRNA mimic.

These data combined show that the viruses engineered at the beginning of this chapter and tested here which contain microRNA binding site for mir122 are sensitive to mir122 translation inhibition of E1A protein production but replicate with Ad5 WT kinetics in cells not containing mir122.

Chapter Five: Mir122 control of adenovirus and toxicological assessment

5.1. Introduction

Virus particle toxicity

It is well established that the injection of particulate matter intravenously into both humans and rodents can cause rapid, systemic and sometimes fatal stimulation of the reticulo-endothelial system. Capture of both non-opsonised and opsonised macromolecular structures, such as viruses, occurs predominately in the liver and spleen and is the major clearance mechanism for viruses from the circulation. The engulfment of virus particles by cells of the reticulo-endothelial system, including resident liver macrophages (Kupffer cells), causes the rapid release of multiple cytokines which can cause local and systemic toxicity. This response, which is common following the injection of large doses of adenoviral particles, is observed in both animal models and human clinical trials following intravenous injection. However, this toxicity should not be confused with virus replication related toxicity. The immune response to virus particles is rapid with flu-like symptoms beginning within less than 30 minutes. The symptoms usually last less than 24 hours and are normally self-limiting. Virus replication induced toxicity does not occur within this time window and typically begins no earlier than 48 hours. The pathological symptoms are also substantially different and described in detail below.

Virus replication induced toxicity

Wild type adenovirus is known to cause significant replication associated hepatic toxicity in murine models following intravenous (iv) administration. This toxicity, which typically develops at 72 hours post injection, is believed to reflect rapid influx of virus particles

into the space of disse within the liver, followed by infection of hepatocytes. Onset of pathological complications is dependent on dose, with higher doses resulting in more severe disease pathology at earlier time points (circa 48 hours). Typically, symptoms include increased alanine and aspartate transaminase levels, weight loss and lethargy. Pathologically, infected livers present with reduced whole protein content when compared to non-infected controls and show high levels of cellular vacuolation and internal haemorrhaging. The boundaries between defined liver strata are also less visible suggesting breakdown of vascular capillaries and loss of intercellular connectivity. Virus replication in hepatocytes, although ultimately incomplete and resulting in no secondary release of virus, does involve an increase in viral genomic DNA which is believed to reflect efficient expression of the E1A promoter in hepatocytes, which allows downstream viral promoters to be activated and genome replication to occur.

The toxicity observed in murine models can be severe, and doses below the maximum tolerated dose can still induce one or many of these complications. Therefore, the initial purpose of the studies conducted here was to ascertain if viral induced toxicity was reduced when adenovirus was engineered to contain microRNA binding sites which should abrogate E1A protein production and DNA replication in hepatocytes.

MicroRNA related toxicity

The microRNA regulatory network is a complicated biological system in which any individual microRNA can subtly influence the quantity of protein translated from an average of >200 different mRNA transcripts. The addition of new mRNA target for a specific microRNA into this network could therefore alter the transcript levels of many

mRNAs which may lead to unpredictable toxicological complications. To determine if such a toxicity is occurring it was necessary to monitor multiple points in the microRNA pathway including events specific to the microRNA in question but also to assess broader, less predictably, genome-wide intracellular changes.

Maximum tolerated dose

Previous studies with wild type adenovirus type five have shown that the maximum tolerated dose is approximately $1x10^9$ plaque forming units (Duncan *et al.* 1978). The toxicity induced above this dose is a major limiting factor for efficacy for using wild type adenovirus as an anti-cancer agent in murine models and would likely represent a dose limiting toxicity if observed in human trials. Therefore, determining the virus induced toxicity mediated by microRNA regulated adenovirus above the maximum tolerated dose of wild type adenovirus would be important for later efficacy studies.

Primary scientific aims:

1. Determine whether microRNA control of adenovirus can significantly reduce toxicity in mice following intravenous administration.

2. Assess the ability of microRNA controlled adenovirus to alter mir122 levels or influence the quantity of known mir122 regulated proteins.

3. Quantify intracellular changes induced by adenovirus wild type and microRNA regulated adenovirus in comparison to a non-replicating adenoviral vector to determine whether microRNA regulation mediates non-specific toxicity.

5.2. Results:

Endotoxin characterisation of virus preparations

The purpose of the following studies was to assess the toxicological consequences of injecting high quantities of adenoviral particles intravenously. It has previously been shown that contaminating lipopolysaccharide (LPS) within an adenovirus preparation can induce toxicity (Cotten *et al.* 1994). It was therefore important to determine the quantity of LPS in the virus preparations used in these studies in order to confirm that any toxicity measured was due to virus mediated effects and not due to contamination. Endotoxin characterisation of all four viruses used for further analysis demonstrated that all viruses were free of endotoxin, containing <1 endotoxin unit per litre of preparation (Figure 5.1). Data also showed that the residual endotoxin in each preparation was equal across all virus stocks. A positive control preparation, known to contain high quantities of bacterial contaminants, produced readings >8 units per millilitre which confirmed the integrity of the assay (Figure 5.1).

MicroRNA binding sites decrease activity of Ad5-mir122-Luc virus in mir122–positive cells in vivo

To assess the *in vivo* activity of the viruses encoding luciferase and to observe the effects of time on E1A expression over 96 h, 5×10^{10} vp of Ad5-WT-Luc and Ad5-mir122-Luc were injected i.v into Balb/C mice. Animals were imaged at 6, 24, 48, 72 & 96 h (Figure 5.2 A-E). After 6 h, Ad5-WT-Luc showed an average luminescence signal of 1.6×10^{8} RLU whilst Ad5-mir122-Luc showed only 3.0×10^{6}, a differential of 52-fold. Interestingly,

the signal from the Ad5-WT-Luc treated mice showed a major increase (2.5×10^9 RLU) between 48 and 72 h (Figure 5.2 F), possibly reflecting a wave of viral genomic replication that provided increased transcription template for further E1A mRNA and protein production. At the same time the microRNA regulated virus showed only a relatively small increase (a rise of 3.4×10^7 RLU). After 96 h the differential expression between the viruses with and without microRNA sites had reached 80-fold (Figure 5.2 F).

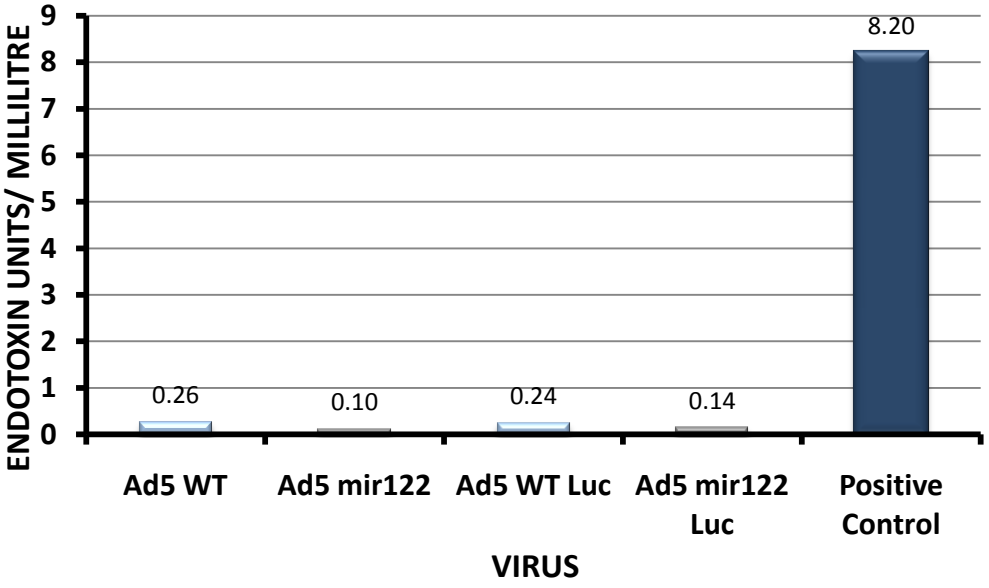
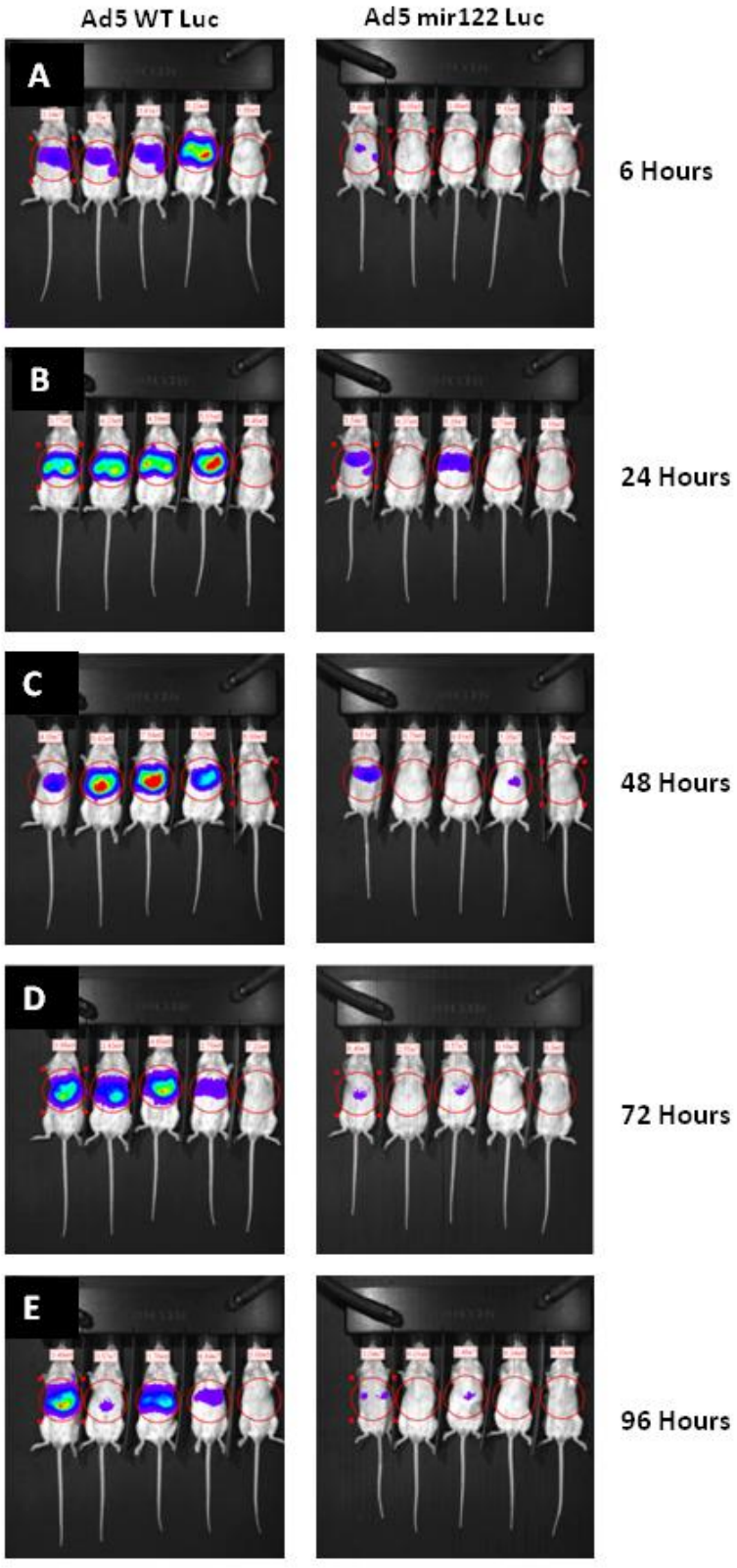


Figure 5.1: Endotoxin quantification of adenovirus preparations. The endotoxin concentration in each of the virus preparations used in this thesis was calculated using a chromogenic assay in order to ensure any toxicological readings were due to virus mediated effects and not due to immune-stimulatory contaminants. Results showed that whilst a sample known to contain endotoxin (marked positive control) produced significant readings, all adenovirus preparations were free of endotoxin (classified as <1 EU/ml).


Ad5 WT Luc
Ad5 mir122 Luc
A
6 Hours
B
24 Hours
C
48 Hours
D
72 Hours
E
96 Hours

F:

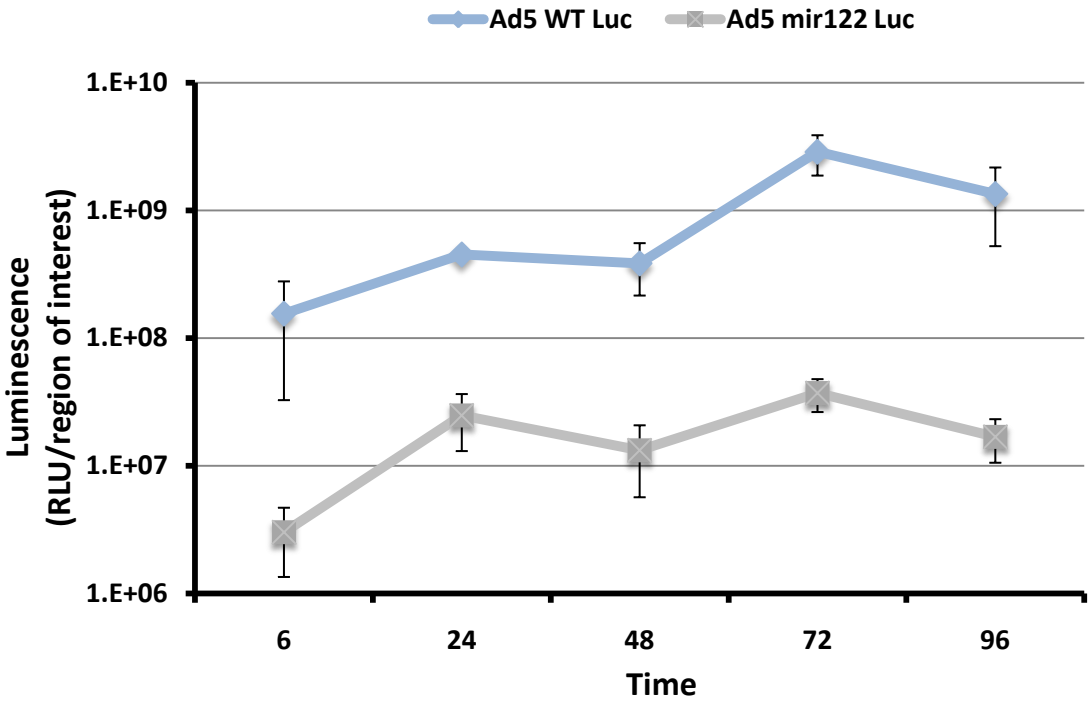


Figure 5.2: Luciferase transgene expression following intravenous administration of reporter viruses to mice *in vivo*. A-E: Groups of four mice were administered intravenously $5x10^{10}$ virus particles of Ad5-WT-Luc (left hand image in each pair of images) or Ad5-mir122-Luc (right hand image in each pair of images) and luminescence was quantified using an IVIS100 imaging system after 6, 24, 48, 72 and 96 h. The mouse on the right of all images is an untreated control, mock injected with luciferin for background levels. Images within pairs can be directly compared, although the scaling is different between time points. F: Graph to summarise liver luciferase expression profile as a function of time.

MicroRNA regulation of Ad5 WT reduces genomic replication and hepatic toxicity

In order to assess the effects on hepatic toxicity of including microRNA mir122 binding sites within wild type adenovirus, $5x10^{10}$ vp of Ad5 WT and Ad5-mir122 were injected intravenously to Balb/C mice. One mouse in the study which received Ad5 WT became hunched and immobile, and was sacrificed after 60 h with macroscopic hepatic pathology. Remaining mice were exsanguinated under anaesthesia 72 h post-injection and blood was allowed to clot. Serum from both groups was tested for Alanine Aminotransferase (ALT) levels and Aspartate Aminotransferase (AST) to assess hepatic damage. Mice administered Ad5 WT showed significantly increased ALT levels (90 times higher than control mice treated with PBS; Figure 5.3) suggesting substantial liver damage had occurred. Mice administered Ad5-mir122 showed approximately 15-fold less serum ALT (5 times normal) demonstrating that less liver toxicity had occurred with this virus. AST results were similar with 17-fold lower AST levels in serum from mice administered Ad5-mir122 compared to serum from mice receiving Ad5 WT (Figure 5.3).

To evaluate viral replication and tissue damage, livers were cut in half and one half of each was used for histological analysis, while QPCR was performed on the other half. Livers taken from mice administered Ad5 WT showed an average of $2x10^9$ ($\pm$ $4.3x10^8$) genomes/mg liver (wet weight; Figure 5.4). In the total liver this represents approximately 60-fold more genome copies compared to the total amount of virus originally injected, suggesting significant viral genome replication. In contrast, livers from mice administered Ad5-mir122 showed only $8x10^7$ ($\pm$ $6.2x10^7$) virus genomes per mg liver, representing less than a doubling compared to the input dose. These data confirm that microRNA

suppression of E1A is capable of significantly reducing replication of the virus genome in murine liver *in vivo*.

Histological analysis showed a substantial difference between animals administered Ad5 WT and those administered Ad5-mir122. Ad5 WT induced vacuolation, haemorrhaging and abnormal nuclear morphology, while livers from mice administered Ad5-mir122 showed very little pathology, with some mice showing no aberrant morphology in any liver section (Figure 5.5). Histological images of liver from a mouse administered $5x10^{10}$ vp of a non-replicating adenoviral vector are presented for comparison, showing similar or slightly greater liver pathology than was induced by Ad5-mir122.

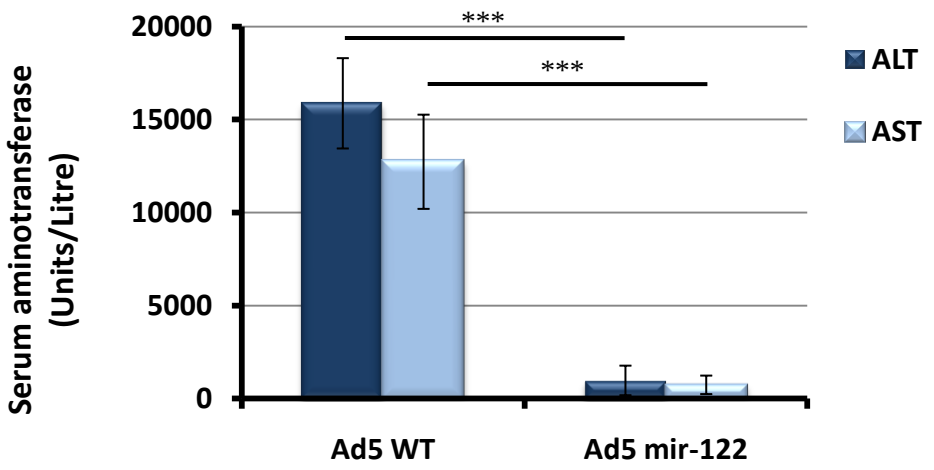


Figure 5.4: Assessment of the hepatotoxicity of Ad5 WT and Ad5-mir122 using Alanine and Aspartate Transaminase (ALT/AST) levels. Measurement of serum ALT (dark blue bars) and AST (light blue bars) released into the blood stream 72h following intravenous administration of $5x10^{10}$ viral particles of Ad5 WT or Ad5-mir122. Data shows that Ad5 WT induces significantly more ALT and AST release from the liver compared to Ad5-mir122. Analysis was performed as described in the methods section (***= P<0.0005).

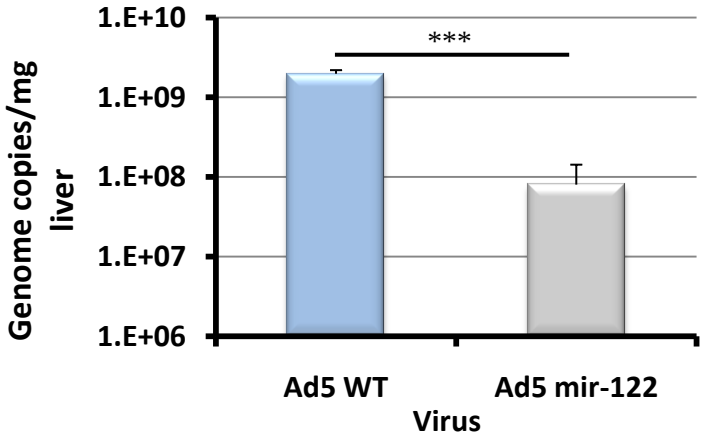


Figure 5.3: Quantitation of genomic replication of Ad5 WT and Ad5-mir122 in murine liver. Adenovirus genomes in murine liver were measured by real time PCR 72h following intravenous administration of $5x10^{10}$ viral particles of Ad5 WT or Ad5-mir122, as described in the methods section. Ad5-mir122 shows significantly reduced genomic replication when compared to Ad5 WT (*** = P<0.0005). The standard curve used to calculate these vales is shown in figure 5.6.

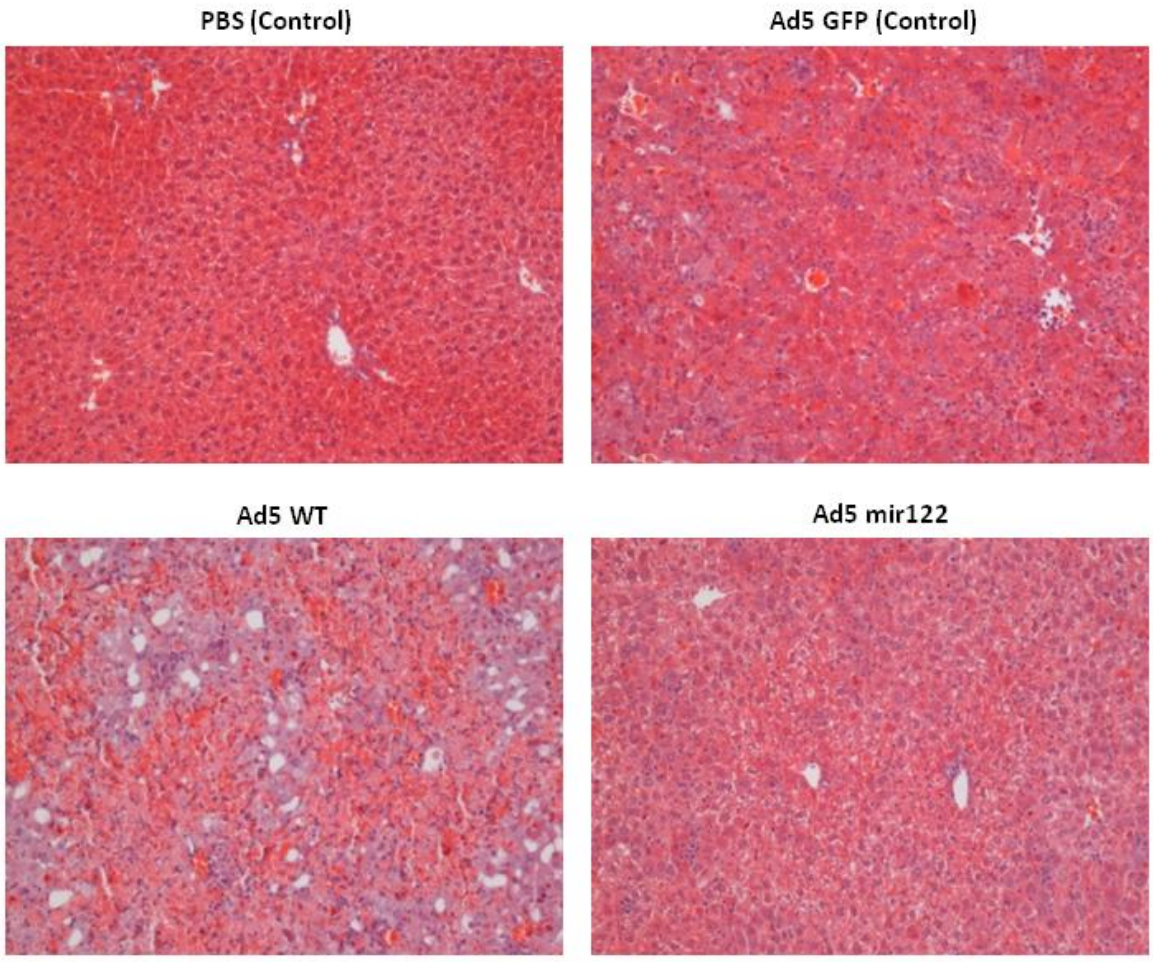


Figure 5.5: Assessment of liver histology. Mice were treated with PBS or $5x10^{10}$vp of either a non-replicating E1, E3-deleted Ad5 expressing GFP (Ad5-GFP), Ad5 WT or Ad5-mir122, as indicated. Mice were euthanised at 72 hours and the left liver lobe from each mouse was immersed in 10% buffered formalin overnight at room temperature, embedded in wax and sectioned using a vibratome. Sections were stained with haematoxylin and eosin and analysed by light microscopy at x 40 magnification. Each image is from an independent mouse, was chosen at random, and is representative of all images obtained for all mice in each group. The damage induced by Ad5 WT is clearly visible whilst Ad5-mir122 shows little to no hepatic pathology.

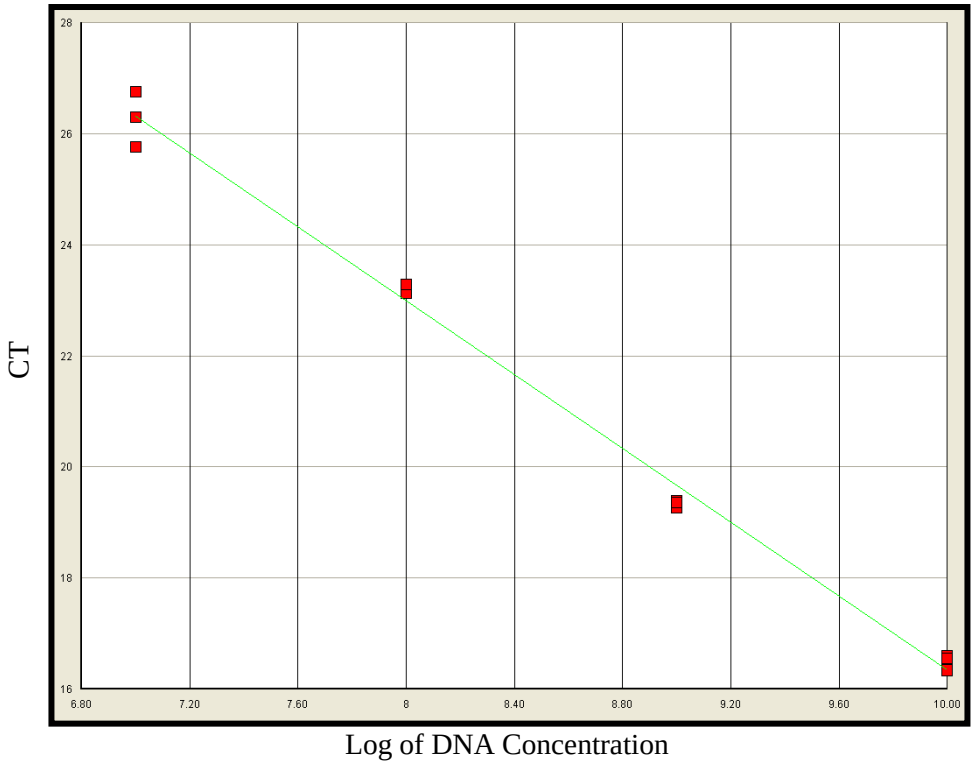


Figure 5.6: QPCR standard curve. Serial dilutions of known concentrations of adenovirus genomic DNA were spiked into samples of murine liver homogenate and extracted using the method identical to all samples tested. Data shows that the efficiency of the reaction was 100.16% with an amplification factor of 2 and a slope of -3.318.

Assessment of intra-hepatic Ad5-mir122 E1A activity

To quantify both the E1A mRNA and protein produced by Ad-mir122 in comparison to Ad5 WT, Balb/C mice were injected i.v with $2x10^{10}$ vp and the livers were harvested after 48 hrs. As further controls, mice were administered PBS or an E1A deleted non-replicating adenovirus type five encoding luciferase (AdLuc) at the same dose. RNA was extracted and the major E1A 13S transcript was measured by RT-QPCR. Ad5-mir122 showed 29-fold lower levels of 13S E1A transcript copies when compared to Ad5 WT. E1A mRNA copies per nanogram of total RNA were $4.57x10^{6}$ and $1.54x10^{5}$, respectively

(Figure 5.7A). This confirmed that the stability of the mRNA is itself directly affected by microRNA regulation, as would be expected given the high degree of homology between mir122 and the binding sites within Ad5-mir122 E1A mRNA.

To confirm that the reduced level of E1A mRNA resulted in decreased E1A protein production, and to confirm that the knockdown was not specific to E1A 13S, liver protein extracts were analysed by western blot for E1A protein. Figure 5.7B shows that whilst infection with Ad5 WT resulted in significant E1A protein production, infection with Ad5-mir122 resulted in almost undetectable levels. In Ad5 WT infected livers, the major species of E1A protein correlating with the 36kDa (13S) and 26kDa (12S) proteins are clearly visible with a smaller band that may represent the protein product of the 10S or 11S mRNA. No E1A signals were detected in control mice receiving PBS or a non-replicating E1A deleted AdLuc vector. Equal protein loading and blot transfer were confirmed by Ponceau staining (Figure 5.8).

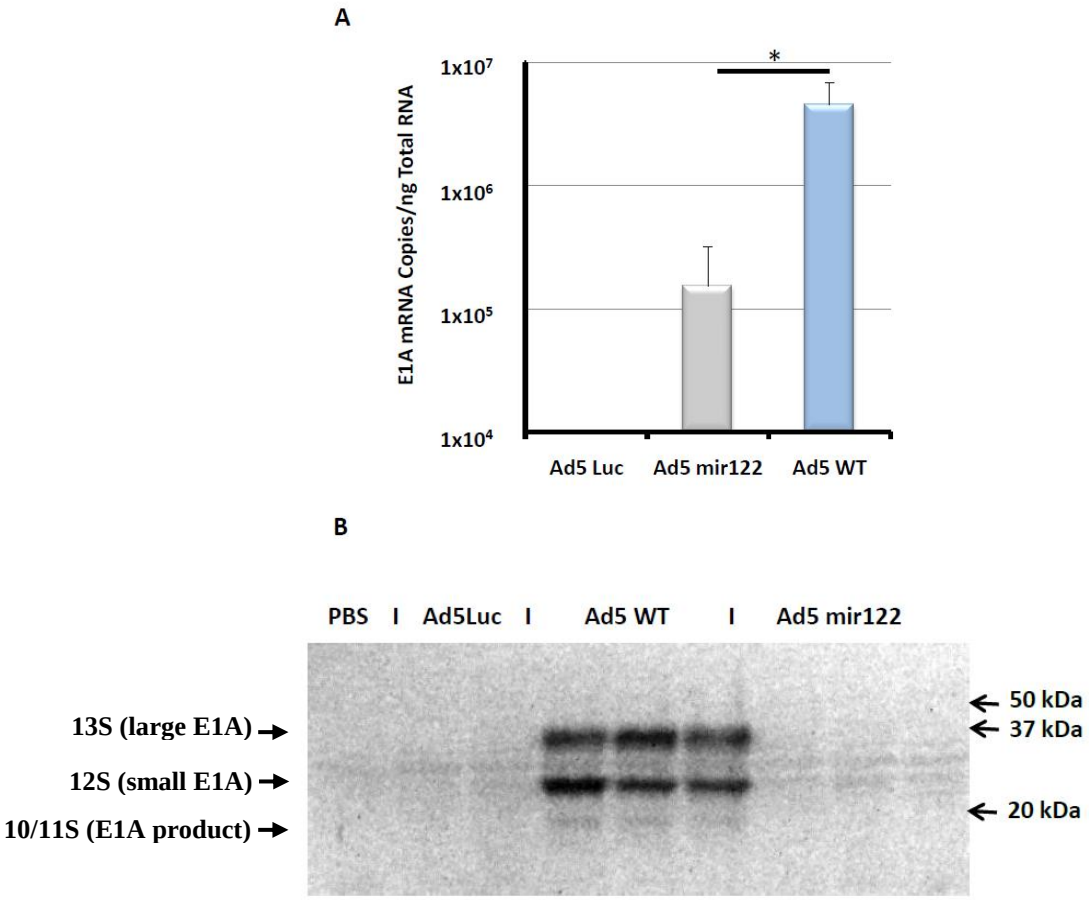


Figure 5.7: MicroRNA mediated knockdown of E1A mRNA and protein measured by RT-QPCR and western blot. A) RT QPCR for the 13S E1A mRNA transcript in the livers of mice 48 hrs after intravenous injection with $2x10^{10}$ vp of Ad5-mir122, Ad5 WT, AdLuc or PBS. Ad5-mir122 shows significantly reduced E1A mRNA compared to Ad5 WT (N=3). Statistical analysis was performed using a two tailed student T-Test (*P=<0.05). B) Western blot of liver extracts for all E1A proteins variants. Each lane represents protein from an individual mouse. Control lanes from mice treated with an E1A deleted AdLuc vector or PBS show no E1A signal. Ad5 WT treatment shows three clearly defined bands corresponding to proteins produced from the 13S (36 kDa), 12S (26 kDa) and a smaller fainter band which may represent the 11S or the 10S E1A transcript product. Treatment with Ad5-mir122 shows significant knockdown in E1A protein levels for all splice variants.

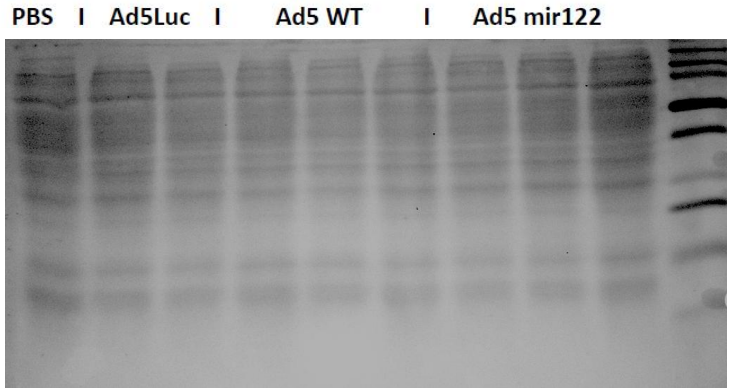


Figure 5.8: Ponceau stain of E1A western blot. Liver was homogenised and standardised against protein content as described in the methods section. Successful transfer and equal protein loading onto the nitrocellulose membrane was confirmed by Ponceau stain as described in the methods section and imaged using an Alpha Innotech gel documentation system using the light image function.

Mir122 levels remain unaffected by Ad5-mir122 infection

The production of E1A mRNA within hepatocytes that contains multiple microRNA binding sites could lead to the sequestration of mir122 away from its endogenous mRNA targets or depletion of the total mir122 content in the cell. The livers screened in Figure 5.7 by E1A western blot and RT-QPCR were analysed to determine the quantity of mature mir122 RNA. All results were standardised against the highly abundant microRNA let7a which should be unaffected by Ad-mir122 treatment. Comparative analysis of the level of mir122 found in mice treated with Ad5 WT, Ad5-mir122 or AdLuc showed that there was no difference in the levels of mir122 following any virus administration when compared to the level of mir122 in mice administered PBS (Figure 5.9). The ratio of mir122 levels in mice administered Ad5 WT, Ad-mir122 and AdLuc when compared to the amount in mice receiving PBS were 1.04 (±0.188), 1.06 (±0.163) and 1.14 (±0.14), respectively. Standard curves used to calculate these data are visible in Figures 5.10 and 5.11. Results from the quantification of Let7a used to correct this data are shown in Figure 5.12. These data proved that the level of mir122 was unaffected by the presence of Ad5-mir122 infection, however, it did not confirm if mir122 was being sequestered away from its endogenous mRNA targets by direct competition.

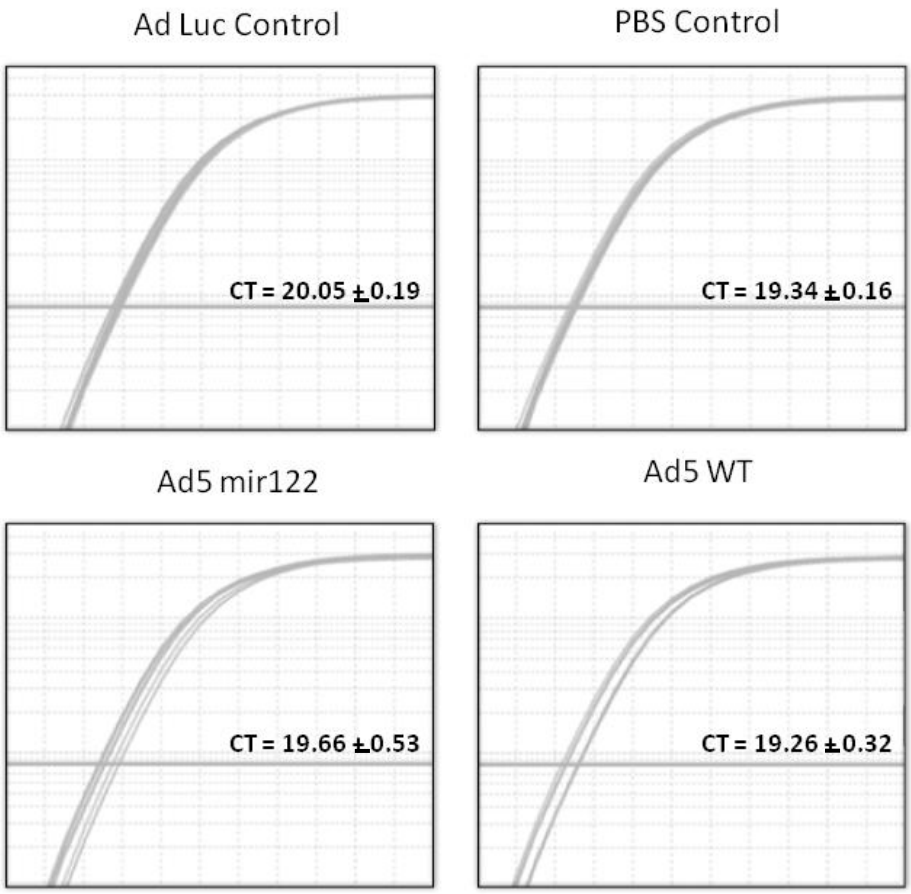


Figure 5.9: The quantity of mature mir122 *in vivo* is unaffected by Ad5-mir122. Mice were injected with $2x10^{10}$ vp of either Ad5-mir122, Ad5 WT, an E1A deleted AdLuc vector or PBS. Quantification of mir122 mature RNA levels was performed using a Taqman microRNA assay specific for mir122. CT values were corrected against the levels of the microRNA, let7a, as a reference gene using the method published by (Pfaffl 2001). Panels show nine superimposed RT-QPCR amplification curves (from three mice) for mir122 in each treatment group, before correction against let7a. Samples were reverse transcribed using equal amounts of total RNA (5 ng) and RT-PCR was performed using equal amounts of cDNA. CT values shown here represent the average of the nine reactions from three mice plus or minus standard deviation in each group.

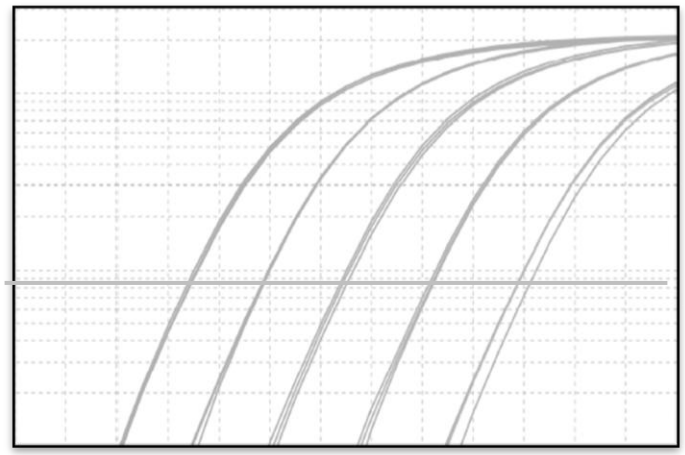


Figure 5.10: Let7a standard curve. In order to ensure that the conditions used to quantify Let7a were within the linear range of the primer and reaction conditions it was necessary to perform a serial dilution relative standard curve. Samples were serially diluted from neat stock by 10-fold. Curve properties are as follows slope 3.268, 102.29%, efficiency 2.01. This confirms that test reactions were conducted within the linear range of the PCR reaction.

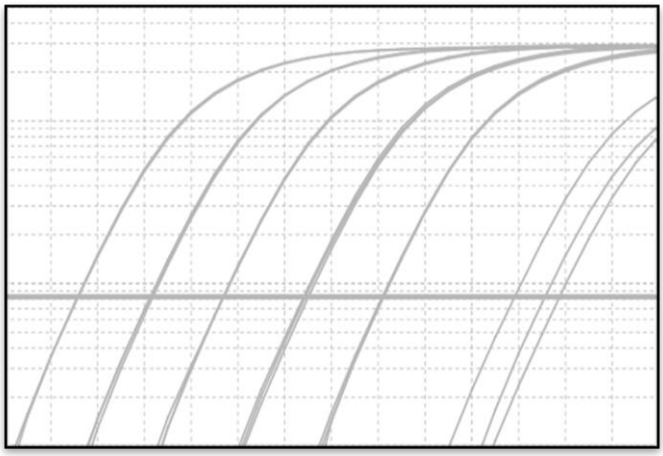


Figure 5.11: Mir122 standard curve. In order to ensure that the conditions used to quantify mir122 were within the linear range of the primer and reaction conditions it was necessary to perform a serial dilution relative standard curve. Samples were serially diluted from neat stock by 10-fold. Curve properties are as follows: slope 3.28, 101.78%, efficiency 2.02. This confirms that test reactions were conducted within the linear range of the PCR reaction.

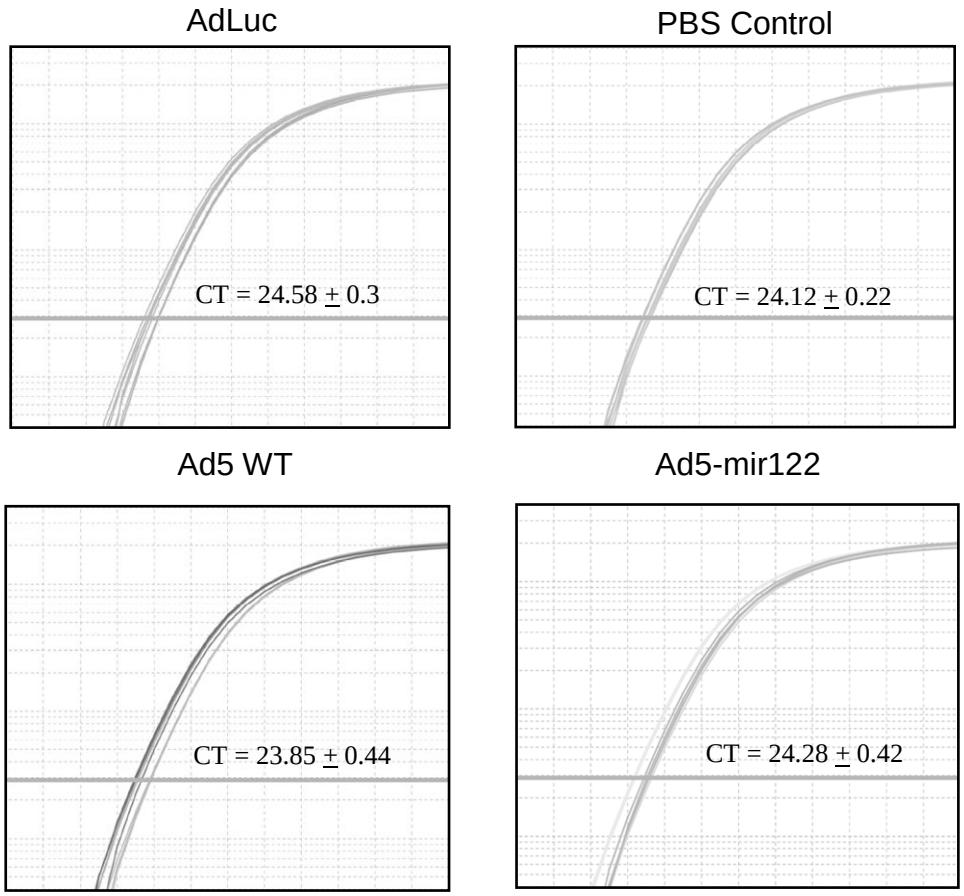


Figure 5.12: Let7a quantitation as a novel internal standard. The use of another microRNA, in this case Let7a, as an internal standard for quantifying a different microRNA (mir122) had not been shown before. This approach compensates for differences in the total microRNA content between mice which are not taken into account when using 18S or GAPDH controls. Panels show nine superimposed RT-QPCR amplification curves (from three mice) for Let7a in each treatment group in each panel. Samples were reverse transcribed using equal amounts of total RNA (5 ng) and RT-PCR was performed using equal amounts of cDNA. CT values shown here represent the average of nine reactions from three mice plus or minus standard deviation.

Ad5-mir122 reduces genome wide hepatocyte mRNA changes

Microarray genome wide mRNA profiling was performed on livers of mice 48 hrs after the injection of either $2x10^{10}$ vp of Ad5-mir122, Ad5 WT, an E1A deleted non-replicating AdLuc vector or PBS. This assessment allowed the quantitation of mRNA changes due to microRNA regulation of Ad5-mir122, but also served to highlight any mRNA differences induced by infection with either Ad5-mir122 or Ad5 WT. The maximum change observed in any mRNA was 111-fold (Table 5.1), although any mRNA profile change that was greater than 2-fold was considered significant and highlighted for further analysis. Data from the three independent animals in each group was remarkably consistent and can be viewed at the NCBI's Gene Expression Omnibus database under accession GSE23854. The most striking result between the different viruses was the total number of mRNAs changed. Whilst Ad5 WT altered 3946 mRNAs $\geq$ 2-fold, Ad5-mir122 altered only 668 mRNAs (Figure 5.13). The E1A deleted AdLuc vector altered a total of 564 mRNAs $\geq$2-fold. This shows that the technique can detect many changes of varying magnitude but that infection of murine liver with Ad5-mir122 causes significantly fewer intracellular changes than infection with Ad5 WT.

The data obtained by microarray profiling was sorted to compare the highest fold changes observed between treatment with Ad5 WT and PBS. The most significant 20 changes in mRNA quantity can be seen in table 5.2. Interestingly, within this list, most mRNAs that have been significantly altered by Ad5 WT have also been altered by Ad5 mir122 but not by treatment with the non-replicating AdLuc vector. This suggests that these changes in mRNA are a consequence of either low levels of E1A expression, or low levels of virus genome replication, because previous data had shown that Ad5 mir122 does show

significant genomic replication albeit less than Ad5 WT. It is also interesting to find that many of the mRNAs demonstrating the highest fold change in expression are related to immune activation. These include the interferon induced proteins CXCL10, IFIT2 IFIT3 as well as Interferon Gamma itself. These findings suggest that the innate immune response to adenovirus is significantly enhanced by even low levels of activation of the replication cycle, which could be an important finding for vectored vaccine research.

When the same data are sorted to directly compare the mRNA changes induced by Ad5 WT and Ad5 mir122 many significant differences can be observed, the most significant of these alterations can be viewed in table 5.2. Many of the mRNAs changes are also shared between Ad5 WT and AdLuc or between Ad5 WT and PBS treatment. This suggests that the differences observed between Ad5 WT and Ad5 mir122 are related to Ad5 WT replication, rather than changes induced by treatment with Ad5 mir122 that are not also observed in control mice. Therefore, these changes, which appear to correlate with no particular pathway or biological response, are likely to reflect the toxicity of Ad5 WT, rather than any unique toxicity induced by Ad5 mir122.

The presence of additional microRNA regulated mRNA could lead to sequestration of mir122 molecules away from their endogenous targets. Therefore, any mRNA change observed between mice administered PBS and Ad5-mir122 that was not shared between Ad5-mir122 and either Ad5 WT or AdLuc was considered Ad5-mir122 specific and was investigated further. These mRNAs, which numbered only 21 in total (Table 5.3), were compared with the predicted mir122 targeted mRNAs in the European Bioinformatics Institutes (EBI) Microcosm database. Sequence analysis showed that no transcripts were predicted to contain mir122 binding sites. The list of mir122 microcosm predicted targets

was then compared to all the signals from the microarray profiling to make sure no important signals had been omitted by filtering the results as described above. 143 Predicted mir122 targets were altered in the microarray analysis. Of those 143 mRNAs, 108 were altered only by Ad5 WT, 20 were shared by Ad5-mir122 and Ad5 WT but were altered to the same extent and in the same direction (up- or down-regulated). The remaining signals showed AdLuc specific changes. This data shows that during the window of initial infection with Ad5-mir122 the presence of microRNA regulated viral mRNA does not alter the level of the endogenous microRNA mRNA targets.

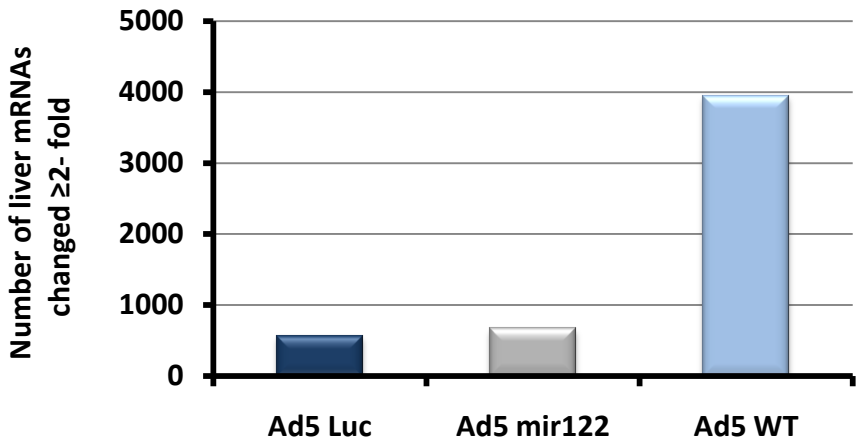


Figure 5.13: Genome wide mRNA microarray screening. Mice were injected intravenously with $2x10^{10}$ vp of either Ad5-mir122, Ad5 WT, AdLuc or PBS and euthanised after 48 hrs. Results show the total number of positive signals in which the median mRNA level changed by ≥ 2-fold in all mice in each group in comparison to the median mRNA level in mice treated with PBS (n=3 for all groups). The total number of genes altered was calculated and is shown using the total number of genes changed in all three mice, therefore, no error bars are shown.

PBS VS Ad5 WT Fold change	*AdLuc VS Ad5 WT* Fold change	*PBS VS Ad5 mir122* Fold change	*AdLuc VS Ad5 mir122* Fold change	*Ad5 WT VS Ad5 mir122* Fold change	*AdLuc VS PBS* Fold change	*Gene Symbol*	*Gene Name*	*Accession*
111.0 ↓	117.8 ↓	70.6 ↓	74.9 ↓	1.6 ↑	1.1 ↓	Cxcl10	Chemokine (C-X-C motif) ligand 10 (Cxcl10), mRNA.	NM_021274.1
71.4 ↓	53.5 ↓	37.7 ↓	28.2 ↓	1.9 ↑	1.3 ↑	2510004L01Rik	Radical S-adenosyl methionine domain containing 2 (Rsad2), mRNA.	NM_021384.2
68.7 ↓	84.5 ↓	65.9 ↓	81.1 ↓	1.0 ↑	1.2 ↓	Gbp1	Guanylate nucleotide binding protein 1 (Gbp1), mRNA.	NM_010259.1
62.2 ↓	60.0 ↓	22.8 ↓	22.0 ↓	2.7 ↑	1.0 ↑	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2 (Ifit2), mRNA.	NM_008332.2
51.9 ↓	37.2 ↓	26.5 ↓	19.0 ↓	2.0 ↑	1.4 ↑	Tyki		NM_020557.3
47.3 ↓	57.4 ↓	29.7 ↓	36.0 ↓	1.6 ↑	1.2 ↓	Ubd	Ubiquitin D (Ubd), mRNA.	NM_023137.2
46.1 ↓	51.6 ↓	32.5 ↓	36.4 ↓	1.4 ↑	1.1 ↓	Serpina3g		XM_354694.1
45.0 ↓	45.7 ↓	1.8 ↓	1.8 ↓	24.7 ↑	1.0 ↓	Spred2	Sprouty-related, EVH1 domain containing 2 (Spred2), mRNA.	NM_033523.2
44.1 ↓	64.1 ↓	45.0 ↓	65.3 ↓	1.0 ↓	1.5 ↓	Cxcl9		NM_008599
42.2 ↑	25.6 ↑	4.8 ↑	2.9 ↑	8.7 ↓	1.6 ↓	Pte2a		NM_134246.2
41.4 ↓	40.9 ↓	14.8 ↓	14.6 ↓	2.8 ↑	1.0 ↑	Ifng	Interferon gamma (Ifng), mRNA.	NM_008337.1
41.2 ↓	45.7 ↓	15.6 ↓	17.3 ↓	2.6 ↑	1.1 ↓	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2 (Ifit2), mRNA.	NM_008332.2
36.4 ↓	43.6 ↓	33.7 ↓	40.3 ↓	1.1 ↑	1.2 ↓	G1p2	ISG15 ubiquitin-like modifier (Isg15), mRNA.	NM_015783.1
31.3 ↓	38.7 ↓	35.3 ↓	43.8 ↓	1.1 ↓	1.2 ↓	Gbp2	Guanylate nucleotide binding protein 2 (Gbp2), mRNA.	NM_010260.1
30.7 ↑	10.8 ↑	5.6 ↑	2.0 ↑	5.5 ↓	2.8 ↓	8430408G22Rik	RIKEN cDNA 8430408G22 gene (8430408G22Rik), mRNA.	NM_145980.1
29.8 ↑	24.5 ↑	3.3 ↑	2.7 ↑	9.1 ↓	1.2 ↓	Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1), mRNA.	NM_007824.2
29.4 ↓	25.6 ↓	20.2 ↓	17.6 ↓	1.5 ↑	1.1 ↑	Ly6a	Lymphocyte antigen 6 complex, locus A (Ly6a), mRNA.	NM_010738.2
28.5 ↓	34.9 ↓	36.5 ↓	44.7 ↓	1.3 ↓	1.2 ↓	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3 (Ifit3), mRNA.	NM_010501.1
27.9 ↓	28.3 ↓	11.1 ↓	11.3 ↓	2.5 ↑	1.0 ↓	Ifng	Interferon gamma (Ifng), mRNA.	NM_008337.1
27.8 ↑	22.3 ↑	1.4 ↑	1.1 ↑	19.7 ↓	1.2 ↓	Keg1	Kidney expressed gene 1 (Keg1), mRNA.	NM_029550.1

Table 5.2: The twenty most significant liver mRNA changes found by microarray profiling between mice infected with Ad5 WT and Ad5mir122

Ad5 WT VS Ad5mir122 Fold change	*AdLuc VS Ad5 WT* Fold change	*PBS VS Ad5 WT* Fold change	*AdLuc VS Ad5mir122* Fold change	*PBS VS Ad5mir122* Fold change	*AdLuc VS PBS* Fold change	*Gene Symbol*	*Gene Name*	*Accession*
24.7 ↑	45.7 ↓	45.0 ↓	1.8 ↓	1.8 ↓	1.0 ↓	Spred2	Sprouty-related, EVH1 domain containing 2 (Spred2), mRNA.	NM_033523.2
19.7 ↓	22.3 ↑	27.8 ↑	1.1 ↑	1.4 ↑	1.2 ↓	Keg1	Kidney expressed gene 1 (Keg1), mRNA.	NM_029550.1
13.3 ↓	15.5 ↑	19.1 ↑	1.2 ↑	1.4 ↑	1.2 ↓	Nudt7		NM_024437
12.8 ↓	12.9 ↑	23.1 ↑	1.0 ↑	1.8 ↑	1.8 ↓	Slco1b2	Solute carrier organic anion transporter family, member 1b2 (Slco1b2), transcript variant 1, mRNA.	NM_020495.1
12.5 ↓	11.0 ↑	15.0 ↑	1.1 ↓	1.2 ↑	1.4 ↓	Csrp3	Cysteine and glycine-rich protein 3 (Csrp3), mRNA.	NM_013808.3
11.7 ↓	12.5 ↑	8.4 ↑	1.1 ↑	1.4 ↓	1.5 ↑	G6pc		NM_008061.2
11.7 ↓	14.3 ↑	15.5 ↑	1.2 ↑	1.3 ↑	1.1 ↓	Nudt7	Nudix (nucleoside diphosphate linked moiety X)-type motif 7 (Nudt7), transcript variant 1, mRNA.	NM_024437.1
10.5 ↓	2.0 ↑	5.6 ↑	5.2 ↓	1.9 ↓	2.8 ↓	Upp2	Uridine phosphorylase 2 (Upp2), mRNA.	NM_029692.1
10.0 ↓	9.5 ↑	11.8 ↑	1.0 ↓	1.2 ↑	1.2 ↓	Csrp3	Cysteine and glycine-rich protein 3 (Csrp3), mRNA.	NM_013808.3
9.5 ↓	7.5 ↑	15.7 ↑	1.3 ↓	1.7 ↑	2.1 ↓	Ugt3a1	UDP glycosyltransferases 3 family, polypeptide A1 (Ugt3a1), mRNA.	NM_207216.1
9.4 ↓	11.0 ↑	12.8 ↑	1.2 ↑	1.4 ↑	1.2 ↓	Cyp2a5		NM_007812.1
9.4 ↓	14.4 ↑	11.2 ↑	1.5 ↑	1.2 ↑	1.3 ↑	Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1 (Cyp8b1), mRNA.	NM_010012.2
9.3 ↓	2.3 ↑	5.0 ↑	4.0 ↓	1.9 ↓	2.2 ↓	Upp2	Uridine phosphorylase 2 (Upp2), mRNA.	NM_029692.1
9.3 ↓	7.1 ↑	14.8 ↑	1.3 ↓	1.6 ↑	2.1 ↓	2810007J24Rik	RIKEN cDNA 2810007J24 gene (2810007J24Rik), mRNA.	NM_175250.3
9.1 ↑	10.0 ↓	11.0 ↓	1.1 ↓	1.2 ↓	1.1 ↑	LOC329521		XM_194054.2
9.1 ↓	24.5 ↑	29.8 ↑	2.7 ↑	3.3 ↑	1.2 ↓	Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1), mRNA.	NM_007824.2
8.7 ↓	25.6 ↑	42.2 ↑	2.9 ↑	4.8 ↑	1.6 ↓	Pte2a		NM_134246.2
8.5 ↓	3.8 ↑	9.4 ↑	2.2 ↓	1.1 ↑	2.5 ↓	Dio1		NM_007860
8.5 ↓	2.1 ↑	5.1 ↑	4.1 ↓	1.7 ↓	2.5 ↓	Upp2	Uridine phosphorylase 2 (Upp2), mRNA.	NM_029692.1
8.4 ↓	15.4 ↑	18.5 ↑	1.8 ↑	2.2 ↑	1.2 ↓	Dbp	D site albumin promoter binding protein (Dbp), mRNA.	NM_016974.1

Gene Symbol	Compared to PBS		Compared to Ad5Luc		Compared to Ad5 WT		Gene Name	Accession
LOC381748	4.9	?	5.6	?	2.7	?		XM_355738.1
2310016F22Rik	4.6	?	5.2	?	2.6	?	RIKEN cDNA 2310016F22 gene (2310016F22Rik)	NM_173743.2
Irf1	4.3	?	5.0	?	2.3	?	Interferon regulatory factor 1 (Irf1)	NM_008390.1
Trim21	3.8	?	4.2	?	2.2	?		NM_009277.2
A630097K09Rik	3.5	?	3.7	?	2.5	?		AK042505
C2ta	3.4	?	3.3	?	2.2	?		AK040723
LOC435565	3.4	?	5.2	?	2.4	?	Similar to interferon-inducible GTPase (LOC435565)	NM_001013828.1
Trim34	3.1	?	3.4	?	2.2	?	Tripartite motif protein 34 (Trim34)	NM_030684.1
Tapbpl	3.1	?	3.3	?	2.1	?	TAP binding protein-like (Tapbpl)	NM_145391.1
Trim34	3.0	?	3.3	?	2.0	?	Tripartite motif protein 34 (Trim34)	NM_030684.1
ligp-pending	2.9	?	3.4	?	2.0	?		AK089702
H2-T23	2.8	?	3.5	?	2.1	?	Histocompatibility 2, T region locus 23 (H2-T23)	NM_010398.1
Scotin	2.6	?	2.8	?	2.2	?	Scotin gene (Scotin), transcript variant 2	NM_026381.1
Saa1	2.4	?	2.5	?	2.0	?		NM_009117.1
EG240327	2.4	?	2.8	?	2.4	?	Predicted gene, EG240327 (EG240327)	NM_001033767.1
LOC280487	2.3	?	2.1	?	3.6	?		XM_203701.3
1200013B22Rik	2.3	?	3.0	?	2.2	?	NUAK family, SNF1-like kinase, 2 (Nuak2)	NM_028778.2
Tapbp	2.2	?	3.0	?	2.9	?	TAP binding protein (Tapbp), transcript variant 1	NM_001025313.1
0710001B24Rik	2.1	?	3.0	?	2.0	?		NM_175118
LOC244882	2.1	?	2.2	?	2.2	?		XM_146997.2
Tnfaip8l3	2.0	?	2.0	?	2.1	?	Tumor necrosis factor, alpha-induced protein 8-like 3 (Tnfaip8l3)	NM_001033535.1

Table 5.3 Total mRNA profiling produced 4562 signals in which the mRNA levels were altered ? 2-fold after administration of either $2x10^{10}$ vp of Ad5 WT, Ad5-mir122, an Ad5luc control vector or PBS. The mRNAs in this table are those that show at least a 2-fold change in quantity following Ad5-mir122 infection that are not altered when compared to mice administered PBS, Ad5luc or Ad5 WT. Mir122 predicted target mRNAs (produced using the Microcosm database) were compared to this list but no positive matches were found.

Mir122 regulated Adolase A levels remain unaffected by Ad5-mir122

Although no differences had been observed in the levels of known mir122 regulated mRNAs, it was unclear if the quantity of protein produced from these transcripts was altered. MicroRNA suppression has been shown to decrease the protein level produced from some mRNAs without changing the mRNA steady state levels (Mathonnet *et al.* 2007). Therefore, mice were injected with $2x10^{10}$ vp of Ad5-mir122, Ad5 WT, AdLuc or PBS. After 48 hours, livers were analysed by western blot for the levels of Aldolase A protein, a known mir122 target (Esau *et al.* 2006; Fabani *et al.* 2008). Inhibition of mir122 function is known to increase Aldolase A protein levels although the western blot showed no difference between mice in the different treatment groups (Figure 5.14). This data suggests that mir122 regulation of Aldolase A is maintained despite the presence of Ad5-mir122 infection.

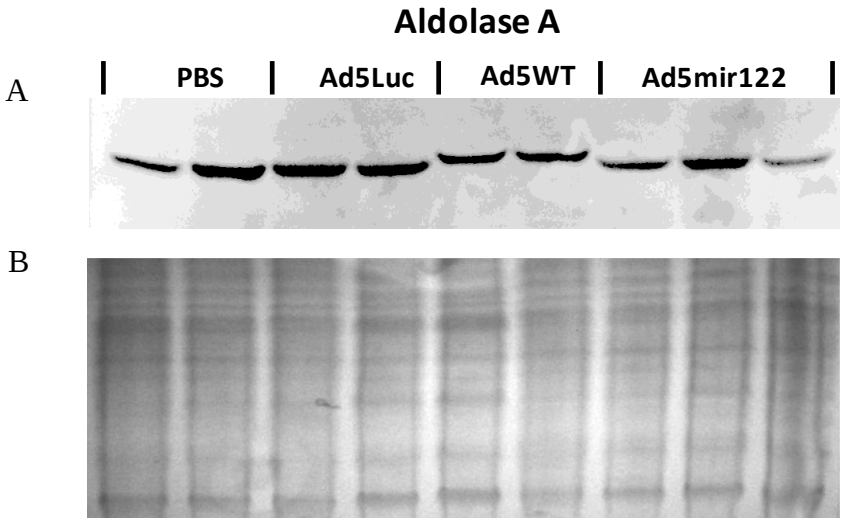


Figure 5.14: Regulation of the mir122 target protein Aldolase A is unaffected *in vivo* by Ad5-mir122. Western blot analysis of the mir122 regulated protein Aldolase A in mice treated as above. Liver protein extracts were subjected to a BCA protein assay and equal transfer was confirmed by Ponceau stain (B).

5.3. Concluding discussion:

The effectiveness of microRNA regulation of adenovirus using hepatic specific microRNA binding sites in the E1A mRNA was assessed *in vivo* to determine if this approach could reduce virus toxicity. Results showed that this technique is capable of substantially preventing protein production from E1A mRNA even at doses that are well above the normal maximum tolerated dose of the wild type strain. Successful knockdown of E1A was measured by both western blot and RT-QPCR and data showed that all transcript variants were successfully controlled by mir122. The role of E1A protein as a key viral replication initiation factor was predicted to reduce viral replication and therefore also reduce hepatic toxicity. This was confirmed by measuring both adenoviral genomes and the release of Alanine and Aspartate Transaminase into the blood. Both were significantly lower when compared to Ad5 WT demonstrating that the use of microRNA binding sites to control adenovirus can reduce viral genome amplification and thereby significantly decrease toxicity.

The microRNA network has evolved to ensure that the quantity of each microRNA that is expressed is sufficient to control the quantity of mRNA it needs to target and regulate. However, if additional mRNA molecules are fed into this system the quantity of microRNA available for silencing the endogenous mRNAs may become limiting. This may induce unpredictable toxicities that can only be quantified by analysing multiple points in the microRNA pathway.

The addition of exogenous mRNA into the mir122 regulatory pathway could have caused increased turnover of the microRNA itself. Therefore the quantity of mir122 was

calculated after exposure to Ad5-mir122 by RT-QPCR. This analysis showed that mir122 levels remained unaltered by Ad5-mir122 infection and the addition of mir122 regulated exogenous mRNA (E1A mRNA).

Despite this finding it was unclear if the microRNA was being sequestered away from its endogenous mRNA targets and thereby causing an increase in mir122 regulated proteins. Western blot for the known mir122 regulated protein Aldolase A showed that this was not occurring with Aldolase A levels remaining constant during infection with Ad5-mir122. Further analysis screened for both specific and non-specific intracellular changes mediated by Ad5-mir122 to attempt to monitor off-target effects. Genome wide microarray screening showed that almost no intracellular changes are induced by the presence of Ad5-mir122 that are not also induced by either a replicating un-regulated (Ad5 WT) or a non-replicating adenovirus vector (AdLuc).

Those limited number of signals which were specifically induced by Ad5-mir122 were of unknown origin. Most of the changes induced showed a less than 5-fold difference when compared to the data produced from the other groups in the study (Ad5 WT, AdLuc and PBS). These results may represent artefacts of the microarray screen given that >40 000 transcripts were screened and only 21 were unique to Ad5-mir122, an artefact rate of 0.05%. This is well below 5% significance cut-off which was used to identify positive signals and therefore these results may reflect false-positive readings. Interestingly, however, these changes were consistent across three independent mice and it is therefore also possible that these changes represent changes that are only induced when low levels of E1A are produced. These changes may not occur at high, non-limited E1A levels as seen with Ad5 WT or in the absence of E1A, as seen in the non-replicating Ad Luc

vector. The mRNAs altered appear to belong to no single biochemical pathway or correspond to any particular toxicological response. Therefore, given the limited number and the small size of the changes induced, and the lack of apparent toxicity at the doses used, it is unlikely that Ad5-mir122 at these doses induces any microRNA specific toxicity.

Within this chapter the concept of exploiting microRNA regulation to reduce virus replication was assessed. The results suggest that this approach is effective at preventing virus replication and toxicity and no signs of any toxicity unique to this method of regulation were detected. As such the approach is a safe alternative to other forms of virus regulation and offers the potential to maintain wild type virus replication kinetics in cells not expressing the microRNA exploited for regulation.

Chapter Six: Optimal dosage assessment and anti-cancer efficacy of Ad5-mir122

6.1. Introduction:

Secondary liver metastasis

The complex vasculature and nutrient rich environment of the human liver make it a common site of secondary cancer metastasis for many neoplasias. This complication is common in the development of advanced colorectal carcinoma with 12-14000 new cases reported in the United Kingdom each year, approximately 50% of all colorectal patients (Burke *et al.* 1996). The only curative treatment of colorectal secondary metastasis to the liver is surgery but this is only possible in less than 20% of patients. Secondary treatments include chemotherapy based on fluorinated pyrimidine bases (e.g. 5-fluorouracil) or thymidylate kinase inhibitors (e.g. folinic acid) but also the direct injection of ethanol or cryotherapy using liquid nitrogen.

The prognosis for patients diagnosed with colorectal carcinoma liver metastasis is usually poor with an overall median survival in untreated patients of seven months. Therefore, the development of therapeutic agents which exploit the differential expression of genes between primary hepatocytes and cancerous cells should lead to targeted agents with a significantly improved therapeutic index. The restricted expression of microRNA mir122 within hepatocytes makes this gene a promising candidate for the development of new genetically targeted treatment modalities.

Primary hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is formed by the genetic alteration of hepatocytes which leads to cellular transformation and the development of cancerous lesions within the liver.

Each year in excess of 700 000 new patients are diagnosed with HCC with an overall relative 5 year survival rate of around 10% (IARC 2011). The overall median survival is approximately 8 months (Schoniger-Hekele *et al.* 2001). Factors which promote the development of HCC include excessive alcohol consumption, chronic Hepatitis B and C infections, fungal derived aflatoxin, haemochromatosis and cirrohosis of the liver. The genetics of HCC are complex with no single identifiable genetic lesion shared in all patients, perhaps reflecting the wide variety of factors responsible for inducing the disease. Proteins in the p53, Wnt (β-catenin), pRb, Ras, TGF-β and DNA methylation pathways are often mutated in HCC although the contribution each pathway makes to HCC development varies widely. This spectrum of genetic changes and biochemical alterations makes it difficult to design broad acting therapies that are targeted to exploit the genetic changes found within HCC and is a major limiting factor in treatment design.

MicroRNA mir122 in hepatocellular carcinoma

The discovery of the microRNA network has led to the screening of many cancers in an attempt to identify unique targets for therapeutic intervention. Screening of the microRNA content of primary HCC and HCC derived cell lines compared to normal human hepatocytes has identified one possible target. MicroRNA mir122 expression has been found to decrease as the stage and aggressiveness of the HCC tumour increases. This observation is reflected in the finding that most human HCC derived cell lines demonstrate either no, or reduced, mir122 expression. The high level of expression of mir122 within hepatocytes is a major determinant of hepatocyte phenotype and function. It is therefore unsurprising that as HCC cells de-differentiate away from the phenotype of

a normal hepatocyte they lose those important markers responsible for hepatocyte identity, including mir122 expression. This finding has the potential to offer a significant therapeutic index between normal mir122 positive hepatocytes and mir122 negative hepatoma cells if this expression differential can be exploited.

The design of viruses that are selectively activated in the absence of mir122 should therefore exploit a substantial difference between the cancerous cell population and the normal surrounding liver tissue when compared to an un-regulated virus. The virus designed here, Ad5-mir122, shows selective virus replication in all cells negative for mir122 and it was the purpose of this chapter to prove the utility of this virus in either a xenografted mir122 negative carcinoma or hepatoma murine model whilst demonstrating reduced toxicity to normal hepatocytes.

Modelling mir122 negative carcinoma and hepatoma

The design of useful, reliable and robust cancer models of HCC and secondary liver metastasis is necessary in order to accurately assess treatment interventions. Established models of HCC include the subcutaneous injection of HCC cell lines from either humans (xenografted) or mice (syngeneic) into either immuno-deficient nude (nu -/-) mice or the strain of mice from which the cell line was derived, respectively. Alternatively, primary HCC can be induced in mice using either genotoxic or non-genotoxic (epigenetic) chemical treatments including diethylnitrosamine (DEN) or clofibrate. Mouse models of HCC have also been developed which involve the over-expression of oncogenes which are activated in HCC. Unfortunately, for the studies conducted herein, feasible animal

models are very limited. Adenovirus does not replicate in murine tissues and therefore syngeneic models could not be used. However, xenografted murine models are often criticised for not accurately recapitulating primary HCC or secondary metastasis. One main concern is that the location in which the tumour is engrafted, usually subcutaneously onto the murine flank, does not imitate the microenvironment of the liver. Therefore, an alternative approach was attempted within this chapter to try and generate more authentic human xenografts in mice using the hydrodynamic delivery of cells via the tail vein. This approach uses the injection of large volumes (10% fluid volume/total mouse weight) into the superficial veins of mice leading to the overload of the heart and backflow into the hepatic vein. This inflates the liver and should allow tumour cells to become lodged in the liver parenchyma.

Optimal Dosage of Ad5-mir122

In order to maximise the therapeutic efficacy of targeted treatments it is necessary to deliver the optimal quantity of therapeutic agent whilst causing only minimal, or acceptable levels of toxicity. Clinically this is normally performed during a phase one trial in human patients to determine the maximum tolerated dose to be used in further studies. Although the maximum tolerated intravenous dose of Ad5 WT in mice had already been determined by other groups ($8.8x10^8$ pfu) (Duncan *et al.* 1978) the maximum tolerated dose of Ad5-mir122 was unknown and needed to be assessed in order to perform efficacy analysis at the optimal dose. Ad5-mir122 had shown substantially lower toxicity when compared to Ad5 WT following intravenous injection and it was therefore expected that the MTD of Ad5-mir122 would be significantly higher than that of the wild type

adenovirus strain. This hypothesis was tested using a novel dose escalation study in which both the maximum tolerated dose and optimal treatment dose of Ad5-mir122 were calculated.

Primary scientific aims:

1. Develop a xenograft model of either mir122 negative colorectal liver metastases or mir122 negative hepatocellular carcinoma to allow the anti-cancer activity of Ad5-mir122 to be assessed.

2. Determine the maximum tolerated dose and optimal therapeutic dose of Ad5-mir122 using a pharmacodynamics led dose escalation study.

3. Assess the oncolytic potency of Ad5-mir122 in comparison to Ad5 WT using a murine xenograft model.

6.2. Results:

Establishing a mir122 negative colorectal liver metastasis xenograft mouse model

Murine models of either hepatocellular carcinoma or secondary hepatic metastasis of human disease should attempt to reflect the condition pathologically. To try and generate an authentic murine metastasis xenograft model, varying quantities of mir122 negative HT-29 colorectal carcinoma cells expressing luciferase were administered by hydrodynamic injection intravenously to CD1 nude mice. Previous pilot studies using B16-F10 melanoma cells had shown that these quantities of cells were non-toxic whilst doses above $1x10^6$ cells per mouse were acutely toxic and represented a dose limiting toxicity for this procedure. Injection doses of $1x10^3$ B16-F10 cells per mouse formed tumours efficiently in the murine liver (data not shown). Luciferase expression of the HT-29 cells was confirmed prior to injection (data not shown) of $1x10^4$, $1x10^5$ and $1x10^6$ cells per mouse (Figure 6.1).

Initial imaging results of mice administered $1x10^4$ or $1x10^5$ HT-29 luciferase cells were variable and no clear liver localisation could be observed (Figure 6.1). This was quite different from earlier studies using plasmids encoding luciferase using the same procedure. The reason for poor liver engraftment of HT-29 cells is unclear and may be due to size constraints within the liver fenestrae or because the luciferase levels produced by the cells were below the limit of detection. Later time points also showed variable, and consistently low, luciferase expression with little to no liver localisation. Luciferase levels gradually decreased in most mice although some mice showed extra-hepatic luciferase foci (Figure 6.1). Conversely the highest dose of $1x10^6$ cells per mouse showed luciferase

foci in the liver in two of three mice but luciferase expression was also present in extra-hepatic locations. This dose also caused some toxicity following injection with mice showing transient reduced mobility. Mice showing strong luciferase signals were euthanised when the luciferase expression suggested significant tumour deposits and organs were assessed for luciferase expression. Expression was observed in the liver, lung and multiple distal sites but results were variable between the two mice in which signals were detected and no tumour deposits were macroscopically visible. These data suggest that the administration of HT-29 cells by hydrodynamic injection does not allow the reproducible establishment of xenografted tumour deposits and the system was therefore not taken further.

A second attempt to develop a xenografted liver metastasis model was attempted using hydrodynamic injection of the mir122 negative colorectal cancer cell line LoVo encoding luciferase. Luciferase expression was confirmed prior to injection via luciferase assay (data not shown). Cell quantities administered per mouse included $1x10^4$, $1x10^5$ and $1x10^6$ cells. Luciferase imaging results again showed little to no liver localisation and uniformly low luciferase levels were observed at all doses throughout study, similar to the lower doses of HT-29 cells. No discernable tumours developed in any mouse throughout analysis (Figure 6.2). Accordingly the use of hydrodynamic delivery of tumour cells to form a hepatic metastatic model was abandoned.

Establishment of a xenografted mir122 negative hepatoma model and preliminary assessment of Ad5-mir122-Luc tumour infection

The inability of human cell lines to establish tumours in murine liver following hydrodynamic intravenous injection required another approach. It is well established that HCC cell lines can form tumours following subcutaneous injection into the flank of immune-deficient nude mice. Therefore, a pilot study was conducted in which three CD1 nude mice were injected subcutaneously with $5x10^6$ HepG2 tumour cells and tumours were allowed to establish. One mouse showed no sign of tumour growth even after four weeks although the remaining mice developed HepG2 tumours of roughly equal volume. This showed that although the initial establishment efficiency of the model might be low, the model could succeed if this was compensated for by inoculating sufficient numbers of mice.

To determine if adenovirus was capable of successfully infecting HepG2 tumours grown on the flank of CD1 nude mice one mouse was injected with $5x10^{10}$ vp of Ad5-mir122-Luc and imaged at 24, 72 and 120 hours. Initially significant liver transduction and transgene expression was observed although this quickly declined to background levels. Conversely, luciferase expression within the tumour was initially low but increased substantially during successive imaging events suggesting tumour selective virus replication (Figure 6.3). Importantly, the tumours produced using this model appeared to express the hepatic specific protein bilirubin and were well vascularised suggesting the model may reflect HCC. Combined this data showed that subcutaneously grown xenografted HepG2 tumours could serve as a useful model to monitor virus anti-cancer activity with Ad5-mir122.

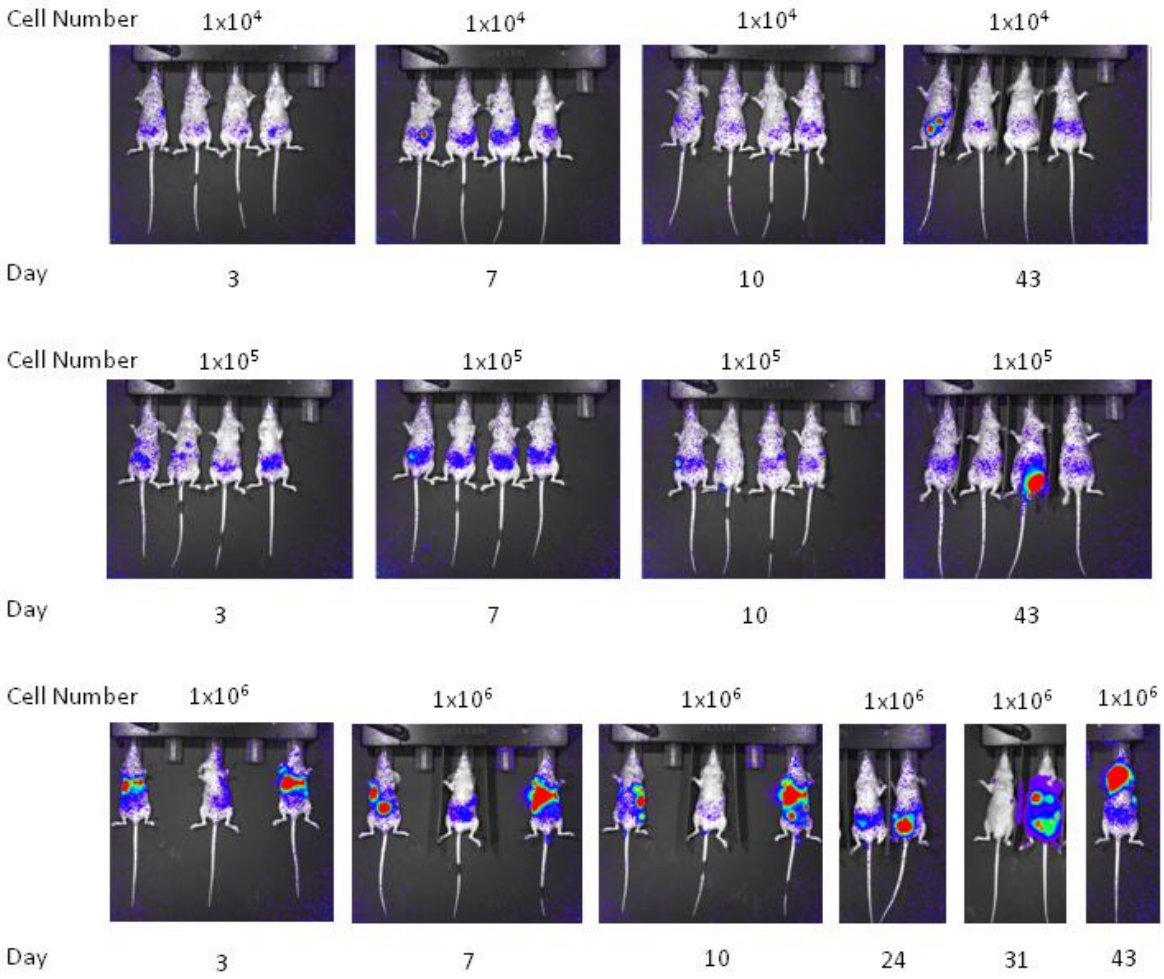


Figure 6.1: Mir122 negative HT-29 hydrodynamic xenografted model. CD1 nude mice were injected with varying quantities of HT-29 colorectal carcinoma cells expressing luciferase. Cell numbers injected are shown above each image and the number of days post cell implantation are shown below each image. Mice were imaged four minutes following injection of luciferin and all images are scaled to allow direct comparison between time points.

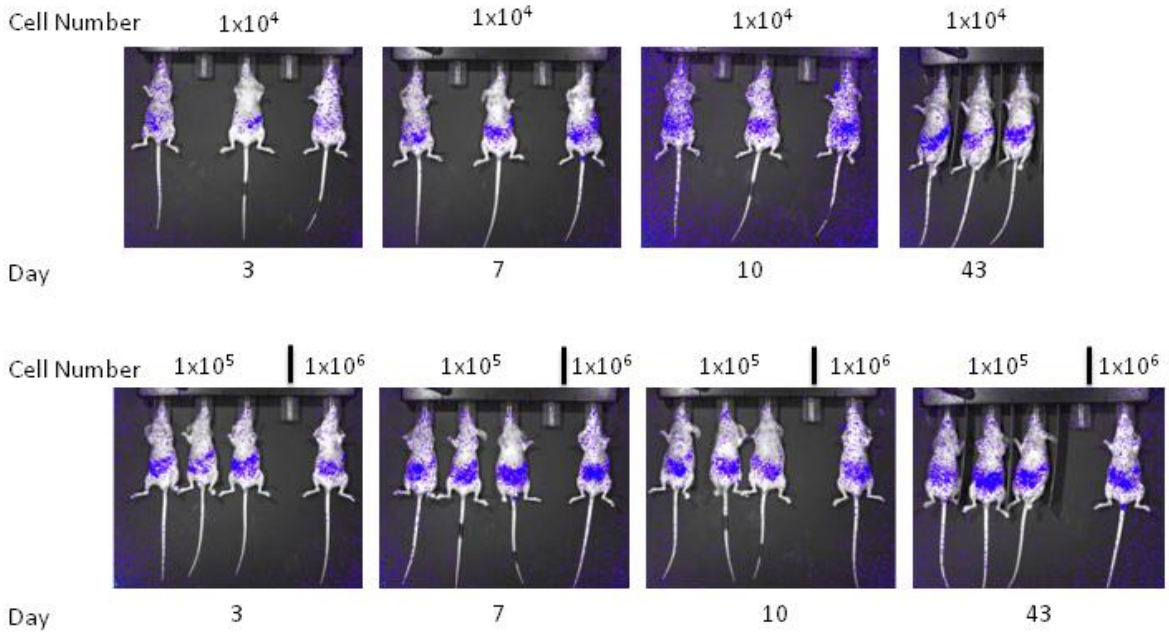


Figure 6.2: Mir122 negative LoVo hydrodynamic xenografted model. CD1 nude mice were injected with varying quantities of LoVo colorectal carcinoma cells expressing luciferase. Cell numbers injected are shown above each image and the number of days post cell implantation are shown below each image. Mice were imaged following injection of luciferin and all images are scaled to allow direct comparison between time points.

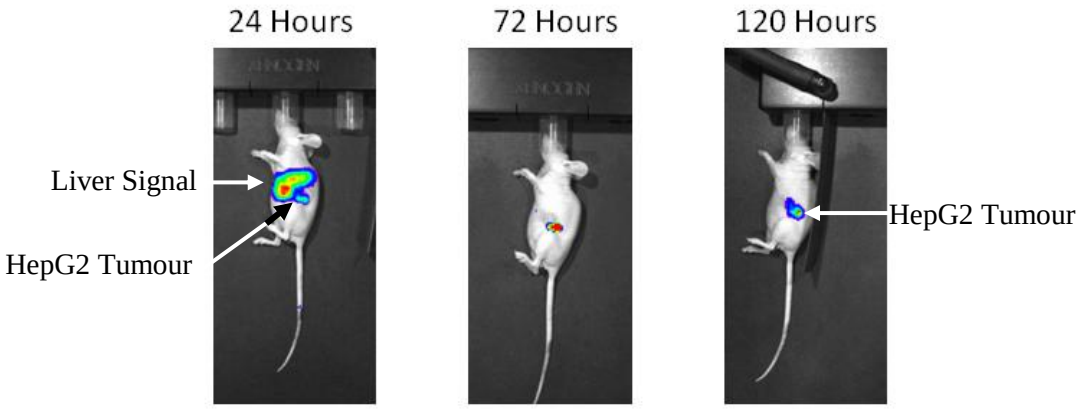


Figure 6.3: Pilot assessment of intravenous infection of Ad5-mir122 in a xenograft HepG2 hepatoma model. CD1 nude immuno-deficient mice were injected subcutaneously with $5x10^6$ HepG2 cells per mouse in the left flank. After two weeks one mouse with a tumour deposit of approximately 20-30 mm^3 was injected intravenously with $5x10^{10}$ vp of Ad5-mir122-Luc and imaged following injection of luciferin at 24, 72 and 120 hrs. Arrows show liver and tumour signals. All images are present on equal scale and are directly comparable.

Pharmacodynamics-led determination of optimal virus doses

The optimal doses of Ad5-mir122 and Ad5 WT were determined in order to define a protocol that would allow repeat delivery in a mir122 negative hepatoma efficacy model. Although it had previously been established that $5x10^{10}$ vp of Ad5-mir122 by single injection was well tolerated for up to three days in wild type immune-competent Balb/C mice it was unclear if xenograft bearing nude mice would tolerate similar doses or if a different treatment regimen would be required. To address this question, and to determine an optimum treatment dose, a pharmacodynamics-led dose escalation study was performed.

Forty CD1 nude mice were injected subcutaneously with $5x10^6$ HepG2 cells and tumours were allowed to establish. The tumour establishment rate was 87.5% (35 mice). Five mice were omitted from study due to absence of tumour growth or insufficient tumour load (<10 mm^3) and the remaining mice were blind grouped into three groups of 10 mice.

The primary readouts of the pharmacodynamic dose escalation study were (i) tumour infection, measured by luciferase imaging and (ii) liver toxicity, measured by alanine transaminase (ALT) release and luciferase expression. The secondary read out was efficacy which was measured by assessing tumour volume at the end of study.

Ad5 WT, Ad5-mir122 and PBS (100 µl total injection volumes) were administered by intravenous injection to the CD1 nude mice bearing established HepG2 xenografts. In order to monitor virus gene expression each dose of virus injected contained a 10% spike of the corresponding virus encoding luciferase, either Ad5-WT-Luc or Ad5-mir122-Luc. Five mice were imaged for luciferase and serum ALT levels were determined in three

mice from each group two days after each injection (Figure 6.4). The same individual mice were monitored from each group throughout.

The starting dose for both viruses was $6x10^9$ vp/mouse, corresponding to $8.8x10^8$ pfu for Ad5 WT, slightly lower than the published maximum tolerated dose (MTD, $1x10^9$ pfu) (Duncan *et al.* 1978). Two days following intravenous administration of $6x10^9$ vp of Ad5 WT, imaging showed high levels of hepatic luciferase expression, confirming virus activity in the liver with little to no tumour signals at this dose (Figure 6.5). ALT levels were significantly elevated (>1000 Units/L) suggesting significant hepatic toxicity had occurred (Figure 6.6). Although the ALT values fell steadily over days 2-8 (Figure 6.6), several animals (6 out of 10) showed significant weight loss (>5%) and one was put down (weight loss >15%). Accordingly this dose of Ad5 WT was taken as the MTD, and treatment was not escalated.

In contrast, two days following administration of the same dose ($6x10^9$ vp) of Ad5-mir122, mice showed ALT readings similar to PBS controls (Figure 6.6). Imaging data confirmed this result with very little hepatic luciferase (Figure 6.5). No substantial tumour infection was observed with this dose.

The dose of Ad5-mir122 was therefore increased by a half log to $2x10^{10}$ vp, three days after the first injection. Imaging at day five (two days after the second injection) showed some luciferase activity (indicating expression of viral E1A) in both tumour and liver, although absolute values varied between mice with two mice showing little to no expression (Figure 6.5). Average ALT values showed an increase to 222 ALT units/L compared to 42 ALT units/L from controls (Figure 6.6). On day six the dose was further increased by a half log to $6x10^{10}$ vp. Imaging at day eight (two days after the third

injection) showed significant luciferase activity in the tumours, however, this was coupled with measurable luciferase activity also in the liver (Figure 6.5). ALT readings showed an increase in all mice compared to mice administered PBS but only a small peak increase from the previous dose (Figure 6.6). This study confirmed that $6x10^{10}$ vp of Ad5-mir122 was an acceptable dose but the high level of hepatic luciferase expression in some mice suggested that it was close to the MTD and maybe be toxic if delivered repeatedly.

Assessment of tumour size in these animals was performed 41 days after the start of treatment. In the surviving mice the single dose at MTD of Ad5 WT ($6x10^{9}$vp) had no significant effect on tumour volume (Figure 6.7). However, the three escalating doses of Ad5-mir122 caused a significant reduction in tumour size compared to controls (P=0.02) and mice receiving Ad5 WT (P=0.05). These data show that the reduced toxicity of Ad5-mir122 allowed the use of doses more than 10-fold above the maximum dose of Ad5 WT, which increased anti-cancer efficacy in this study.

Repeat administration of adenovirus can cause cumulative toxicity. For assessment of efficacy it was desirable to administer virus on multiple occasions, hence for repeated administration doses were selected that were half a log lower than the estimated MTDs of Ad5-mir122 (MTD = $6x10^{10}$ vp/mouse, estimated optimal dose = $2x10^{10}$ vp/mouse) and Ad5 WT (MTD = $6x10^{9}$ vp/mouse, estimated optimal dose = $2x10^{9}$ vp/mouse).

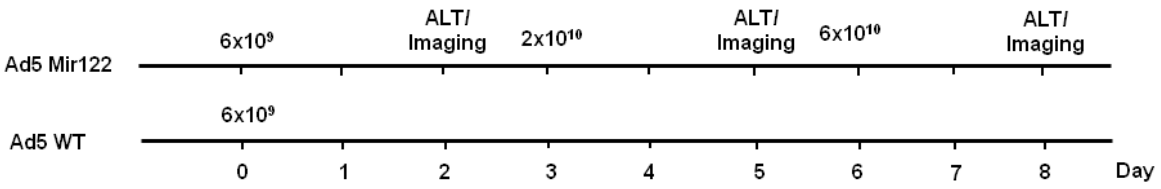


Figure 6.4: Dosage and treatment schedule for Ad5-mir122 and Ad5 WT. Viral doses are indicated above each line and include 10% of the corresponding equivalent luciferase reporter virus. All mice were treated with bisphosphonate liposomes at day -1 to remove Kupffer cells from within the liver.

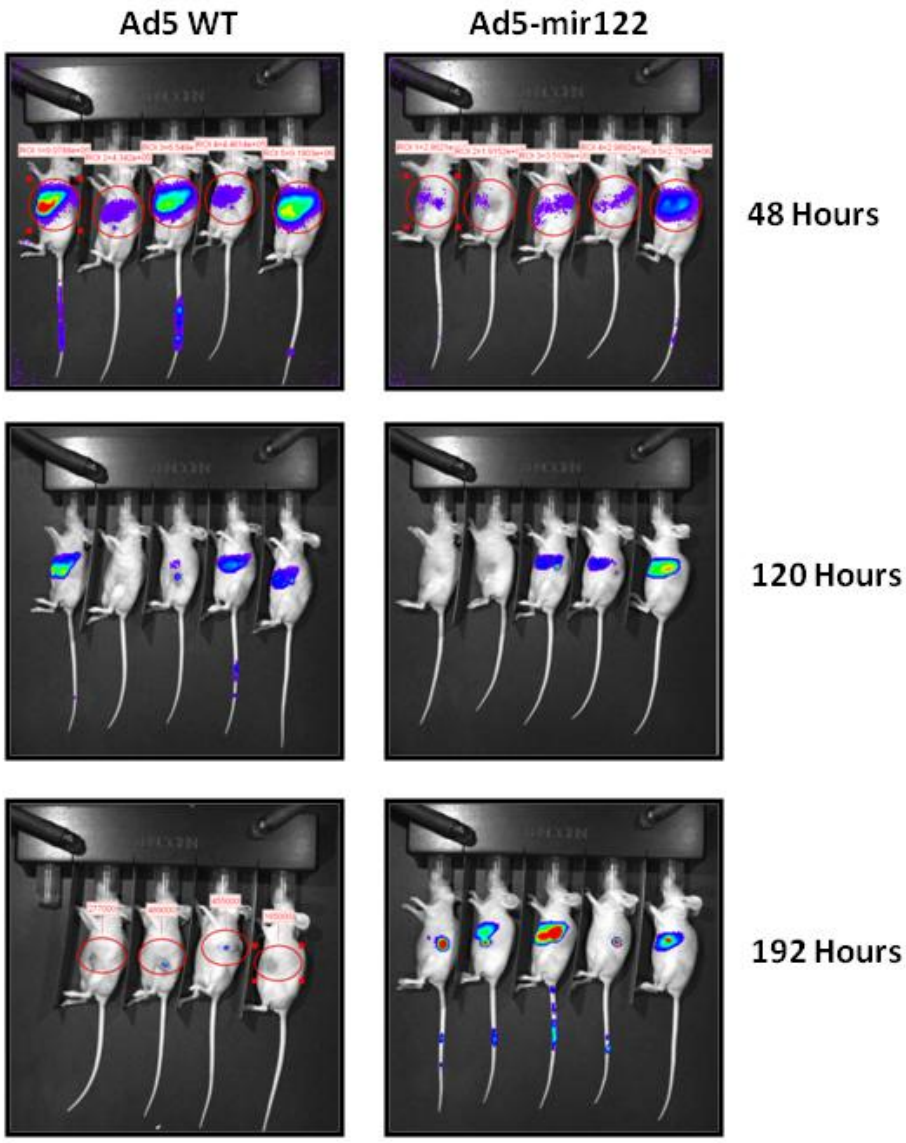


Figure 6.5: Pharmacodynamics led dose escalation study to determine the optimal treatment dose with Ad5-mir122 and Ad5 WT in tumour bearing mice. Luciferase imaging of mice injected with either Ad5 WT (left panel) or Ad5-mir122 (right panel) at days 2, 5 and 8. For doses see figure 6.4. Mice which received a single injection of $6x10^9$ vp of Ad5 WT show significant liver luciferase expression. Mice receiving a combined dose of $8.6x10^{10}$ vp (14-fold more than Ad5 WT) of Ad5-mir122 do not show this level of hepatic luciferase. Image pairs are all presented on the same scale but vary between time points.

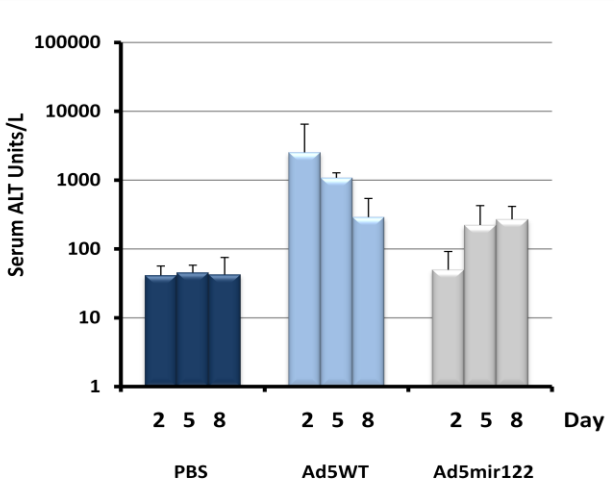


Figure 6.6: Measurement of hepatic toxicity of Ad5 WT and Ad5-mir122 during dose escalation. Serum Alanine Transaminase (ALT) levels from mice receiving intravenous Ad5-mir122, Ad5 WT or PBS. ALT values for each group are shown at days 2, 5 and 8 (n=3) after the first injection. Data is presented as ALT units per litre using the equation described in the materials and methods. Error bars represent standard deviation.

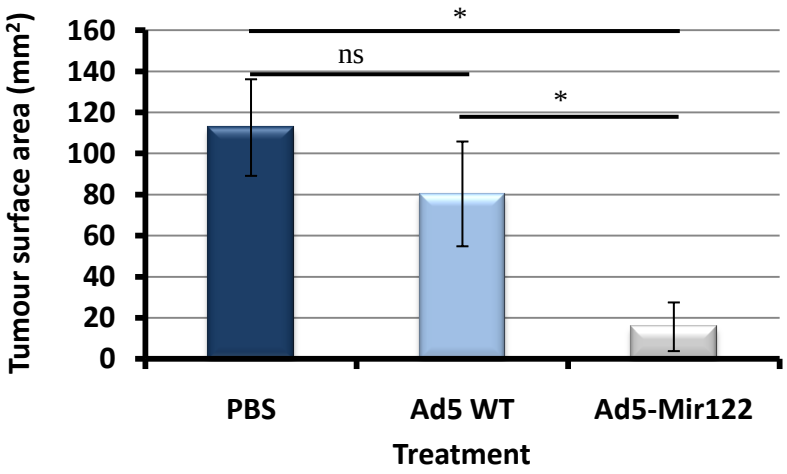


Figure 6.7: Tumour size calculations. The tumour burden of mice receiving either PBS, Ad5 WT or Ad5-mir122 during the pharmacodynamic dose escalation study was calculated at the end of study. Results showed that whilst the single injection of Ad5 WT ($6x10^9$ vp/mouse) had no significant effect on tumour volume, the repeat injection of Ad5-mir122 ($6x10^9$, $2x10^{10}$, $6x10^{10}$ vp/mouse) had significant therapeutic benefit. Error bars are shown as standard deviation. Statistical analysis is using two-way ANOVA. (*=P<0.05, ns=not significant, n=10 for Ad5-mir122 and PBS but n=9 for Ad5 WT).

Efficacy of Ad5-mir122 and Ad5 WT in a hepatoma xenograft model

To determine the anti-cancer activity of Ad5-mir122 used at a single, repeated, dose level, fifty nude mice were injected subcutaneously with $5x10^6$ HepG2 cells and tumours were allowed to establish. Mice bearing tumours with volumes in excess of 10 mm^3 were blind grouped into groups of 10. Mice were administered $2x10^{10}$ vp of Ad5-mir122 intravenously on days 0, 3, 19 and 22 while control animals received either $2x10^9$ vp of Ad5 WT or PBS at days 0, 3, 19 and 22 (100µl total injection volumes). All virus doses contained a 10% spike of the corresponding luciferase encoding virus, either Ad5-WT-Luc or Ad5-mir122-Luc. Tumour growth was monitored for efficacy and a survival endpoint (for the Kaplan Meier analysis) was introduced when individual tumour volume reached 400 mm^3. Animals showing the fastest tumour growth were allowed to continue beyond this point (up to 1000mm^3) to allow the group average tumour size to reach 400 mm^3.

Mice administered Ad5-mir122 showed significantly reduced tumour volume and efficacy from day 20 compared to PBS controls (Figure 6.8). However, mice receiving the lower dose of 4 injections of $2x10^9$ vp Ad5 WT also demonstrated significant anti-cancer efficacy which is in contrast to the tumour volumes obtained during the pharmacodynamic dose escalation study. This is perhaps unsurprising given that Ad5 WT has been shown to have potent anticancer activity (Bazan-Peregrino *et al.* 2008) following repeat administration but importantly it suggests that repeat administration is more important than dose.

It is essential to highlight that the doses of Ad5-mir122 and Ad5 WT were not equitoxic. After 4 days in treatment with Ad5 WT one mouse was put down due to weight loss

(>15%) and hepatic toxicity (Figure 6.9). Multiple other mice treated with Ad5 WT also showed transient weight loss up to 10% of their starting body weight. No such adverse events occurred in the group receiving Ad5-mir122 at a 10-fold higher dose.

Kaplan Meier analysis of mice administered Ad5-mir122 showed increased survival with all mice surviving longer than all controls (Figure 6.9). Light images of all the mice in study are shown in figure 6.10. The reduced tumour burden of mice receiving both Ad5-mir122 and Ad5-WT when compared to mice receiving PBS is visible as the primary subcutaneous tumours are significantly reduced. These data show that the repeat administration of Ad5-mir122 at doses above the MTD of Ad5 WT can have significant anti-cancer efficacy without significant toxicity.

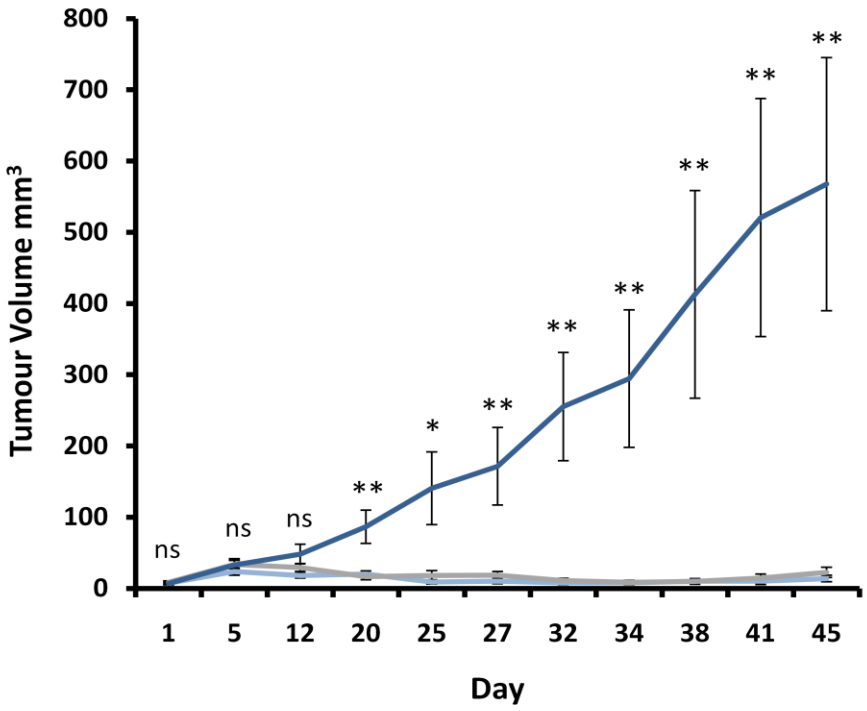


Figure 6.8: Ad5-mir122 has potent anti-cancer efficacy in HepG2 tumour bearing mice. Repeat administration of Ad5-mir122 (light grey line, N=10 mice) at the treatment dose determined from the pharmacodynamics led dose escalation study ($2x10^{10}$vp) on days 0, 3, 19 and 22 by intravenous injection. Control animals received repeat administration of $2x10^{9}$ vp Ad5 WT (light blue line) or PBS (dark blue line) by intravenous injection (n= 10 mice) on days 0, 3, 19 and 22. Data shows the tumour volume of mice receiving PBS or Ad5-mir122 or Ad5 WT and was calculated as the volume of an ellipsoid (Tomayko *et al.* 1989). Statistical analysis was performed using one way ANOVA with a Bonferroni post test (ns = not significant, * = $P<0.05$, ** = $P<0.01$) at each time point. Error bars are shown as standard error of the mean.

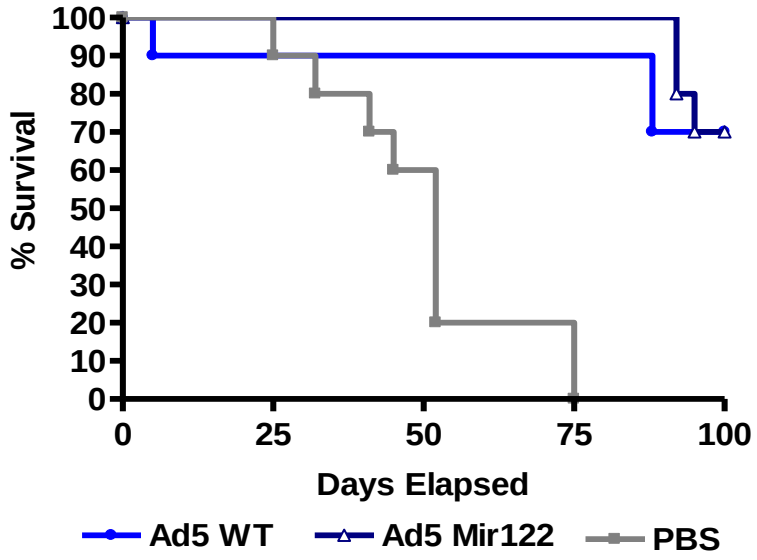


Figure 6.9: Ad5-mir122 enhances overall survival of HepG2 tumour bearing mice. Kaplan Meier survival analysis shows increased survival of mice receiving Ad5-mir122 or Ad5 WT compared to control mice receiving PBS. After 4 days in treatment with Ad5 WT one mouse was put down due to toxicity. No treatment related toxicities were observed during Ad5-mir122 treatment or treatment with PBS. Days shown on the horizontal axis represent the number days since the first virus injection.

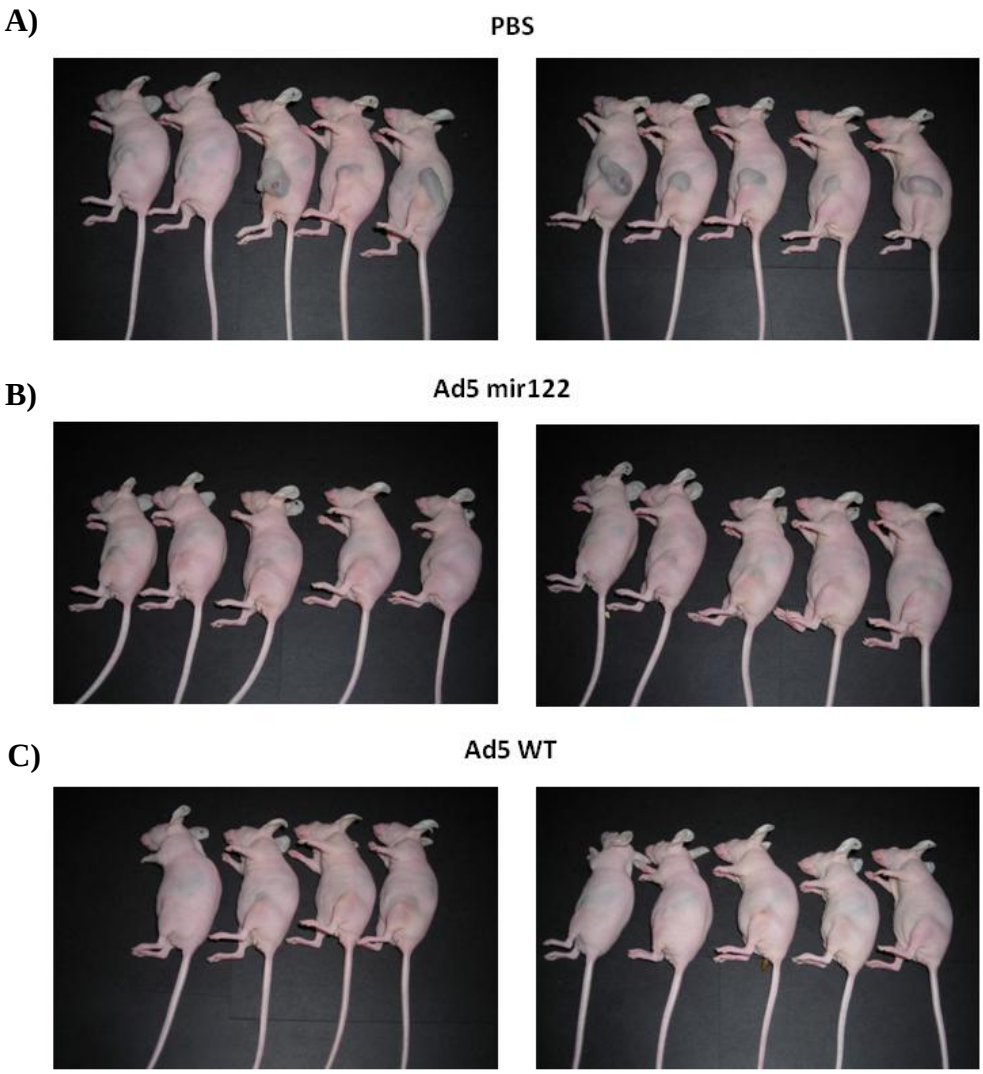


Figure 6.10: Light images of all mice in the efficacy study showing tumour burden in each group. All tumours are subcutaneous on the left flank. A) Mice receiving PBS show substantial tumour burden in all mice in study. B) Mice receiving Ad5-mir122 showing reduced tumour load. C) Mice receiving Ad5 WT showing reduced tumour load but only nine are pictured because one mouse was put down early in treatment due to viral toxicity.

6.3. Concluding discussion:

The initial work conducted in this chapter intended to develop a novel xenograft tumour model using the hydrodynamic injection of mir122 negative tumour cells into CD1 nude immuno-deficient mice. This model was intended to allow the establishment of tumours specifically within the liver because previous data using DNA delivery had shown that the plasmid was almost exclusively delivered to the liver. Similarly, early syngeneic pilot studies using B16 melanoma cells in C57 Black/6 immuno-competent mice had proved successful in establishing multi-focal lesions within the liver.

The delivery of mir122 negative HepG2 and LoVo cells by hydrodynamic injection at three different doses ultimately proved unsuccessful, with no mice showing any signs of tumour development. There are many possible reasons for this observation. The hydrodynamic technique requires that cells are disaggregated into single cell suspension in order to prevent blocking of capillaries which results in toxicity. Moreover, following injection the cells are likely to be dispersed throughout the liver. This requires that individual cells are capable of forming a tumour independently without paracrine influence from surrounding cells. Melanoma is known to be highly metastatic and a large fraction of melanoma cells have been shown to be capable of forming tumours independently (Quintana *et al.* 2010), this may explain the success of the B16 hydrodynamic model. Moreover, normal untransformed melanocytes exist as independent cells within the skin and are derived from neural crest cells which are known to have independent migratory properties (Dupin *et al.* 2003). The HepG2 cells and LoVo cells used in this chapter may not share these properties. Most of the cells from each of these lines are phenotypically similar to hepatocytes and epithelial gut luminal lining

respectively and patients with these conditions do not demonstrate the same level of secondary metastases as melanoma patients of equal stage. Therefore, perhaps a deficiency in autocrine signaling and/or the lack of paracrine signals from surrounds cells prevented the tumours from establishing. It is also possible that the cells were delivered in insufficient quantity due to the limitations of the technique. The maximum tolerated dose of cells delivered was $1x10^6$ cell per mouse and even at this dose some adverse events occurred. Subcutaneous implantation of the same tumour cell lines is normally performed at $1\text{-}5x10^6$ cells per mouse and therefore the procedure itself may limit tumour development for less aggressive tumour types. It may also be possible that the success of the B16 model is a consequence of the functional immune system of the C57 Black/6 mouse compared to the CD1 nude mice used in the xenograft models. To establish the reason for the lack of success of the xenograft model would require further investigation but it is likely given the success of the B16 model that this technique could be successful if a more aggressive cell line is used which can form independent tumours from single isolated cells. It is also possible that a component of the immune system still remaining within CD1 nude mice prevented the establishment of the HepG2 and LoVo cells or, conversely, an immune component that was absent in the CD1 nude mice that was present in the C57/Black 6 mice, enabled the establishment of the B16 tumours.

The use of Ad5-mir122 for cancer therapy in a murine model required that the optimal dose of this virus was first calculated. Therefore, a pharmacodynamics led dose escalation study was performed to determine the maximum safe dose which caused virus accumulation within the tumour whilst inducing minimal side effects. This dose was determined as $2x10^{10}$ vp of Ad5-mir122. Repeat administration of this dose to HepG2

tumour bearing mice mediated substantial anti-cancer efficacy and allowed enhanced survival, with animals receiving Ad5-mir122 performing better than all controls receiving PBS. This is the first anti-cancer efficacy produced using a microRNA regulated oncolytic DNA virus. The same efficacy study also builds on previously published evidence that Ad5 WT also has potent anticancer efficacy (Bazan-Peregrino *et al.* 2008), even at low doses. Interestingly one single dose of Ad5 WT at $6x10^9$ yielded little therapeutic benefit in the dose escalation study but repeat injection of $2x10^9$ Ad5 WT in the efficacy study had significant anti-cancer, even after the second infection. This suggests that repeat administration may be far more important for anti-cancer activity with an oncolytic virus than the dose administered.

Whilst the repeat injection of Ad5 WT did have significant, comparable therapeutic benefit in the efficacy study when compared to Ad5-mir122 it is important to highlight that Ad5 WT was still more toxic even at 10-fold lower doses. One mouse was put down in the Ad5 WT group early in treatment due to excessive weight loss whilst no mice in the Ad5-mir122 group experienced such toxicity. It was also important to note that the two viruses behave identically in mir122 negative cells and replicate with equal kinetics. Therefore, the viruses are equally potent if successfully deposited within the tumour. The observation that both viruses induce similar efficacy suggests that perhaps the important aspect of the treatment regimen used was repeat delivery and not the dose administered. However, as was clear in our studies, the toxicity caused by Ad5 WT, even at low doses, is a major limitation to its use in animal models.

Chapter Seven: Synopsis

7.1 Synopsis:

Molecular engineering of replicating viruses to avoid pathology whilst maintaining potency in therapeutic sites would provide an important new platform for the design of viral vaccines and oncolytic therapies. In this work I explored the possibility of achieving this using the wild type strain of the DNA virus adenovirus, engineered to avoid its major toxicity in murine liver by including four binding sites for the hepatocyte-specific microRNA mir122 within the 3′ UTR of E1A. This approach was adopted because it offered the ability to allow wild-type replication in all non-hepatic cells which did not restrict the oncolytic potency of adenovirus to a specific cell type or a cell type with a particular transforming mutation.

Preliminary testing of mir122 activity using CMV promoter driven plasmids

Initially, the regulatory capacity of mir122 was undetermined and therefore CMV promoter driven luciferase plasmid vectors were engineered into which four microRNA mir122 binding sites were inserted into the 3' UTR. Results from preliminary testing of these plasmids were encouraging, with all constructs behaving as expected by showing mir122 specific knockdown in luciferase levels following both *in vitro* and *in vivo* testing. Importantly, the expression level of luciferase from plasmids with and without mir122 binding sites was equal in cells not expressing mir122. This suggested that in tissues other than hepatocytes this method of control allowed 'wild-type' levels of protein to be expressed without any attenuation. Interestingly, the use of eight microRNA binding sites

for mir122 was more effective at reducing luciferase expression through translation inhibition.

Preliminary testing of mir122 activity using luciferase encoding E1A promoter driven plasmids

The positive results obtained through the testing of CMV driven plasmids was followed by the development and analysis of plasmid vectors in which the E1A coding sequence was fused to the luciferase coding sequence under the control of the E1A promoter. This allowed the effect of mir122 regulation to be assessed from the adenovirus E1A promoter which was useful for virus design. Results were encouraging, with significant mir122 mediated down-regulation of luciferase observed both *in vitro* and *in vivo*. This data, combined with that obtained using CMV promoter driven vectors, suggested that mir122 was capable of regulating substantial quantities of mRNA. This was particularly evident from the efficient control (over 120-fold) of mir122 regulated plasmids within the murine liver following hydrodynamic injection of plasmid DNA. The luminescence signals measured in this study were also higher than any that had previously been observed using any viral vector.

Design and engineering of microRNA mir122 responsive viruses

The observation that eight microRNA binding sites were more effective at controlling gene expression from plasmid experiments was an important finding, however, the incorporation of eight repeat sequences into a viral genome was anticipated to encourage

reversion to the wild-type strain through deletion or recombination. Therefore, although slightly less effective, four microRNA binding sites were selected as the optimum number to ensure significant E1A knockdown with minimal chance of reversion.

Ad5 WT was engineered to contain four mir122 binding sites in the 3' UTR of the E1A transcription cassette, however, it was unclear how the influence of microRNA regulation on E1A production could be measured both temporally and non-invasively *in vivo*. To achieve this it was necessary to engineer a virus strain in which the luciferase coding region was introduced 3′ to the E1A coding region of wild-type adenovirus in order to produce a contiguous E1A-luciferase expression cassette, where E1A splicing would produce a series of E1A-luciferase fusion proteins. This novel virus, called Ad5-WT-Luc, was then modified further to contain four mir122 binding sites in the E1A-luciferase 3′ UTR. This virus was termed Ad5-mir122-Luc.

Before testing it was unclear if the ligation of the luciferase protein to the E1A protein would prevent both luciferase and/or E1A from functioning correctly. Surprisingly, neither function appeared to be inhibited with both Ad5-WT-Luc and Ad5-mir122-Luc growing in E1A deficient cells (A549 lung carcinoma), confirming the ability of E1A to initiate viral replication. This is perhaps unsurprising, given that E1A protein has been shown to still operate despite significant deletions and insertions and lacks both enzyme activity and significant secondary structure (Frisch *et al.* 2002). However, it was clear during *in vitro* virus preparation that the presence of the luciferase sequence slightly decreased the rate of cell killing, compared to the corresponding virus without luciferase, but a complete cytopathic effect was still achieved in permissive cells after one extra day.

The luciferase expression from these viruses was also high and appeared to be stable over repeated passage. These viruses reported E1A protein levels accurately and enabled non-invasive real-time assessment of protein translation inhibition and also indirectly measured the effect of microRNA control on virus genome replication and gene expression.

The infection of mir122 negative cells with either Ad5-WT-Luc or Ad5-mir122-Luc showed that both viruses replicated with equal kinetics. Similarly, the non-luciferase encoding viruses (Ad5 WT and Ad5-mir122) induced equal cell kill in mir122 negative cells using a single time point cell kill assay. However, in the mir122 positive hepatoma cell line Huh7 the luciferase expression between Ad5-WT-Luc and Ad5-mir122-Luc were significantly different. Mir122 knocked down E1A-luciferase expression more than 29-fold from Ad5-mir122-Luc compared to Ad5-WT-Luc. Comparative results were also obtained following infection of both primary human hepatocytes and murine liver following intravenous injection of both viruses where up to 80-fold lower hepatic luciferase expression was observed from Ad5-mir122-Luc compared to Ad5-WT-Luc. The successful control of viral gene expression in primary human hepatocytes was also the first demonstration that microRNA control of virus gene expression was possible in primary human cells.

Ad5-mir122 shows reduced toxicity compared to Ad5 WT

Ad5 WT is normally capable of an abortive genome replication cycle in murine liver *in vivo*, where it mediates considerable and sometimes lethal hepatotoxicity (Duncan *et al.* 1978). It was unclear whether microRNA regulation could successfully control Ad5 WT,

since the DNA genome is not a direct target for microRNA recognition and it is known that even small amounts of E1A translation can lead to genomic replication, which would then provide a template for more transcription providing an ever increasing challenge for microRNA control.

Hepatic replication and toxicity of Ad5 WT was assessed compared to Ad5-mir122 by injecting $5x10^{10}$ vp of both viruses intravenously to Balb/C mice. After 72 h the serum ALT was 15-fold lower in mice administered Ad5-mir122 compared to Ad5 WT, hepatic morphology also showed far less evidence of toxicity (most sections appearing normal with Ad5-mir122) and the number of viral genomes found in liver was decreased by a factor of 25. These findings are consistent with those using the E1A-luciferase reporter viruses, and suggest that the inclusion of the mir-122 binding sites had a dramatic effect on the hepatic activity and toxicity of the virus. It is also of note that, in this study, the viruses were applied at a dose *in vivo* (5×10^{10} vp/mouse) which was well above the lethal dose for wild-type Ad5 (circa $6x10^{9}$ vp) (Duncan *et al.* 1978), hence this regulatory strategy appears capable of controlling the activity of significant quantities of virus. This also suggests that the doses of virus used in this study did not exceed the regulatory capacity of mir122.

Mir122 knockdown of E1A mRNA and protein

To determine the extent to which mir122 was down-regulating E1A the mRNA and protein were quantified by RT-PCR and western blot. Since microRNA regulation is known to affect protein translation via multiple pathways, it was unclear if the mRNA

would be significantly degraded. However, given that the microRNA target sites I inserted were precisely complementary to mir122 it was likely that Argonaute 2-mediated RNA cleavage was responsible for the majority of the knockdown of E1A. Data showed that the E1A mRNA was down-regulated by 29-fold and the protein levels were knocked down to background levels. The protein levels were not quantified because no E1A signals were detected from gel lanes containing protein extracts from Ad5-mir122 infected livers. Therefore, a comparison to the high levels detected from protein extracts from Ad5 WT infected livers would have been an unfair comparison. The efficiency of E1A knockdown observed by western blot was high and explains the high degree of attenuation of Ad5-mir122 in murine liver even at doses above the maximum tolerated dose of Ad5 WT.

MicroRNA mediated toxicity

MicroRNA mir122 is highly abundant, representing in excess of 70% of the microRNA content in primary hepatocytes. The data shown here suggests that mir122 is capable of regulating more mRNA transcripts than are regulated under normal conditions in hepatocytes and that it is probably expressed in excess of its regulatory requirements. This is evident from the lack of virus mediated toxicity and escape following intravenous injection to mice. This finding was an advantage in my studies as it allowed the tight regulation of adenoviral E1A without affecting the quantity and activity of mir122. This was determined by measuring mature mir122 levels by RT-QPCR and the level of a known mir122 regulated protein (Aldolase A) in Ad5-mir122 infected murine livers. Genome wide mRNA profiling also showed that not only does Ad5-mir122 cause

significantly fewer intracellular mRNA changes when compared to Ad5 WT but also that Ad5-mir122 does not alter the mRNA level of any known mir122 target mRNA.

The ability of mir122 to regulate adenovirus and simultaneously maintain its endogenous inhibitory function is likely to be due to its high abundance, however, other microRNAs may not be as successful at controlling additional exogenous mRNA, especially if they are expressed at lower quantities. The direct competition for microRNA-mRNA binding between endogenous (cellular) and exogenous (viral) mRNA could induce changes in endogenous protein expression if the quantity of the microRNA required for normal regulation was only just met by its expression level. This concept has been exemplified by the use of microRNA binding sites to control lentivirus gene expression. By infecting cells with non-replicating lentiviral vectors at high multiplicity of infections, typically above >200 infectious units per cell, Gentner *et al* (Gentner *et al.* 2009) discovered that it was possible to influence the regulatory capacity of specific microRNAs. These lentiviruses were engineered to express transgenes containing multiple binding sites for specific tissue selective microRNAs and it was demonstrated that the quantity of the endogenous proteins normally repressed by these microRNAs was increased, suggesting dysregulation of the specific microRNA in study. Whilst this technique was developed and proposed as a method of controlling and understanding microRNA activity, toxicity is a major concern. The alteration of the level of a single microRNA could have significant consequences for many cellular processes and may induce unpredictable toxicity. Importantly, it has been reported that the use of replication competent RNA viruses has no effect on the microRNA levels within infected cells (Kelly *et al.* 2008). The data

presented in this thesis corroborates these findings by showing that mir122 and its normal regulatory functions are unaltered by infection with Ad5-mir122.

It has also been reported that toxicity can be induced in murine liver if the microRNA network is overloaded with new exogenous short hair-pin RNA that requires nuclease cleavage and processing (Grimm *et al.* 2006). In these studies the toxicity observed was lethal and was found to result from dysregulation of the microRNA RISC and Dicer machinery which caused impairment of the microRNA network and increased protein expression of many microRNA regulated genes. The addition of new exogenous mRNA target, as was performed in this thesis, should not induce this toxicity. MicroRNAs systems are not reported to contain feedback mechanisms that increase the quantity of microRNA in response to an increase in target mRNA. Therefore, the addition of new mRNA molecules to be degraded by a particular microRNA should not induce over-expression of microRNAs and the RISC and Dicer systems should remain unaffected. The results obtained by genome wide microarray screening suggest that such toxicity does not occur following infection by Ad5-mir122 with the number of genome wide changes being induced by Ad5-mir122 closely matching the number induced by a non-replicating, non-toxic control viral vector.

Xenografted tumour model

The data described showed that Ad5-mir122 was a tightly regulated, well tolerated and safe virus in murine liver. Results had also shown that in mir122 negative cells Ad5-mir122 showed equal cell kill and replication kinetics to Ad5 WT, however, the oncolytic potency of Ad5-mir122 *in vivo* remained untested. It was originally hoped that a novel

metastatic liver xenograft model could be established by the hydrodynamic injection of tumour cells via the tail vein. This model could have served as more accurate model of secondary colorectal liver metastatic disease compared to subcutaneously implanted tumours and would have allowed efficacy testing of Ad5-mir122 in a novel cancer model. Initial studies injecting B16-F10 tumour cells into C57 Black/6 mice revealed a dose limiting acute toxicity for this procedure at doses above $1x10^6$ cells/mouse. Importantly B16-F10 tumours were efficiently established at doses as low as $1x10^3$ cells/mouse almost exclusively in the liver of mice.

Injection of both HT-29 and LoVo cell lines expressing luciferase yielded disappointing results with very few mice showing luciferase expression more than 40 days after injection. This model was therefore abandoned in favor of a xenografted subcutaneous hepatoma model. Interestingly, subcutaneously grown mir122 negative HepG2 tumours were visibly well vascularised and preliminary pilot studies showed that Ad5-mir122-Luc could infect HepG2 tumours following intravenous injection.

To assess anti-cancer efficacy of Ad5-mir122 and Ad5 WT both viruses were repeat injected intravenously into HepG2 tumour bearing mice. Results showed that both Ad5-mir122 and Ad5-WT mediated significant anti-cancer efficacy and caused tumour regression in all mice in treatment when compared to mice receiving PBS. This data showed that Ad5-mir122 has Ad5 WT oncolytic potency however the anti-cancer efficacy mediated by Ad5-mir122 was no better than that mediated by Ad5 WT even though 10-fold higher doses of Ad5-mir122 were injected. This may reflect delivery limitations following intravenous injection, however, it should also be noted that all animals in

treatment responded well with some animals showing complete remission. Perhaps, therefore, the quantity of Ad5 WT injected was already sufficient to mediate maximum therapeutic benefit, with the additional Ad5-mir122 administered having little to no effect.

These results were the first anti-cancer efficacy reported using a microRNA regulated oncolytic DNA virus and it was a significant finding that Ad5-mir122 maintained Ad5 WT potency. This data also builds on previously published evidence that Ad5 WT has potent anti-cancer efficacy (Bazan-Peregrino *et al.* 2008), however, it was also clear that toxicity is a major limitation.

Chapter Eight: Discussion

8.1: Discussion

MicroRNA regulation compared to other regulatory strategies

MicroRNA regulation of viruses and virus transgenes is an emerging field which allows the selective de-activation of viruses in sites of unwanted replication. This technique has already been applied to a broad variety of viruses from a range of virus families. In each virus in which the method has been attempted it has been shown to successfully regulate and control virus protein translation in microRNA expressing cells, although the degree of control appears to range significantly dependent on the virus in question and the microRNA exploited (Barnes *et al.* 2008; Edge *et al.* 2008; Kelly *et al.* 2008; Cawood *et al.* 2009; Perez *et al.* 2009; Schaefer *et al.* 2009; Kelly *et al.* 2010).

The use of microRNA regulation to control virus replication has many advantages over existing methods of control. One of these is the broad utility of microRNA regulation for regulating every known virus. All viruses must produce protein in order to replicate and all must contain the genetic material essential for encoding the proteins they require. MicroRNA regulation functions by preventing protein synthesis from cytoplasmic RNA containing complementary binding sites. Given that all viruses must translate their mRNA in the cytosol, even nuclear RNA viruses such as Influenza. Therefore, unlike other regulatory mechanisms, such as transcriptional targeting, microRNA regulation can be used to control any virus from any viral family.

The broad array of microRNAs that have been discovered in many tissues of both humans and animals is another major benefit to using microRNA regulation. Extensive screening of both normal tissue samples and cancers has also discovered many microRNAs which

are either unique to specific tissues or absent from certain forms of cancer (Landgraf *et al.* 2007). This allows the identification of multiple candidate microRNAs which can be used for tissue-specific virus attenuation. Other methods of regulation, such as complementing mutations, are often dependent on cancer associated alterations which are not always found at high frequency or involve broadly attenuating the virus to prevent toxicity. The use of microRNA binding sites prevents virus attenuation, provided that the microRNA is absent from the cancerous cells. This allows the virus to replicate with the same kinetics as the wild type strain in any cell not expressing the microRNA. This is of critical importance when designing oncolytic viruses which are required to demonstrate maximum potency in neoplastic cells.

Another benefit is the size of the genetic insertion that is necessary for microRNA control. Many viruses are size restricted, particularly if the wild type virus strain is the candidate to be explored. Therefore, the addition of 21-23 nucleotides should have little to no effect on packaging capacity, although it is likely that more than one binding site would be used. If four binding sites are added then the addition would be approximately 120 nucleotides, which is still small when compared to the size of a promoter or gene.

The conserved evolutionary sequence identity shared within many microRNAs also makes it possible to test viruses in pre-clinical animal models. For example, microRNAs mir122 and mir124 are conserved between both mice and humans (Griffiths-Jones *et al.* 2008). This allows microRNA control to be assessed in animals before human testing. MicroRNA regulation is also highly efficient and able to down-regulate significant quantities of mRNA. Alternative approaches such as tissue specific promoters often demonstrate significant off-target expression which can cause unwanted toxicity. These

advantages make the exploitation of the microRNA network an attractive approach for controlling viral gene expression. Consequently, it is likely that many potent new oncolytic vectors will be generated that are engineered to show wild type replication in the therapeutic site, with little to no toxicity, using microRNA control.

Comparative assessment of mir122 controlled wild-type adenovirus with other microRNA controlled viruses

The data presented here has shown consistent knockdown of microRNA regulated wild-type adenovirus replication in many experimental models using mir122. In order to determine if the level of microRNA mediated knockdown observed in these studies is either better, or worse, than similar approaches with other viruses many issues must be considered. The most important consideration is likely to be the level of expression of the microRNA which is to be used to suppress viral replication. For most studies using other viruses this has not been mir122 and, therefore, it is immediately difficult to compare in detail the effectiveness of the approach described here with other vectors. However, a similar approach to that shown here, using three microRNA binding sites for mir122, has also been evaluated for regulating the activity of adenovirus serotype five *in vitro* (Ylosmaki *et al.* 2008). This work suggested that mir122 control of adenovirus was less effective at reducing viral replication than the data produced within this thesis. Therefore, Ylosmaki *et al* concluded that further attenuation of the virus was necessary in order to prevent CPE in Huh7 cells. Their decision to further attenuate this vector without testing it in models other than Huh7 was probably scientifically poor, given that Huh7 cells were used as a mir122-positive cell line, yet these cells show such a significant reduction in

mir122 levels compared to normal hepatocytes (Chang *et al.* 2004). The superior performance of the virus reported here may also reflect the presence of four microRNA binding sites, rather than three, in the 3′ UTR of E1A. The absence of *in vivo* testing by Ylosmaki *et al* has also made direct comparisons difficult. This problem is further compounded by the observation that the virus modified by Ylosmaki *et al* was also not the wild-type strain. Instead, the authors inserted the mir122 binding sites into an Ad5 based virus in which the CR2 region of the E1A protein was deleted between amino acids 922-947. Whilst these issues make direct comparisons difficult, the observation that mir122 could down regulate E1A, and reduce adenoviral replication, in the work presented by Ylosmaki *et al* helps to validate the research presented herein, even though the models the authors used suggested the microRNA control was less efficient than that reported here.

RNA viruses have also been shown to be susceptible to microRNA regulation. Perez *et al* demonstrated that microRNA regulation of Influenza virus replication could attenuate the virus by more than 2-logs following *in vivo* challenge to mice (Perez *et al.* 2009). Similarly, Barnes *et al* demonstrated that Polio virus could be attenuated by up to 3-logs following *in vivo* challenge in mice (Barnes *et al.* 2008). Interestingly, whilst Perez *et al* could not isolate virus from the peripheral blood of mice after infection with microRNA attenuated Influenza virus, Barnes *et al* reported that Polio virus isolated from the spinal cords of mice often showed substitutions within the microRNA binding sites, although these mutations did not lead to the generation of escape mutants.

The finding that RNA viruses could be efficiently controlled by microRNA regulation was further confirmed by studies using Coxsackie virus strain 21 (CAV21). This virus

could be attenuated by up to 5-logs *in vitro* following the artificial introduction of microRNA mimics into cell lines. However, whilst the virus could be efficiently controlled *in vitro* it was found that lethal escape mutants could emerge *in vivo*. In total two out of eleven mice developed lethal myositis after 45 days post infection (Kelly *et al.* 2008).

Combined, the data which has been published regarding the microRNA regulation of RNA viruses has consistently shown that the principle is highly effective at reducing replication, however, the finding that escape mutants can arise quickly, and with lethal toxicity, suggests that the low-fidelity of RNA-dependent-RNA-polymerases is a major limitation to the use of microRNA control.

The microRNA control of DNA viruses has also been shown using Herpes Simplex virus 1. Lee *et al* demonstrated that Herpes virus could be attenuated by controlling the expression of the gene ICP4 to allow cancer selective replication (Lee *et al.* 2009). The introduction of microRNA mimics into cell lines showed that virus replication could be reduced up to 5-fold *in vitro*. The *in vivo* therapeutic index of the parental Herpes virus strain compared to the microRNA regulated virus was not calculated and it is therefore difficult to assess how efficiently the virus was controlled in mice. The *in vitro* data suggests that microRNA regulation is less efficient at regulating a DNA virus. It is interesting to note that data presented herein for adenovirus showed significantly higher levels of attenuation, up to 30-fold *in vitro*. This may reflect the central importance of the E1A protein for adenoviral replication. ICP4 protein in Herpes virus, whilst serving

important functions, is not responsible for all Herpes virus gene transcription (Wu *et al.* 1996). This may explain the increased control seen in the work presented here.

The published examples of other studies using microRNAs to control virus replication have shown that this technique can be applied to a wide variety of virus families. It is interesting to find, however, that the usefulness of the approach may depend more on the replicative fidelity of the virus itself rather than the level of control mediated by the microRNA. This is exemplified by the absence of any report describing the reversion or deletion of microRNA binding sites for any DNA viruses even though they are less effectively controlled than their RNA counterparts. The ability of microRNAs to prevent the replication of RNA viruses so effectively is likely to be due to the ability of the microRNA to directly target the viral genome for degradation. In the case of DNA viruses, the genome cannot be directly degraded, therefore the microRNA must be sufficiently potent to prevent enough protein from being produced from the regulated mRNA to prevent viral genomic replication. In the example presented in this thesis for adenovirus, it was anticipated that if mir122 could not knockdown E1A protein production sufficiently then viral replication may proceed to 'wild-type' levels after an initial lag phase. This would occur because the E1A protein that was still produced could act on the DNA genome to allow some genomic replication. This, in turn, would produce more E1A protein and allow more virus genomes to be produced, and make microRNA control more difficult. In the work shown here this did not occur, but it is feasible that, if sufficient quantities of virus were used, the regulatory capacity of the microRNA could be overcome.

Reversion and loss of microRNA control

Virus genomes are often devoid of non-essential genetic material and can be size restricted. Moreover, the mutation rate of many RNA virus polymerases is high due to a lack of proof-reading capability. The reversion and loss of microRNA binding sites from a modified replication competent virus is therefore a major safety concern. This is particularly important where the wild-type strain of the virus has the capacity to induce significant pathology in humans. As described above, most published studies using microRNA regulation of RNA viruses have reported some level of reversion within viral preparations after multiple passage (Kelly *et al.* 2008; Kelly *et al.* 2010). Typically RNA viruses have mutation rates of $1x10^{-4}$ per nucleotide which is orders of magnitudes lower than all DNA viruses (Lauring *et al.* 2010). The mutation or deletion of sequences which are not essential for replication is therefore likely to occur relatively quickly following extensive growth *in vitro* or *in vivo*. RNA viruses encoding insertions of approximately equal size to the luciferase coding sequence have been reported to lose such sequences in less than five passages (Pugachev *et al.* 1995). As described above, RNA viruses encoding microRNA binding sites have also been shown to revert to their wild type form and cause lethal toxicity (Kelly *et al.* 2008). This has not been reported for DNA viruses which have much higher replicative fidelity (Lauring *et al.* 2010). In the case of adenovirus, the virus encodes a high-fidelity DNA polymerase which has proof reading capacity (Field *et al.* 1984). This decreases the chance of base mis-incorporation through 3'-5' exonuclease activity (Field *et al.* 1984). If these viruses are engineered to encode luciferase, no appreciable drop in flux levels is observed over 50 passages despite luciferase serving no essential function for viral replication.

The inclusion of microRNA binding sites into the genome of a DNA virus should also have few consequences for packaging of the viral genome. The size of the insertion is small and has no secondary structure, because the DNA of most DNA viruses is double stranded. It is also possible, in DNA viruses, to insert the microRNA binding sites within close proximity to key regulatory elements such as poly-adenylation signals and promoters. This principle limits the possibility of large deletions, containing the microRNA binding sites, producing viable virus clones.

In the case of RNA viruses many of these principles and techniques are not possible, or are only poorly effective. RNA viruses typically depend on significant nucleic acid secondary structure as a major determinant of viral translation, replication and packaging, and the availability of insertion points is often limited, due to only small 3' and 5' UTRs which are sensitive to modification. The pressure to lose such insertions is, therefore, great and any loss is enhanced and expedited by the lack of RNA polymerase proof-reading capacity. It is therefore desirable to use a DNA virus where possible when exploiting microRNA mediated virus control to limit reversion, maintain microRNA suppression and prevent toxicity.

Clinical development

The field of virotherapy has developed several oncolytic viruses that have progressed into clinical trials, including adenovirus, reovirus, vaccinia, Newcastle Disease Virus and herpes simplex. Adenoviruses have seen the greatest clinical development, leading to product licensure in China for the oncolytic virus H101. Generally, oncolytic adenovirus clinical trials have shown only limited toxicity, and it can be distinguished into acute

inflammation arising from input virus particles interacting with the immune system (flu like symptoms observed in a large percentage of patients), leukopenia and toxicities arising from virus infection and replication (Nemunaitis *et al.* 2001; Reid *et al.* 2002; Laurie *et al.* 2006; Hotte *et al.* 2007). Elevated circulating transaminases are sometimes seen in patients receiving oncolytic adenoviruses (Nokisalmi *et al.* 2010), and this is assumed to reflect hepatic infection of the adenovirus. Although hepatic toxicities have not been serious so far (Au *et al.* 2007), all adenovirus clinical studies so far have employed the common serotype Ad5, to which most patients have significant pre-existing immunity. As we move into clinical studies using rarer serotypes (for example a group B oncolytic virus, ColoAd1, is currently scheduled for clinical trial in Oxford) viruses may survive longer in the body and we may see increased hepatic infection. Accordingly it is an attractive possibility to engineer such viruses by incorporating mir122-binding sites to decrease potential liver toxicity without abrogating anticancer activity.

Governmental regulations surrounding clinical trials are both complex and detailed. As such if the virus designed and tested within this thesis was to be used clinically, many properties of the virus would need to be examined, including the testing of the stability of the inserted genetic material and demonstration of consistent microRNA mediated knockdown between separate adenovirus batches. To assess the latter of these issues, a reproducible model for analysing microRNA mediated knockdown would have to be established. Given the variability between individual batches of primary human hepatocytes it is unlikely that these cells would allow the level of consistent testing required for batch release to good manufacturing process (GMP) guidelines. An alternative approach could be the mir122 positive cell line Huh7, however, the level of

microRNA within these cells could potentially drift over time, or change, dependent on the environmental conditions in which the cells are cultured, although this has not been experimentally demonstrated. Although this eventuality could be controlled for by continual RT-QPCR analysis it is unlikely that this model would reflect the high level of knockdown observed in primary hepatocytes, in part due to the 88% reduction in mir122 levels within Huh7 cells. Stem cell-derived human hepatocytes would comprise a very attractive model, if they were properly validated and consistent. However the tissue engineering technology required for this is still in development (Takata *et al.* 2011). As a realistic, practical and consistent model for the analysis of adenovirus batches, the transfection of microRNA mir122 mimics into non-liver derived cells may represent the most suitable model. This model allows the transfection mir122 into cells and could be controlled by the creation of cell line carrying a mir122 sensitive reporter construct. For clinical development this model is likely to reflect the most accurate and reproducible system with the highest level of microRNA mediated knockdown *in vitro*. *In vivo* testing in murine models could also be performed, and safety (but not anticancer efficacy) could be assessed in the permissive immune-competent Syrian Hamster (Thomas *et al.* 2006), and compared with other Ad5-based agents. ALT and AST plasma levels could be used as an accurate indicator of liver pathology, as was performed in this thesis.

In order for any genetically modified virus to be tested clinically, the stability of the genetic modifications must be properly tested. The assays used to determine this property vary depending on the vector to be analysed. For testing the stability of microRNA binding sites inserted into adenovirus the use of deep DNA sequencing methods would allow the number of adenoviral genomes containing mutated binding sites to be calculated

in relation to the number containing intact binding sites. Another approach which could be employed could involve the serial dilution of adenovirus particles down to a dilution of 1 viral plaque forming unit per well in 96 well plates. By using the transfection of mir122 mimics, as described above, it would be possible to quantify the number of viruses showing a wild-type phenotype in relation to the number showing the expected level of mir122 mediated knockdown. Readouts could include cell survival (measured using a MTS or Resazurin assay) and/or by measuring either E1A production or viral genome replication. This method would be more laborious than sequencing analysis and the number of particles that could be tested would be minimal, but this method would make it possible to determine the phenotypic and biological consequences of particular mutations within the microRNA binding sites.

Limitations and future work

Whilst the adenovirus designed in this thesis shows promise as an anti-cancer agent, there are still many limitations to its use clinically. One such issue is the potent neutralising capacity of human blood against adenovirus serotype five. This issue has been circumvented by other groups by using other human serotypes or non-human adenoviruses. Although this approach does potentially decrease the amount of virus which is neutralised following initial delivery, many other serotypes are susceptible to anti-adenovirus cross-neutralising antibodies, and the host will still rapidly mount an immune response against the new virus, limiting its future utility. Other approaches have involved the genetic or chemical modification of the virus surface, but these are both limited by similar issues. All of these approaches allow more time for initial infection and viral

replication but most strategies do not allow repeat delivery and/or do not avoid immune clearance. Both of these issues represent important limitations to current virotherapy.

Whilst anti-viral immunity is a major obstacle to successful treatment, the use of microRNAs to control virus replication also has its limitations. Once such issue is that of reversion through the deletion and loss of the binding sites. The absence of any proof reading RNA polymerases makes it difficult to imagine how the fidelity of RNA viruses could be improved. This issue alone could make it impossible for microRNA regulated RNA viruses to be clinically translated. In the case of DNA viruses, the chances of reversion occurring is lower but it is still a major safety concern. As described above, this problem could be monitored carefully for clinical development but this would only monitor the effect, not decrease the chances of it occurring. To decrease the chances of reversion within a DNA virus, it might be possible to optimize the sequences surrounding, and between, the microRNA binding sites to reduce recombination or alternatively use multiple microRNA binding sites for multiple different microRNAs which are enriched in a particular tissue. This latter method would help to eliminate the chances of homologous recombination, because it would remove any homologous DNA sequences from within the viral genome. It may also be possible to alter coding sequences within conserved regions of viral genes within DNA viruses. This could limit the chances of deletions and base pair substitutions leading to viable virus production. This approach was attempted by Perez *et al* for Influenza but could also be used to regulate DNA virus replication.

One other major issue, that has not been previously addressed, is the microRNA control of mRNAs that have been produced from strong viral promoters without turning the promoter itself off. In the case of potentially oncogenic viruses such as retroviruses, the

insertion of the viral genome in close proximity to mammalian genes within the genome could cause over expression of particular cancer associated genes and lead to transformation. Given that viral expression would be silenced in the cytoplasm by microRNA regulation, and no structural genes would be produced, it would be impossible for the immune system to detect the virus and clear potentially transformed cells. In the case of adenovirus, the control of structural genes rather than E1A could have similar consequences by allowing long term expression of the oncogenic proteins E1A and E1B. The virus designed in this thesis should not have this effect because the virus does not integrate into the host genome and the potentially oncogenic proteins are silenced efficiently by preventing E1A expression.

Conclusions

Genetic modification of adenoviruses to allow replication selectively within tumour cells often relies on their activation by cellular mechanisms, which can be weak compared to intrinsic viral mechanisms. Accordingly such viruses, while providing good safety, often show decreased anti-cancer potency compared to wild type strains, particularly *in vivo* (Bazan-Peregrino *et al.* 2008). The exploitation of the microRNA regulatory network is enabling the design of many new viruses for cancer virotherapy, vaccination and gene therapy that are modified to allow selective replication and expression only in specific cell types and represents a new, safe and effective method of controlling viral gene expression.

The research presented here has proven that it is possible to effectively control the replication of a DNA virus, adenovirus, using microRNA control within the livers of mice and also within primary human hepatocytes. It was anticipated that the regulation of a DNA virus might be inefficient due the inability of microRNAs to degrade the DNA genome, however, the results have shown that this is not the case. MicroRNA control is a highly efficient method of tissue specific virus attenuation that harnesses the therapeutic potency of the wild type virus strain at the therapeutic site. As such, Ad5-mir122 represents a potent oncolytic virotherapy candidate with significant therapeutic potential, and the use of microRNA regulation may well yield a new generation of effective yet safe oncolytic viruses.

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Appendix