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Adenoviral Vectors Targeted to Hepatocellular Carcinoma

by

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third leading cause of cancer-related deaths. Due to the low response to current HCC therapy, many studies have been done to identify novel HCC therapeutics. Non-replicating first generation adenovirus (Ad) vectors and conditionally replicating oncolytic Ads have been extensively studied as potential HCC therapies. Several mechanisms have been utilized to target these Ads to HCC cells in order to reduce the potentially high liver toxicity associated with this therapeutic approach. Since the liver-specific miR-122 is downregulated in many HCC cells, the use of RNA interference-mediated post-transcriptional targeting of therapeutic genes by insertion of miR-122 targeted sequences is a promising targeting modality for Ads.

Although miR-122-mediated targeting of FGAd vectors encoding pro-apoptotic genes may have potential as therapies for HCC, amplification of these vectors is currently challenging due to the induction of premature apoptosis of the packaging cells. In this study, we have attempted to circumvent this difficulty through post-transcriptional silencing of the apoptotic gene in packaging cells expressing miR-122.

We have also utilized miR-122-mediated targeting modality to increase the specificity of oncolytic Ads for HCC cells. Furthermore, we show that HCC selectivity was increased by deletion E1b and VA-RNA genes. While these deletions reduced the activity of the virus, we found that the oncolytic activity of the virus could be enhanced by treatment with 2-aminopurine. Since VA-RNA-

deleted Ads are attenuated in HEK293 cells commonly used for packaging virus, we have identified the HCC cell line Hep3B as a potential alternative for the amplification of these Ads.

In our studies presented here, we have examined systems to increase the concentrations of first-generation and oncolytic Ads that are challenging to amplify. Furthermore, we show that Ads lacking E1b and VA-RNA genes have high potential as a novel targeted HCC therapy.

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List of Abbreviations and Acronyms

2'AP	2-aminopurine
bp	base pair
Ad	adenovirus
ADAR	dsRNA-induced adenosine deaminase
ADP	adenovirus death protein
AmpR	Ampicillin resistance gene
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
CAR	coxsackie-adenovirus receptor
CBP	cAMP-response element binding protein (CREB) binding protein
CDK	cyclin-dependant kinase
CR	conserved region
CREB	cAMP-response element binding protein
CPE	cytopathic effect
DMEM	Dulbecco's modified Eagle's medium
ds	double stranded
E1	adenovirus early region 1
E2F	adenovirus early region 2 promoter binding factor
EBV	Epstein-Barr virus
EBER	Epstein-Barr virus noncoding RNA
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
FGAd	first-generation adenovirus vector
GFP	green fluorescent protein
GFU	green fluorescent units
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
hCMV	human cytomegalovirus immediate early promoter
HEK293	human embryonic kidney 293 Cells
HRP	horseradish peroxidase
HSP	heat-shock protein
HSPG	heparan sulfate proteoglycan
HSV-TK	herpes simplex virus thymidine kinase
hTERT	human telomerase reverse transcriptase
IFN	interferon
IFNAR	IFNα receptor

IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
ITR	inverted terminal repeats
kb	kilobase
KanR	Kanamycin resistance gene
LB	Luria-Bertani medium
MAPK	mitogen-activated protein kinase
mCMV	murine cytomegalovirus immediate early promoter
MDM2	mouse double minute 2
MEM	minimum essential medium
miR-122T	miR-122 targeted sequence
miRNA	microRNA
MLP	major late promoter
MOI	multiplicity of infection
NFkB	nuclear factor kappa light chain enhancer of activated B cells
NS	not significant
OD	optical density
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing 0.1 % Tween-20
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PFU	plaque forming units
PI3K	phosphoinositide 3-kinase
PKR	dsRNA-induced protein kinase
PKRi	dsRNA-induced protein kinase inhibitor
PMSF	phenylmethanesulfonyl fluoride
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA transcript
RID	receptor internalization and degradation
RIPA	radioimmunoprecipitation assay buffer
RISC	RNA-induced silencing complex
RFU	relative fluorescent units
RLU	relative light units
RNAi	RNA interference
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	small hairpin RNA
STAT	signal transducer and activator of transcription

TACE	transcatheter arterial chemoembolization
TENS	Tris-EDTA, sodium hydroxide and SDS solution
TFIID	transcription factor IID
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
UTR	untranslated region
VA1	virus-associated RNA1
VA2	virus-associated RNA2
VA-RNA	virus-associated RNA
VEGFR	vascular endothelial growth factor receptor
VP	virus particles

Chapter 1 - Background

1.1 Liver Disease

1.1.1 Liver Cirrhosis

The liver is one of the largest organs and has many functions, such as blood homeostasis, bile secretion and fat metabolism, as well as immunity (1). The liver consists of a heterogeneous population of parenchymal cells, such as hepatocytes and cholangiocytes, as well as non-parenchymal cells, such as endothelial cells, liver-specific macrophages (Kupffer cells), hepatic stellate cells and immune cells (1).

One of the most common liver diseases is cirrhosis of the liver, which can be induced by chronic inflammation. Cirrhosis is associated with many risk factors, such as chronic infection by hepatitis B and C viruses (HBV and HCV) and aflatoxin B1 exposure. This disease is characterized by the continuous death and regeneration of cells, involving either hepatocyte entry into the cell cycle directly or induction of progenitor cell division (1-5). Furthermore, hepatic stellate cells repair the damaged area by producing large amounts of collagen, inducing fibrosis (6-8). Liver-associated endothelial cells as well as hepatic stellate cells promote angiogenesis to provide blood supply to the new hepatocytes (9). However, chronic liver regeneration can lead to portal vein hypertension, which could further lead to many diseases such as hepatic failure, renal failure and hepatic encephalopathy (10; 11). Furthermore, chronic HBV and HCV infection, as well as cirrhosis, are major risk factors for liver cancer development (11-16).

1.1.2 Liver Cancer

The most common form of liver cancer is hepatocellular carcinoma (HCC), which comprises of about 80% of total liver cancers (17; 18). Although the exact mechanisms of HCC development are not fully established, chronic HCV and HBV infections are known risk factors. Furthermore, patients co-infected with HBV and hepatitis D virus are thought to have a higher risk of HCC development (19). Additionally, patients with chronic HBV infection exposed to aflatoxin B1, which is strongly associated with promotion of p53 mutations, were found to have an increased risk of HCC development, compared to patients with chronic HBV infection alone (20; 21).

Activation of several oncogenic pathways have been observed in HCC, such as the phosphoinositide 3 kinase (PI3K) (22) and mitogen-activated protein kinase (MAPK) (23) pathways. These pathways are activated through the deregulation of many receptor tyrosine kinases, such as the epidermal growth factor receptor and the transforming growth factor β receptor (24-28). Other research has focused on determining the role of the RNA interference (RNAi) pathway in HCC development and progression (29; 30).

1.2 Current HCC therapy

1.2.1 Surgery, Radiation and Chemotherapy

As with most localized cancers, surgery is the most effective treatment for HCC. This treatment is mostly through partial liver resection. However, many HCC patients also have liver cirrhosis and low liver function, and therefore, resection may not be a viable option. Furthermore, the location of the tumour, as

well as the presence of metastasis, may contraindicate liver resection (31; 32). Another surgical option is liver transplantation, which has shown improved progression-free survival (33). However, due to the low availability of donors, this treatment option may not be available for many patients. In addition to surgical resection, radiofrequency ablation and percutaneous ethanol injections are often treatment strategies for patients with high liver function (32).

Chemotherapy is another therapeutic approach for HCC. In order to preserve liver function, the drugs are often administered directly to the tumour through transcatheter arterial chemoembolization (TACE). However, in patients with advanced metastatic disease, chemotherapy is often administered systemically or by hepatic arterial infusion. Unlike surgery, chemotherapy is not associated with long progression-free survival, partly due to the high resistance developed to the drugs (32; 34).

1.2.2 Antiviral Therapy

While HBV vaccines are likely to reduce future incidence of HCC, there are approximately 300 million individuals with chronic HBV infection, of which approximately 25% may develop HCC (35-37). Furthermore, there has been an increase in HCV-positive carriers, and therefore, the numbers of HCV-related HCCs are predicted to increase in the future (38). Thus, reducing chronic inflammation in HCV- or HBV-infected patients is an actively researched area. One of the main therapies for both HCV and HBV is interferon therapy in combination with nucleoside analogs.

The interferon (IFN) response is one of the main cellular antiviral mechanisms (39-42). Following virus infection, activation of transcription factors, such as NFkB and interferon regulatory factors (IRFs), induce the expression of IFNβ, which activates type I IFN response through binding to IFNAR1 and INFAR2 receptors (43-48). Receptor activation induces the activation of the signal transducer and activator of transcription (STAT) factors, STAT1 and STAT2, which then heterodimerize and bind IRF9 forming the interferon-stimulated gene factor 3 (ISGF3) complex (49; 50). Activated ISGF3 induces the expression of interferon-stimulated genes (ISGs) (51). Additionally, this activity is enhanced by the histone acetyltransferases GCN5 and p300 (52; 53). Similar to IFNβ, IFNα was also found to induce ISG expression through activation of IFNAR receptors (48).

Many viruses, such as HCV and HBV, have evolved mechanisms to downregulate the

IFN response. Interestingly, the IFN signaling pathway is also downregulated in many cancers (54). IFN therapy, which is designed to re-activate the IFN response pathway in infected cells, was found to reduce virus-associated HCC development (55-57). Furthermore, IFNα treatment was found to induce apoptosis of HCC cells in vitro through upregulation of tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) receptor expression (58), suggesting that IFNα therapy may also have a therapeutic effect on non-virus-associated HCCs.

Nevertheless, interferon therapy may not be a viable option for all patients due to development of resistance to the therapy and to side effects severe enough

to result in noncompliance (59-62). As a result, identification of novel therapeutics to block chronic HBV and HCV infection and HCC development are actively researched.

1.2.3 Targeted Therapy

Sorafenib has been shown to improve survival of patients with high liver function or with advanced disease, and has been approved as an HCC targeted therapy (63; 64). Sorafenib is a tyrosine kinase inhibitor, originally identified as an inhibitor of Raf-1, a member of the MAPK pathway (65; 66). Since its discovery, however, it has also been found to inhibit additional tyrosine kinases, such as VEGFR and PDGFR, suggesting that sorafenib may also block angiogenesis (67; 68).

While sorafenib showed great promise preclinically, the increase in survival was found to be less than one year (63; 64). Therefore, many studies are currently performed to determine whether sorafenib activity can be enhanced in HCC cells in combination with other therapies, such as TRAIL (69; 70). Furthermore, additional novel targeted therapies for HCC are being actively pursued, such as through modulation of the RNAi pathway.

1.3 The RNA Interference Pathway and HCC

1.3.1 RNAi Pathway

The RNAi pathway (Figure 1.1) is a highly conserved biochemical pathway that is thought to be part of the cell's innate antiviral immune responses (71). RNAi is activated in the cytoplasm by long double stranded (ds) RNA molecules, which are expressed during the life cycle of many viruses, and this

induces highly specific inhibition of gene expression (Reviewed in (72)). The long dsRNA molecules are cleaved to shorter molecules, termed small interfering RNAs (siRNAs), by the RNase III enzyme Dicer (73; 74) and its accessory proteins, TRBP and PACT (7; 75-78). SiRNAs are approximately 21 base pairs (bps) long and include a guide and a passenger strand (79; 80). Dicer directs the siRNAs to the RNA-induced silencing complex (RISC) (76), which cleaves the passenger strand and retains only the guide strand (81; 82). Targeted transcripts that contain sequences completely complementary to the guide strand will then be degraded by the RISC complex (83-86).

1.3.2 RNAi in Gene Regulation

In addition to the antiviral role of RNAi, another important role of the pathway in many organisms, including humans, is cellular gene regulation (Reviewed in (87; 88)). In this arm of the RNAi pathway, inhibition of gene expression is mediated by endogenously expressed short RNA molecules called microRNAs (miRNAs). Most miRNAs are transcribed by RNA polymerase II (RNA pol II) as long primary miRNAs (pri-miRNAs) containing a stem loop structure (89; 90). Once transcribed in the nucleus, pri-miRNAs are cleaved at the base of the stem-loop by an RNase III enzyme, Drosha, releasing an approximately 70 nucleotide (nt) stem-loop structure called precursor miRNA (pre-miRNA) (91-94). Pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin-5 (95-99), where Dicer binds and cleaves the pre-miRNA to produce the miRNA (73). Similar to siRNAs, miRNAs are short dsRNA molecules, which include a passenger and a guide strand, also called mature

miRNA. RISC binds to the mature miRNA and induces cellular gene expression inhibition.

Most mammalian miRNAs require only 7 nucleotides at the 5' end of the RNA (seed sequence) to be completely complementary to the target site (100-103). In mammalian cells, following the binding of RISC-bound miRNA to target mRNAs, RISC inhibits the translation of the mRNA rather than inducing cleavage (103). The mechanism of translational inhibition is currently not clear but it is thought to occur through the inhibition of either translation initiation or elongation (87; 88). RISC-bound mRNAs are localized in cytoplasmic RNA storage areas called processing bodies (P-bodies), which contain deadenylases and decapping enzymes that reduce the half-life of mRNAs. Therefore, although most mammalian miRNAs do not directly induce degradation of their target mRNAs, they could indirectly lead to their degradation by reducing mRNA stability (104-113).

Since their discovery, miRNAs have been shown to be important in the regulation of many cellular pathways. Certain miRNAs have been found in all cell types, while others are tissue-specific and important in the function of the tissue, such as the hepatocytes-specific miR-122 (114).

1.3.3 The Liver-Specific miR-122

The liver-specific miR-122 was first discovered through cloning and sequencing total miRNAs within mouse liver (114). This study showed that miR-122 accounted for 72% of total liver miRNAs. A subsequent study showed that miR-122 was also expressed in human hepatocytes (115). The same group also

experimentally validated the first miR-122 target, the cationic transporter protein, CAT-1 (116). To identify additional miR-122 targets, a microarray was performed on mouse liver tissue following inhibition of miR-122 using a chemically modified RNA molecule that was completely complementary to miR-122, called antagomir-122 (117). The study found that 363 transcripts were up-regulated while 305 transcripts were down-regulated. From the transcripts found to be down-regulated by antagomir-122, the group identified miR-122 targets important in cholesterol biosynthesis. Interestingly, antagomir-122 was found to indirectly downregulate 3-hydroxy-3-methylglutaryl-Coenzyme A reductase. Furthermore, serum cholesterol was specifically reduced following *in vivo* administration of antagomir-122 (117).

In addition to the regulation of cholesterol and lipid biosynthesis, miR-122 was also shown to be important in promoting apoptosis following cellular stress, through direct inhibition of the pro-apoptotic Bcl-2-like protein Bcl-w as well as through regulation of p53 translation and stability (118-122), suggesting that miR-122 has a role in tumour suppression. Interestingly, the expression of miR-122 was found to be downregulated in HCC samples and cell lines, and this downregulation was correlated with dedifferentiated tumours as well as poor prognosis (123; 124). Furthermore, overexpression of miR-122 *in vitro* and in an HCC tumour model was shown to reduce cell proliferation and survival, confirming the role of miR-122 as a tumour suppressor (125; 126).

MiR-122 was also found to have a role in the host antiviral response through the regulation of the IFN response, where a recent study has found that inhibition of miR-122 activity reduced interferon α (IFNα) and IFNβ-mediated

activation of the interferon response (127). Interestingly, treatment with IFN was found to downregulate miR-122 expression (128), suggesting a negative feedback loop between miR-122 and the IFN response. Furthermore, studies have shown that miR-122 expression may have a direct effect on the replication of HCV and HBV. HCV replication was found to rely on miR-122 binding to the 5' untranslated region (UTR) of the viral RNA genome (129). Thus, miR-122 inhibition is thought to be a promising therapy for patients with chronic HCV infections, and is currently in clinical trials (129; 130). In contrast, miR-122 expression was found to downregulate HBV replication through binding of viral mRNAs (122; 131). Therefore, overexpression of miR-122 has high potential as a therapy for HBV and HCC.

Interestingly, recent studies suggest that miR-122 may sensitize HCC cells to sorafenib, as both inhibit the activity of the insulin-like growth factor 1 receptor (125). These results suggest that sorafenib in combination with miR-122 may have a stronger therapeutic effect than sorafenib treatment alone.

In addition to sorafenib and miR-122, modified adenoviruses (Ads) have been extensively studied as a potential HCC targeted therapy. These modified viral vectors are designed to specifically induce cancer cell death through either delivery of therapeutic genes or through cell lysis mediated by completion of the adenoviral replication cycle.

1.4 Adenoviruses

Adenoviruses (Ads) are icosahedral nonenveloped linear double stranded (ds) DNA viruses with an approximate size of 90 nm, and a genome of approximately

36 kilobases (kb) in size (132). They were originally identified from adenoid tissue in a patient with an upper respiratory infection (133-135). As of 2013, at least 51 human serotypes have been discovered based on their binding to inactivating antibodies (136; 137). These serotypes are grouped into 6 subgroups, A through F (138). Most Ad infections result in non-life threatening diseases, such as upper and lower respiratory tract diseases, gastrointestinal diseases and conjunctivitis. However, in immune-compromised individuals, Ad infections could result in more severe diseases, such as pneumonia and viral hepatitis, and may be fatal (139-142).

Most of the current knowledge of Ad infection and replication was acquired through studies with the members of the subgroup C viruses, Ad2 and Ad5. Therefore, the majority of the studies to determine the potential of Ads for clinical applications have used these serotypes.

The initial step of Ad2 and Ad5 entry into host cells is the binding of the fiber protein to the primary receptor on the cell, the coxsackie-adenovirus receptor (CAR) (143-146). Next, the penton protein binds to the secondary receptors, $\alpha_v\beta3$ or $\alpha_v\beta5$ integrins, which induces virus entry (147; 148). Once the virus enters the host cell, it is shuttled to the nucleus using the microtubule transport system (149-151).

In the nucleus, the first gene to be transcribed is the immediate early gene E1a, which is essential for the activation of the other early and late viral genes as well as for the promotion of cell entry into S-phase (152-156). Entry into S-phase

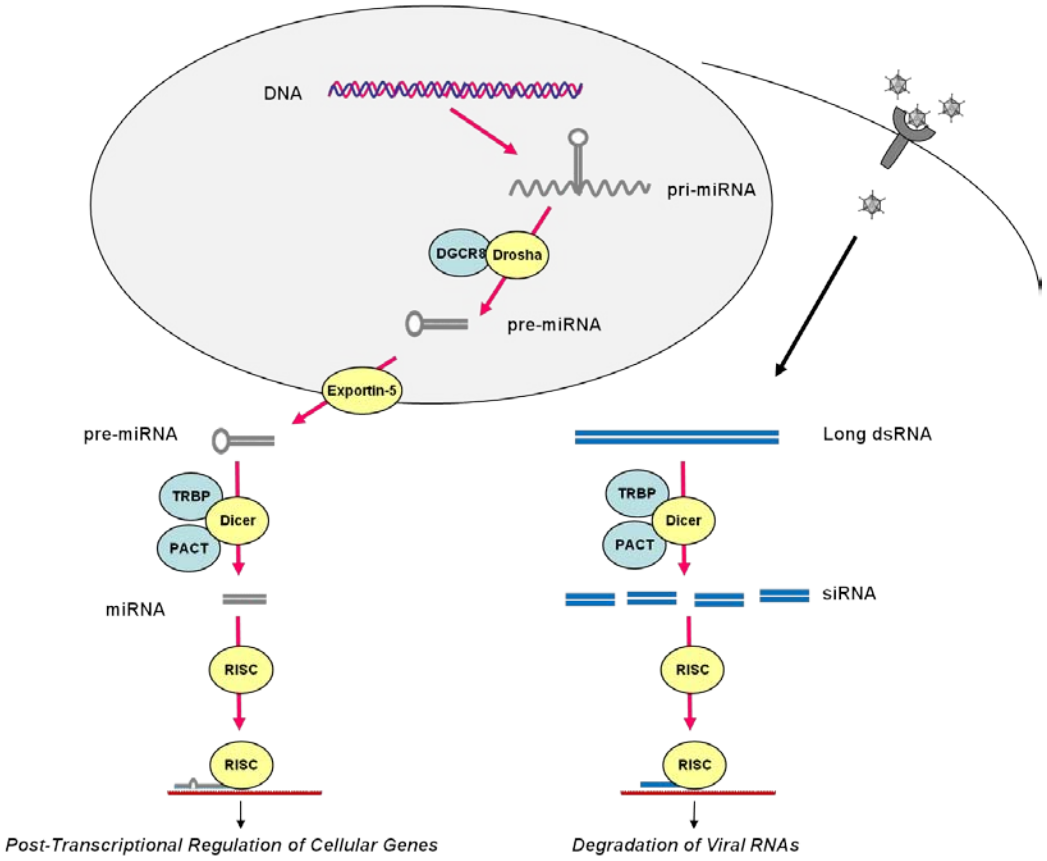


Figure 1.1: Schematic representation of the RNA interference pathway.

The RNAi pathway is an evolutionarily conserved antiviral mechanism, in which viral long dsRNA molecules (blue) are processed into small-interfering RNAs (siRNAs) by key enzymes of the pathway (yellow) and their accessory proteins (light blue) in order to induce the degradation of viral transcripts. The RNAi pathway is also important in cellular gene regulation through processing genes encoding primary-microRNAs (pri-miRNAs) transcripts into small miRNAs.

allows for viral DNA replication, which is directly mediated by the E2 gene products (152-154).

Following DNA replication, the late adenoviral genes are expressed, which mostly encode the structural proteins (157). While the early genes have separate promoters, the late genes are thought to be under the control of a single promoter, the major late promoter (MLP) (158). Through processing of the MLP transcript, 5 transcript families containing different polyadenylation signals are expressed (L1, L2, L3, L4 and L5), which are then further processed to approximately 30 mRNAs (157).

Interestingly, a smaller MLP transcript is expressed early during infection, which codes for the L1-52/55K protein. This protein interacts with viral pIVa2 to regulate MLP activity (157; 159). Furthermore, the switch from early to late MLP expression is thought to be induced by the activity of the adenovirus splicing factor, L4-33K (160).

In addition to early and late gene promoters, pIVa2 and pIX are under the control of intermediate promoters, and are expressed prior to the large MLP transcript (161-163). Furthermore, the Ad genome encodes two noncoding RNA molecules, the virus associated RNA 1 (VA1) and VA2, which are expressed throughout the viral lifecycle, and are under the control of an RNA polymerase III promoter, which is activated by E1a proteins (164-166).

Following viral structural protein expression, the capsid is formed and mature virus is released from the host cell following induction of host cell death. While the mechanism of viral release is not fully established, recent studies have

suggested that virus-mediated cell death occurs through autophagocytosis mediated by caspase activation (167; 168). Furthermore, the E3-encoded adenovirus death protein (ADP; E3-11.6K), which is highly expressed at late timepoints during infection, was found to be important in virus-mediated cell death and spread (169-171). In addition to induction of cell death, viral release may also rely on structural changes of the cell. Studies have also shown that the viral proteinase L3-23K is important in viral release

partly through degradation of cytokeratins (172).

1.5 Role of E1 Proteins in the Promotion of Virus Replication

1.5.1 The E1a Protein

Once the adenoviral genome enters the nucleus, the first transcript to be expressed is E1a, which is translated into at least 5 proteins through alternative splicing (173; 174). The two most studied proteins are the E1a 13S and 12S (also called 289R and 243R) of subgroup C viruses (175; 176). The E1a gene encodes four regions highly conserved between different serotypes, conserved region 1 (CR1), CR2, CR3 and CR4. While the 13S E1a protein contains all regions, the 12S E1a does not contain the CR3 region (154; 177; 178).

1.5.2 E1a as a Transcription Factor

The E1a proteins were found to be essential for initiation of viral DNA replication. This fundamental role in virus replication is due to the ability of E1a to function in both the activation and suppression of cellular and viral gene expression (152-154). Although E1a binds DNA directly, this DNA binding ability was not found to be important for its activity (179; 180). E1a transcriptional

activity is mediated by binding to host transcription factors. The transactivation ability of E1a was mapped to the CR3 domain, therefore, the 13S E1a protein is thought to be most important in transcriptional activation (154; 181). Studies have shown that the CR3 domain associates with many transcription factors such as MED23 and TFIID (182; 183). In addition to binding to transcription factors, E1a interacts with transcriptional coactivators such as CBP/p300 and p400 (184-187).

1.5.3 E1a-Mediated pRB Inhibition

A major role of 12S and 13S E1a is promotion of cell entry into S-phase through inhibition of the tumour suppressor protein, retinoblastoma (pRB) (155; 156). Binding of E1a to pRB allows the release of E2F transcription factor family members from pRB. Released E2F proteins then act as transcription factors to activate many cellular proteins involved in promoting S-phase. In addition to activating host genes, E2F proteins induce viral E2 gene expression, and therefore, viral DNA replication (188; 189).

Another function of E1a is to promote apoptosis. E1a was found to induce p53 activity through direct binding and inhibition of MDM4, an inhibitor of p53 (190). Furthermore, E1a-mediated release of E2F from pRB could also induce apoptosis through transcriptional activation of pro-apoptotic genes, such as APAF-1 and p14ARF (191-194). p14ARF promotes the stabilization of p53 through promoting the degradation of another p53 inhibitor, MDM2 (195-197). p14ARF may also sequester MDM2 to the nucleolus, thereby blocking MDM2 ability to inhibit p53 (198). Furthermore, p14ARF was found to activate p53 in an MDM2-independent mechanism through promoting the degradation of an

additional p53 inhibitor ARF-binding protein 1 (199). E2F1 and p14ARF were found to induce apoptosis in a p53-independent mechanism through downregulation of the anti-apoptotic Bcl-2-like protein, Mcl-1 (200; 201). Interestingly, E1a was also found to inhibit Mcl-1 expression and to promote Mcl-1 protein degradation (202).

Ads have evolved several mechanisms to inhibit E1a-induced apoptosis prior to virus replication. E1b gene products have been shown to inhibit both p53-dependent and independent-apoptosis directly during virus replication.

1.5.4 Role of E1b Proteins in the Inhibition of E1a-Induced Apoptosis

The E1b gene products E1b-19K and E1b-55K were found to inhibit E1a-mediated apoptosis by multiple mechanisms (203; 204). Ads with deletion of E1b-19K were found to produce large plaques as well as to induce DNA fragmentation compared to wild-type Ad, suggesting that E1b-19K expression blocked apoptosis (205; 206). Subsequent studies found that E1b-19K protein contain functional and sequence homology to the pro-apoptotic Bcl-2 protein and interacts with Bax and Bak to inhibit apoptosis (205; 207-209).

Similar to E1b-19K, E1b-55K was found to be an important anti-apoptotic protein (204). E1b-55K associates with the viral E4ORF6 protein to form a ubiquitin ligase complex, which induce the degradation of p53 as well as other host proteins (210; 211).

1.5.5 E1b-55K Role in the Inhibition of the DNA Damage Response

In addition to inhibition of apoptosis, E1b-55K was also found to be

important in the inhibition of the DNA damage response. Due to the linear structure of the Ad genome, host cells are thought to detect the genome ends as dsDNA breaks (212; 213). In an attempt to repair the damage, host proteins may induce concatemerization of viral genomes, producing DNA sequences larger than the packaging limit, which interfere with virus production (213-216). One of the main complexes that detect DNA damage is the MRN complex, comprised of Mre11, Nbs1 and Rad50 (217; 218). Furthermore, Mre11 was found to contain a nuclease activity important in the initiation of dsDNA break repair (219; 220). The MRN complex also was found to recruit the ataxia telageictasia mutated (ATM) and ATM-and Rad3-related (ATR) proteins, which phosphorylate many substrates, including checkpoint proteins, p53 and MDM2, in order to block cell cycle progression (221-228). In order to block the DNA damage response, E1b-55K promotes the degradation of key proteins within the DNA damage response, such as p53, Mre11, DNA ligase IV and Bloom helicase (210; 211; 213; 229-232).

1.5.6 E1b-55K Role in Promotion of Late Virus Gene Expression

The ubiquitin ligase complex containing E1b-55K was also found to have an important role in late virus gene expression through promotion of nuclear export of late mRNAs as well as protein translation (233-240). Interestingly, while L3 and L5 cytoplasmic mRNA export are more dependent on E1b-55K expression than L2 mRNA export is, proteins encoded by all three late transcript families were reduced in E1b-55K-deleted Ad infected cells (235; 240). Furthermore, in addition to promotion of late viral gene expression, E1b-55K and

E4ORF6 have also been shown to have a role in the inhibition of cellular protein synthesis through inhibition of cellular mRNA export and translation which is thought to be important for viral release and spread (236-238; 241).

1.6 Role of Viral Genes in the Inhibition of the Innate Immune Response

In addition to the promotion of virus replication, the E1a and E1b-55K proteins were found to be important in the inhibition of the IFN response. Additionally, other viral genes have been shown to be important in the inhibition of the immune response, such as the E3 and the VA-

RNA genes (Figure 1.2).

1.6.1 Inhibition of Cellular Innate Immunity by E1a

The E1a gene products were found to block the activation of IFN-stimulated gene (ISG) expression following interferon treatment (242; 243). One mechanism of E1a-mediated inhibition of ISG expression occurs through direct binding and inhibition of STAT1 transcription factor activity (244-246). E1a can also inhibit ISGF3 transcription factor complex formation and activity through inhibition of transcription coactivators, p300 and GCN5 (52; 53; 246). NFkB, a key player in host innate immunity, is also inhibited by E1a through the inhibition of IkB kinase (IKK) activity (247). While the mechanism of E1a-induced inhibition was not determined in the study, a recent study showed that E1a may reduce IKK activity through inhibition of miR-520h (248). Interestingly, another study has also shown that E1a reduced IFN-induced histone H2B monoubiquitination through inhibition of hBre1 ubiquitin ligase-complex formation (249). Furthermore, the authors found that E1a-mediated inhibition of

H2B monoubiquitination reduced ISG expression (249).

1.6.2 Inhibition of Cellular Innate Immunity by E1b-55K

Similar to E1a, E1b-55K was found to have transcriptional inhibitory activities. E1b-55K has been reported to inhibit the transcription of p53-induced genes as well as ISGs (250-253). In addition to transcriptional inhibition, E1b-55K was also found to inhibit the activities of several IFN-inducible proteins (254). In particular, E1b-55K was found to sequester the IFN-inducible protein Daxx away from the PML bodies where it commonly resides (254). Furthermore, Daxx-mediated enhancement of p53 transcriptional activity was inhibited by E1b-55K (254). Another recent study has found that E1b-55K blocked the activity of the IFN-inducible protein, PKR (255). PKR, a serine/threonine kinase, binds to dsRNA molecules, leading to PKR autophosphorylation and activation. Active PKR then can phosphorylate several substrates, including eIF2α which was found to inhibit total protein translation upon phosphorylation (256). In addition to eIF2α, p53 was also found to be a substrate of PKR, suggesting that E1b-55K-induced inhibition of PKR might be an additional mechanism of p53 inhibition (257; 258).

1.6.3 Inhibition of Cellular Innate Immunity by VA-RNAs

The VA-RNAs, VA1 and VA2, are RNA molecules of approximately 160 nucleotides that contain high degree of secondary structure. The VA-RNAs contain three domains, the apical stem, the central stem and terminal stem domains (164; 259-261). Both VA1 and VA2 are expressed at low levels early during infection and at high levels at late timepoints following infection (260).

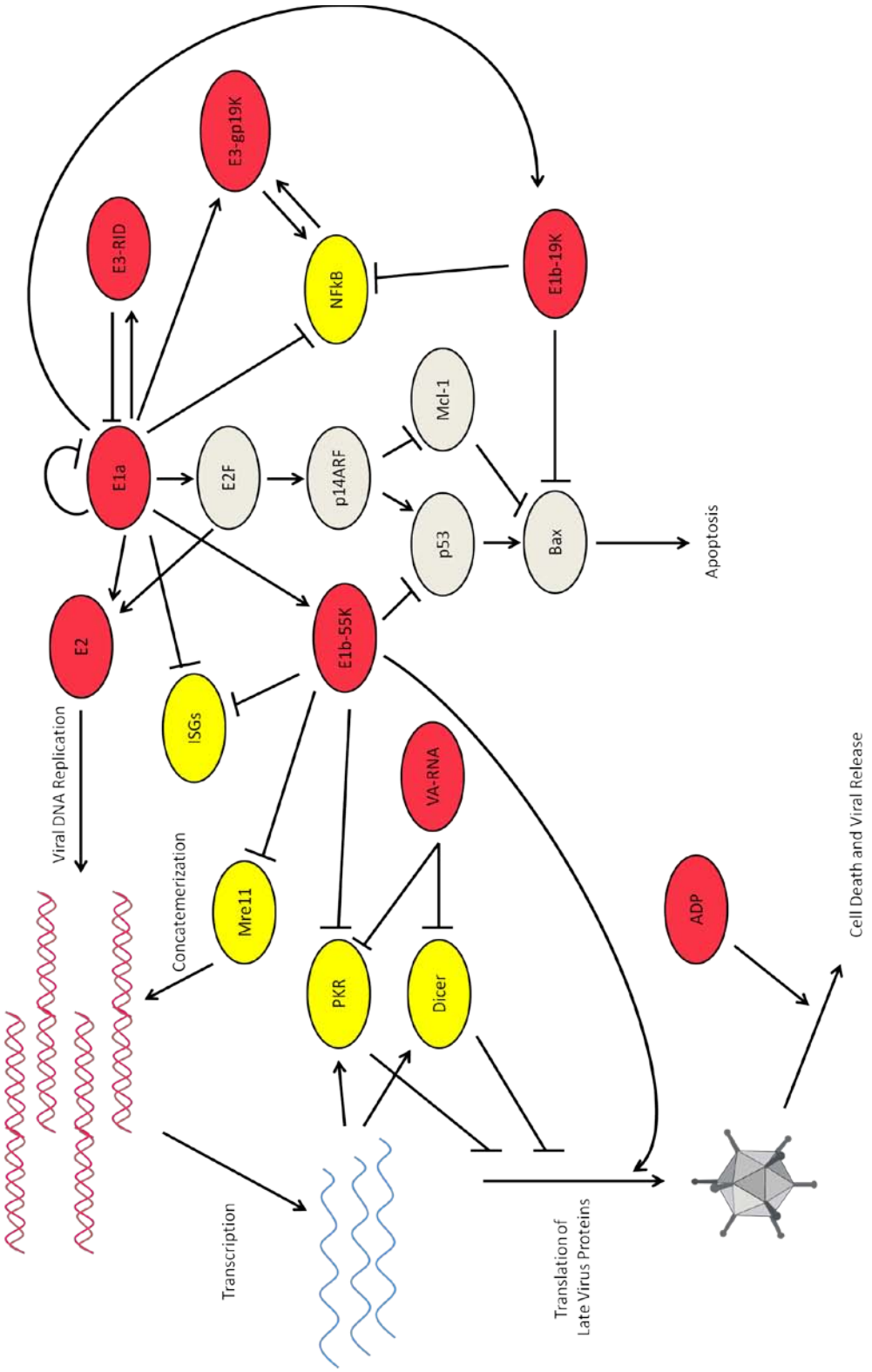

E3-gp19K
E3-RID
NFkB
E1b-19K
E1a
E2F
p14ARF
Mcl-1
p53
Bax
Apoptosis
E2
E1b-55K
ISGs
VA-RNA
Viral DNA Replication
Concatemerization
Mre11
PKR
Dicer
ADP
Cell Death and Viral Release
Transcription
Translation of
Late Virus Proteins

Figure 1.2: Role of early proteins and VA-RNAs in promotion of virus replication.

The interaction of early viral proteins and VA-RNAs (Red) with host proteins allows the inhibition of E1a-mediated apoptosis (grey) as well as the inhibition of intra-cellular antiviral immunity (yellow) to allow the progression of the adenovirus life cycle.

Similar to E1b-55K, one of the major functions of VA1 is PKR inhibition (Figure 1.2) (262-268). Additionally, VA1 is important in the inhibition of an additional IFN-inducible protein, the dsRNA-induced adenosine deaminase (ADAR) (269). The role of VA2 during infection is currently unknown. Studies have shown that deletion of VA2 had little effect on virus growth, whereas deletion of VA1 reduced virus growth. Additionally, further reduction of virus growth was detected with the deletion of both VA-RNAs when compared to the deletion of VA1 alone, suggesting that VA2 may partially compensate for VA1 deletion (262; 267; 270; 271).

Studies have shown that both VA-RNAs are processed by the RNAi pathway into virus-

associated miRNAs (Figure 1.3) (272-275). Furthermore, cellular targets of VA1-associated miRNAs were identified and validated (276). Due to the high levels during virus replication, processing of the VA-RNAs was found to saturate the RNAi pathway (275). Therefore, it is currently unknown whether the general inhibition of the RNAi pathway or the specific inhibition of the virus-associated miRNA targets is important in virus growth. *In vitro* studies have found that inhibition of RNAi enzymes, Dicer or Exportin-5, was sufficient to rescue VA-RNA-deleted Ad replication in HeLa cells (277). In contrast, inhibition of PKR was not sufficient for the rescue of a VA-RNA-deleted Ad, suggesting that the impact of VA-RNAs on RNAi inhibition is more important for virus growth in these cells (278). In contrast, PKR inhibition but not Dicer inhibition rescued VA-RNA-deleted Ad replication in HEK293 cells (279).

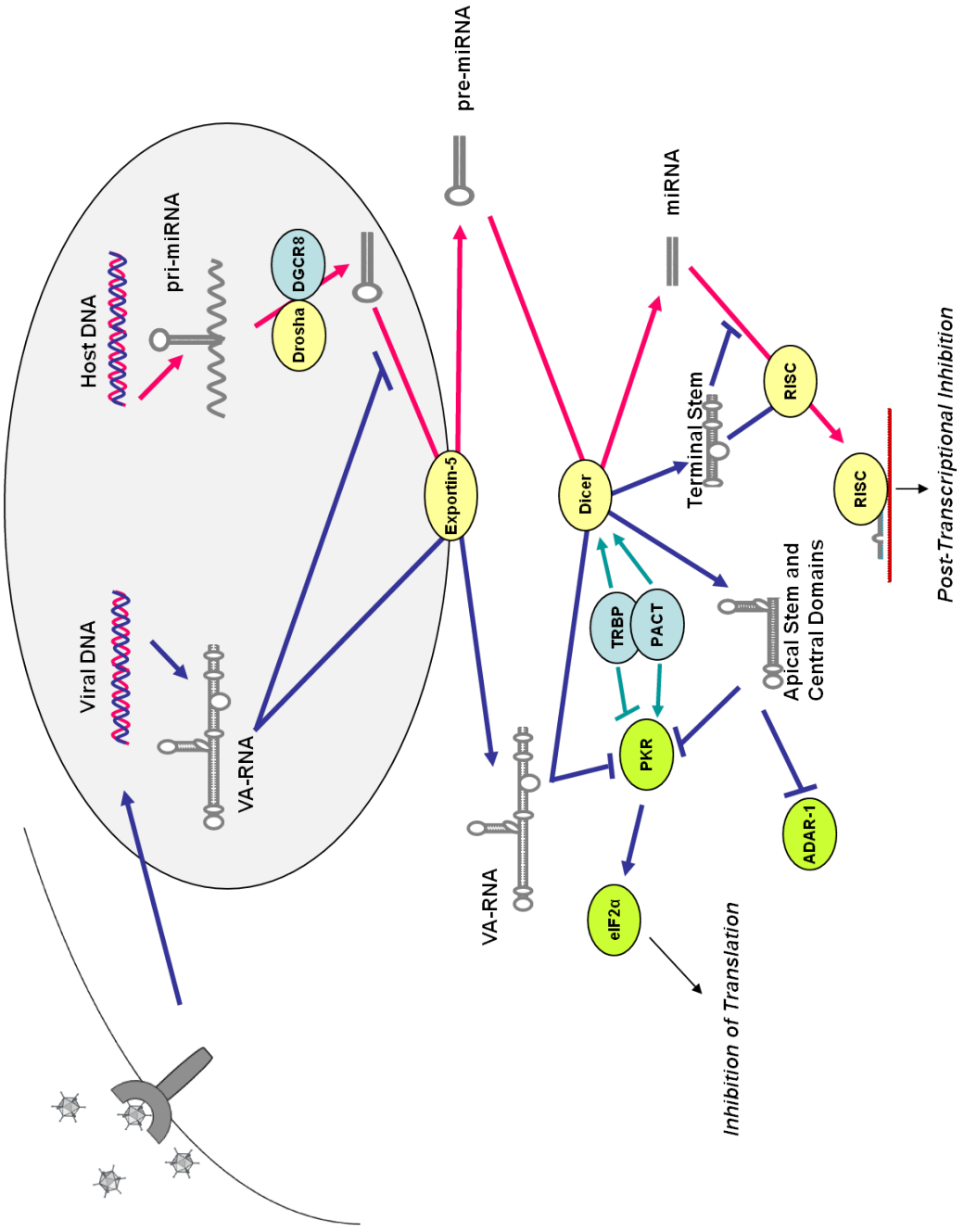

Host DNA
pri-miRNA
Drosha
DGCR8
pre-miRNA
Viral DNA
VA-RNA
Exportin-5
Dicer
miRNA
Terminal Stem
RISC
RISC
Post-Transcriptional Inhibition
TRBP
PACT
Apical Stem and
Central Domains
PKR
ADAR-1
VA-RNA
eIF2α
Inhibition of Translation

Figure 1.3: Mechanism VA-RNA-mediated inhibition of RNAi and interferon signalling pathways.

VA-RNAs inhibit the RNAi pathway by saturating key enzymes within the pathway (depicted in yellow and their accessory proteins in blue). Furthermore, the VA-RNAs inhibit several interferon-stimulated proteins, such as PKR and ADAR-1 (green).

Furthermore, a study has shown that inhibition of PKR through treatment with the PKR inhibitor, 2-aminopurine (2'AP), was sufficient to rescued VA-RNA-deleted Ad replication in HEK293 cells (280; 281). These results suggest that the requirement of VA-RNA activities in virus growth might be host cell dependent.

1.6.4 Inhibition of Immune Response by E3 Gene Products

Most of the E3 gene products were found to be important in the inhibition of antiviral immunity. However, while the E1 proteins as well and the VA-RNAs were also shown to be important in the inhibition of the intracellular antiviral immunity, the E3 proteins are thought to be important in the inhibition of the antiviral immune response mediated by the immune cells.

The E3 promoter is activated by E1a, similar to other viral genes, as well as by NFkB (282-284). The E3 gene products include E3-12.5K, E3-6.7K, E3-gp19K, ADP, receptor internalization and degradation a (RIDα), RIDβ, and E3-14.7K (Figure 1.4). While the function of E3-12.5K is currently unknown, the roles of the other E3 gene products have been studied.

E3-gp19K, a glycoprotein localized to the ER membrane, is important in the inhibition of MHC class I antigen loading and transport to the plasma membrane, thereby blocking antigen presentation and detection by immune cells (285-287). Interestingly, this inhibition was found to activate the NFkB pathway (288), therefore E3-gp19K may be important in further upregulation of the E3 promoter through NFkB activation.

E3-6.7K and the RID complex, which contains RIDα and RIDβ, induce

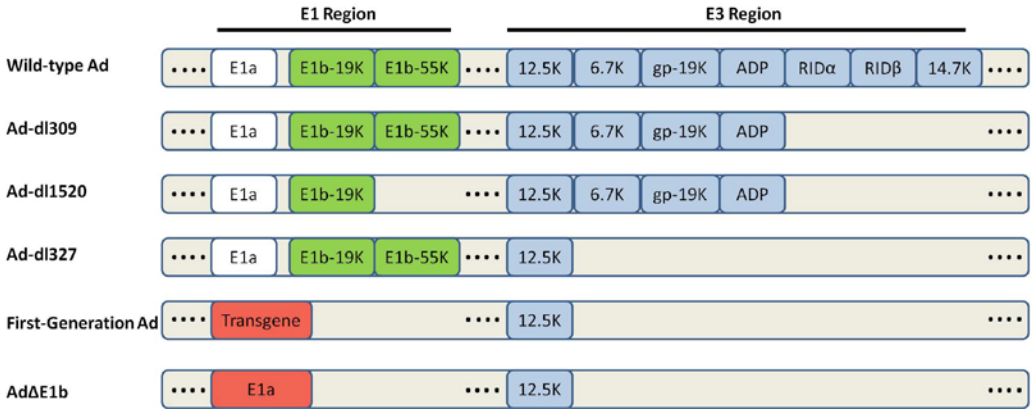


Figure 1.4: Schematic representation of E1 and E3 gene deletions of Ads used in this study.

The E1 region encodes the E1a gene (white) as well as the E1b gene (green), which expresses two major proteins, E1b-19K and E1b-55K. The E3 region encodes 7 proteins (blue), however, a deletion in the E3 region of Ad-dl309 and the E1b-55K-deleted Ad-dl1520 results in the loss E3 sequences coding for RIDα, RIDβ and 14.7K. Ad-dl327 contains a larger E3 deletion than Ad-dl309 resulting in the loss of all but the sequences coding for 12.5K. The same E3 deletion as in Ad-dl327 is often found in E1-deleted first-generation Ad vectors and oncolytic Ads, such as AdΔE1b, in which a therapeutic gene (red) or the E1a gene (red) were inserted in the E1 region, respectively.

the degradation of inflammatory receptors, such as Fas, TRAIL and TNFR, to ultimately block apoptosis mediated by immune cells (289; 290). Similarly, E3-14.7K is important in the inhibition of TNFR-mediated cell death (291-296). E3-6.7K localizes to the ER and maintain calcium homeostasis of the ER, and therefore may reduce ER stress-mediated cell death (297; 298).

Due to the function of the E3 gene products in inhibition of immune cell recognition and induction of cell death, they are not thought to be essential for Ad replication *in vitro*. Therefore, many studies are performed with Ads containing a small deletion, such as in Ad-dl309 as well as a large deletion, such as in Ad-dl327, due to the ease of construction of mutant viruses from these Ads (Figure 1.4).

However, while not essential for Ad production, several E3 gene products were found to enhance virus replication *in vitro*. The E3 protein ADP was found to have a role in virus release and spread (169-171). Furthermore, similar to the E1b proteins, RIDα and/or RIDβ were found to inhibit E1a-induced cell death through downregulation of E1a expression (299-301).

1.7 Adenoviruses as Potential Cancer Therapeutics

Soon after discovery, studies were performed to determine the potential of Ad as a cancer therapy. While cancer regression was observed following treatment with replicating Ads, most patients succumbed to the disease following treatment (302; 303). Much more is now known about Ad replication, as well as virus-host interactions, and technology has greatly improved such that novel Ads have been designed and constructed to increase the efficacy of these cancer

therapeutics.

There are many advantages for the use of Ads as a therapeutic. There is extensive knowledge of Ad infection and biology, which allows researchers to modify the Ad genome to specifically target the virus to cancer cells. Furthermore, there are currently relatively easy systems for the construction and purification of new Ad vectors (304). The frequency of Ad genome integration is extremely low (305; 306), therefore, adenoviral infections have a low risk of insertional mutagenesis. Furthermore, due to the high expression of CAR in many cell types (146; 307), these vectors have broad tropism. Currently, the most studied Ad vector for cancer gene therapy is the first-generation Ad (FGAd) vector. These vectors were originally constructed with a deletion of the E1 region, rendering them nonreplicating (308-310). Furthermore, this deletion allowed for the introduction of a transgene of approximately 5 kb. As E3 gene products mostly inhibit cell death mediated by immune cells (311), these protein were found to be non-essential for virus replication *in vitro*, and therefore, most of the E3 region was also deleted in order to increase the cloning capacity of the vectors to approximately 8 kb (304; 312).

In order to amplify FGAd vectors, different cells have been constructed that express the E1 gene products in *trans*, such as the human embryonic kidney (HEK) 293 cells (313), the human embryonic retinoblasts, 911cells (314) and PerC.6 cells (315).

FGAd vectors have been extensively studied as vehicles for delivery of many therapeutic genes to HCC cells *in vitro*, *in vivo*, and in clinical trials. These

therapeutic genes include genes to activate an anti-tumour immune response, such as interleukin-12 (IL-12) (316; 317). FGAd vectors have also been extensively used to deliver tumour suppressor genes, such as miR-122 and p53 (318; 319). Furthermore, an FGAd vector encoding the p53 tumour suppressor (Gendicine) was approved for the therapy of head and neck cancers in China (320). Additionally, several clinical studies have shown that Gendicine may increase survival of HCC patients when combined with chemotherapy or radiotherapy (321-323). In addition to delivery of tumour suppressor genes, FGAds have also been used to deliver pro-apoptotic genes, such as Bax, to
HCC cells to induce high cancer cell killing (324; 325).

In addition to FGAds, oncolytic Ads have been studied as potential HCC therapeutics. These Ads are often constructed through re-insertion of the viral E1a and/or E1b genes, rendering them replication competent. Due to the ability of oncolytic Ads to replicate, much lower viral load would be required to induce a therapeutic effect when compared to FGAd vectors.

Due to the ubiquitous expression of the CAR receptor, Ads can infect many cell types, and therefore, the therapeutic gene may be transferred to and expressed in normal cells (146; 307). Furthermore, CAR expression is downregulated in many cancer cells, and therefore, high viral loads may be required to obtain a therapeutic effect (326-328). Thus, there has been much research involved in targeting Ads specifically to the cancer cells. One of the main targeting modalities that have been extensively studied is restriction of transgene expression to cancer cells, which can be accomplished through different

mechanisms, such transcriptional and post-transcriptional targeting.

1.7.1 Transcriptional Targeting

Transcriptional targeting can direct transgene expression specifically to the cancer cells by the use of tissue-specific promoters (TSP) that are highly active in cancer cells. One of the most commonly used promoters for targeting therapeutic genes to HCC is the human telomerase reverse transcriptase (hTERT) promoter (329-331). hTERT is highly expressed in most cancer cells but not in normal cells (332). Furthermore, the hTERT promoter was found to be highly upregulated by the oncogenic transcription factor, c-myc, and downregulated by the tumour suppressor p21 (330; 333; 334).

The hTERT promoter was also extensively used in oncolytic Ads to restrict the expression of E1a to HCC cells. For example, hTERT-Ad was constructed to encode the E1a gene under the control of the hTERT promoter (335). This vector was found to specifically replicate in HCC cell lines *in vitro*, as well as reduce tumour growth in an Hep3B mouse tumour model (336). hTERT was also used to control the expression of both E1a and E1b using an internal ribosomal entry site, which allows the expression of both proteins from the same transcript (337; 338). Similar to hTERT-Ad, this vector (Telomelysin) was found to specifically replicate in HCC cells and induce cell death both *in vitro* and *in vivo* (339).

While hTERT expression is repressed in most tissues, several studies have found that proliferating epithelial cells, such ovarian cells, express telomerase (340-342). Furthermore, while studies have shown that hTERT is not expressed in

normal liver, proliferating primary hepatocytes were found to express detectable hTERT levels (343). Furthermore, telomerase promoter activity and expression was increased in normal liver tissue following partial hepatectomy in a mouse model (343). Since hepatocytes proliferation can occur during HCC development, hTERT promoter activity may be high in normal liver tissues of HCC patients. Thus, in addition to transcriptional targeting of Ads using hTERT promoter, other targeting modalities might be required to reduce hepatotoxicity mediated by transgene expression in normal hepatocytes.

1.7.2 Post-transcriptional Targeting

The discovery of oncogenic and tumour suppressor miRNAs has led to post-transcriptional targeting of Ad vectors to many cancers, including HCC. This approach involves the insertion of sequences complementary to tumour suppressor miRNAs in the 3'UTR of the therapeutic gene. For example, since the activity of the tumour suppressor miR-122 is inhibited in many HCC cells compared to normal cells, addition of miR-122 targeted (miR-122T) sequences has the potential to block transgene expression in normal hepatocytes without affecting the expression in miR-122-negative HCC cells (123). A study has shown that the insertion of six miR-122T sites in the 3'UTR of a luciferase gene encoded within an FGAd vector reduced gene expression in miR-122-positive HuH7.5 HCC cells by approximately 10 fold, and even more in normal hepatocytes that have higher miR-122 expression (344). In the absence of miR-122, however, the insertion of these miR-122T sites had no effect on luciferase expression.

Similar results were obtained when 4 miR-122T sites were inserted instead

of 6 sites (345). In this study, the authors also inserted 4 miR-122T sites in the 3'UTR of the HSV-TK gene encoded in an FGAd vector. The insertion of the miR-122T was found to reduce hepatotoxicity following administration of the virus in combination with gancyclovir, when compared to a control vector carrying sequences complementary to the miR-122T sites.

Post-transcriptional targeting was also studied in oncolytic Ads. Several studies have shown that liver toxicity in mouse models was decreased through the restriction of E1a expression by insertion of miR-122T sites in the 3'UTR of E1a (344; 346; 347). Furthermore, miR-122T insertion had no effect on Ad replication and lysis of miR-122-negative HCC cells *in vitro* and *in vivo* (344; 348). A recent study found that miR-122T targeting of E1a inhibited Ad replication in primary human hepatocytes (349), and therefore, miR-122T has high potential as a de-targeting modality to reduce liver toxicity with conditionally replicating Ads therapy for HCC.

While liver toxicity is a major concern due to high virus accumulation in the liver following Ad delivery, low level of Ads were also found to localize in other tissues, such as lung and spleen (350-352). Thus, additional targeting modalities may further improve the efficacy of miR-122-mediated post-transcriptional targeted oncolytic Ads.

1.8 Oncolytic Ad Design Through Deletion of Essential Genes

In addition to restriction of E1a and E1b expression to cancer cells, oncolytic Ads are also designed with deletions within viral genes that can be compensated for in cancer cells but not normal cells, such as viral genes involved

in inhibition of apoptosis and innate immunity.

1.8.1 E1b-deleted Oncolytic Adenoviruses

1.8.1.1 Cancer-Specificity of E1b-55K-Deleted Oncolytic Ads

One of the most commonly studied oncolytic Ad is Ad-dl1520 (Onyx-015), which contains mutations that block E1b-55K expression as well as a similar E3 deletion as Ad-dl309 (Figure 1.4) (353). Since one of the main functions of E1b-55K is the degradation of p53, it was originally thought that the cancer-specificity of Ad-dl1520 was due the loss of p53 in most cancer cells (354-357). However, it was later found that Ad-dl1520 cancer specificity was p53-independent (358-361).

Ad-d1520 replication and lysis were studied in the p53-positive HepG2 and the p53-negative Hep3B HCC cells (362; 363). Since both cell lines supported the replication of the virus, p53 status was not correlated with the Ad-dl1520 replication (358-363). However, cell killing did not parallel virus production, as Hep3B cells were more sensitive to Ad-dl1520-mediated cell death than HepG2 cells *in vitro* (362; 364). Protection of HepG2 might have been due to activation of p21 in a p53-dependent mechanism. Furthermore, Ad-dl1520 was also found to induce a greater anti-tumour activity in a Hep3B tumour model than in a HepG2 tumour model (362). These results suggest that while p53-positive HCC cells support Ad-dl1520 replication, virus-mediated cell death may be reduced in these cells.

Several studies have attempted to identify p53-independent markers that correlate with Ad-dl1520 replication in different cancer cells. It was suggested

that mutations or deletions of the p14ARF gene may result in increased sensitivity to Ad-dl1520 (358; 359), however it was later found that, similar to p53, p14ARF status was not correlated with Ad-dl1520 replication (365). This result is consistent with the fact that Ad-dl1520 is able to replicate in HepG2, which encodes wild-type p53 and p14ARF genes (365).

Several studies have shown that cancer specificity may be due to the E1b-55K function in promotion of late viral mRNA export (360). Interestingly, a study has shown that this function can be complemented by treatment with HSP90 inhibitors, such as geldanamycin (366). This result might be partly due to the increased HSP70 expression observed following geldanamycin treatment as a previous study has found that overexpression of HSP70 sensitizes cells to E1b-55K-deleted Ad replication (367).

1.8.1.2 Contribution of HBV X Protein in E1b-55K-Deleted Oncolytic Ad Activity

The HBx protein encoded by HBV was found to act as a transcription factor that activates several oncogenic pathways, such as NFkB and ras (368-370). Additionally, HBx was found to block activation of the DNA damage response through inhibition of p53 and Mre11 activity (371-374). HBx was also found to block the interferon response through the inhibition of many proteins within the pathway, such as IRF3 and PKR (375; 376). These results suggest that E1b-55K functions may be complemented in HBV-positive cells. Interestingly, a study has found that HBx facilitated lysis of cells infected with E1b-55K-deleted Ads (377). This result may explain the higher Ad-dl1520-mediated lysis of Hep3B cells, which encodes an integrated HBV genome (378), than of the HBV-negative

HepG2 cells.

1.8.1.3 Deletion of E3 and/or E1b-19K Increases the Oncolytic Activity of E1b-55K-Deleted Ads

Although Ad-dl1520 showed promise as a therapeutic agent for HCC, clinical trials have shown a minimal effect in patients treated by Ad-dl1520 therapy (379; 380). Nevertheless, this study showed that Ad-dl1520 therapy had no severe side effects. Interestingly, a virus similar to Ad-dl1520, called H101 (Oncorine), was constructed containing a deletion of the E1b-55K gene, but with a larger E3 deletion, similar to Ad-dl327 (Figure 1.4) (381). Oncorine has been approved in China for the treatment of head and neck cancers (382). In a recent case study, H101 was used in combination with TACE for the treatment of a patient with recurrent HCC, previously treated with surgery and TACE (383). With second line TACE, in combination with H101, no recurrence was detected even 18 months following treatment. Therefore, H101 in combination with common therapies may have a strong therapeutic potential for HCC, perhaps through the sensitization of cancer cells to chemotherapy.

A recent study has found that deletion of the E3 region increased E1b-55K-deleted Ad-mediated cell death without affecting virus replication in Hep3B cells (384). Therefore, these results may partially explain the enhanced clinical effect found in H101, which contains a larger E3 deletion than Ad-dl1520 does.

Although the deletion of the E3 gene may increase the lytic activity of E1b-55K-deleted Ads, several studies have found that the loss of the E3-encoded ADP may result in reduced Ad spread in certain cells (169-171). More recent

studies have shown, however, that deletion of E1b-19K rescued the reduced spread phenotype of E3-deleted Ads (385).

Interestingly, a study has shown that deletion of the E1b-19K gene enhanced replication of an E3-positive Ad as well as cancer cell death in comparison to wild-type virus, suggesting E1b-19K-deleted Ads may have oncolytic properties (386). Furthermore, E3-positive oncolytic Ads with deletions of both E1b-19K and E1b-55K genes (full E1b deletion) were shown to replicate efficiently in HCC cells as well as induce a stronger virus-mediated cell death than a virus with E1b-55K deletion alone (387-389). Therefore, similar to E3 deletion, E1b-19K deletion may increase the oncolytic activity of E1b-55K-deleted Ads. Several studies have also determined the oncolytic activity of Ads lacking the full E3 and E1b genes. While these studies showed that the mutant Ads were highly lytic in HCC cells, the effect of the combined deletions on the replication efficiency was not determined (390-395). Since the combined deletion of the full E1b and E3 genes have reduced ability to block E1a-mediated apoptosis relative to deletion of E1b gene alone, the observed lysis of infected HCC cells might have been due to premature cell death, leading to the degradation of both viral and host DNA, and therefore, attenuation of the virus (396; 397).

1.8.2 VA-RNA-deleted Oncolytic Adenoviruses

Similar to E1b-55K, the VA-RNA molecules have been shown to down-regulate the antiviral immune response (265; 266; 398; 399). Therefore, similar to E1b-55K-deleted Ads, VA-RNA-deleted or -modified Ads are predicted to be

attenuated in normal cells, yet retain their oncolytic potential. Similar to studies with E1b-deleted Ads, a comparison of different studies of VA-RNA-deleted Ads is complicated because the amount of the E3 gene retained in these viruses has been quite variable, from complete deletion to complete retention.

Previous studies have shown that replacement of the adenoviral VA-RNA genes with the

noncoding RNA sequences (EBERs) of the Epstein-Barr virus (EBV) can rescue Ad replication (400; 401). Therefore, a subsequent study determined the oncolytic activity of a VA1-deleted Ad in EBV-associated cancer cells (402). The VA1-deleted virus Ad-dl331, which also has a large E3 deletion (similar to Ad-dl327), efficiently replicated in and induced high cell death of EBV-associated cancer cells, such as the nasopharyngeal carcinoma cell line C666-1. The study also confirmed the results through the use of a gastric cancer cell line, AGT, stably replicating EBV (AGT-EBV). While the parental AGT cell line was found to be highly resistant to Ad-dl331, the Ad replicated efficiently and induced high cell death in AGT-EBV cells. Interestingly, Ad-dl331 was found to induce a higher anti-tumour response in a C666-1 tumour model than the wild-type virus control. Furthermore, the study also found that Ad-dl331 induced less liver toxicity than the wild-type virus.

Ad-dl331 is lytic in cancer cells not associated with EBV as well. Activating ras mutations or other non-ras mutations, which may affect PKR activation, may have roles in sensitizing these cancer cells to VA-RNA-deleted Ads (270; 280; 403-405). A study has reported that certain pancreatic cancer cells

are highly sensitive to Ad-dl331 (280). The same group later showed that an oncolytic Ad with deletion of both VA1 and VA2 (AdVAdel, with an intact E3 gene) had a higher specificity for cancer cells than did Ad-dl331 (403). Interestingly, in normal human hepatocytes, Ad-dl331 replication levels were similar to wild-type Ad levels, but AdVAdel replication was highly attenuated, suggesting deletion of both VA-RNAs might increase the cancer specificity of Ads in comparison to VA1-deletion alone. While expression of the E3 gene products may have contributed to the differences in replication efficiency in this study, a subsequent study has confirmed this observation using VA-RNA-deleted Ads with the same partial E3 gene deletion (similar to Ad-dl309) (270). Interestingly, the latter study also showed that while deletion of both VA-RNAs highly attenuated Ad replication in several normal cell lines, the same deletion had no effect on Ad replication HepG2 cells, suggesting that VA-RNA genes may not be required for Ad replication in HCC cells (270). Furthermore, since VA-RNA and E1b-55K have similar functions in the inhibition of the interferon response, deletion of VA-RNA may increase the specificity of an E1b-deleted Ad.

1.9 Effect of Adenovirus Modifications on Production of High Viral Titers

Oncolytic Ads and FGAd vectors are often constructed and amplified in the packaging cell line, HEK293, which endogenously expresses E1 gene products (313). However, this cell line may not be able to support the replication of Ads containing deletions of essential genes outside the E1 region. For example, VA-RNA-deleted Ads were found to be highly attenuated in this cell line (279; 280). Because oncolytic Ads grow in many cancer cell lines, these cell lines might

be better suited as packaging cells than HEK293. In contrast, FGAd vectors can only be amplified in cells complementing the E1 proteins in *trans*, such as HEK293 cells. However, if additional deletions in the FGAd are detrimental to virus growth, then modifications in HEK293 cells may be required for virus amplification. A recent study has shown that stable expression of VA1 in HEK293 cells increased production levels of a VA-RNA-deleted FGAd vector (406).

In addition to deletions in essential virus genes, insertion of certain transgenes may also hinder virus amplification. While FGAd vectors encoding toxic genes have therapeutic potential for many cancers, amplification of these vectors has been challenging due to the induction of packaging cell death mediated by the toxic gene (407). Therefore, several approaches have been studied to increase the amplification levels of these FGAds through inhibition of toxic gene expression at the transcriptional and translational level.

1.9.1 Transcriptional Inhibition of Toxic Gene Expression in Packaging Cells

A binary Ad system has been designed in order to amplify toxic gene-encoded FGAds (331). The toxic gene was placed under the control of a promoter containing GAL4 DNA binding sites. In order to transcribe the toxic gene, cells would have to be co-infected with an additional FGAd encoding a GAL4-VP16 fusion protein (331). In the absence of the GAL4-VP16 protein, high levels of vector production could be achieved. A similar binary system was designed in which transgene expression was inhibited by an insertion of a loxP-flanked sequence between the promoter and the open reading frame of the transgene. This insertion blocked expression of the toxic gene in cells unless they were co-

infected with an FGAd encoding Cre recombinase, which excised the loxP-flanked sequence (408; 409).

While toxic gene-encoding FGAds were efficiently amplified in packaging cells with these binary systems, cancer cells would have to be co-infected with both FGAds, and therefore, high viral loads would be required to induce a strong therapeutic effect. To circumvent this problem, a single vector was designed to be regulated by the Tetracycline-off system (325; 410). In this system, the FGAd-encoded toxic gene was placed under the control of a promoter containing a tetracycline-response element. An additional gene encoding the VP16-tetracycline repressor fusion protein was introduced at a second site in the same FGAd. This design allowed toxic gene expression to be activated by co-expression of the VP16-fusion protein in infected target cells. However, to reduce toxic gene expression during amplification of the FGAd, packaging cells could be treated with tetracycline, thus inhibiting activity of the VP16-fusion protein.

An alternative system has been designed to block FGAd vector-mediated gene expression in HEK293 cells through the use of the bacterial lac operon system (411-413). In this system, the lac operator sequence was inserted into the promoter controlling the toxic gene expression. To increase the yields of FGAds encoding toxic genes, vectors were amplified in packaging cells expressing the lac repressor that inhibited toxic gene expression at the transcriptional level.

1.9.2 Post-Transcriptional Inhibition of Toxic Gene Expression in Packaging Cells

Post-transcriptional inhibition of FGAd vector-mediated gene expression has also been investigated as a strategy to increase titers in packaging cells. One study has found that the transfection of antisense oligonucleotides targeting the toxic gene allowed for increased FGAd titers (414). However, the major drawback with this method is the requirement for continuous supply of synthetic oligonucleotides during propagation. Interestingly, a recent study has found that high titers of an FGAd vector encoding a nontoxic gene were obtained in HEK293 cells expressing a small hairpin RNA (shRNA) targeting the gene (415) suggesting that RNAi-mediated inhibition of gene expression might also be a potential method to amplify FGAd vectors encoding toxic genes.

1.10 Thesis Summary

Due to the loss of miR-122 expression in many HCC cells (123-126), post-transcriptional targeting of Ads through insertion of miR-122T sites has high therapeutic potential. While insertion of these sites in the 3'UTR of FGAd-encoded toxic genes may reduce toxicity of normal miR-122-positive liver cells, amplification of these Ads is highly challenging due to induction of apoptosis of packaging cells. Therefore, miR-122T sites were utilized to block FGAd-encoded Bax expression in engineered miR-122-positive packaging cells in order to increase the amplification levels. Results from this study suggest that while transient and stable expression of miR-122 was able to reduce FGAd-mediated gene expression, this reduction was not sufficient to allow high amplification

levels of FGAd-encoding Bax gene. Therefore, in addition to miR-122-mediated inhibition of gene expression, further modifications of packaging cells are required to reduce Bax expression in order to efficiently amplify FGAd vectors encoding this toxic gene.

MiR-122T sites were also introduced in the 3'UTR of the E1a gene of an oncolytic Ad in order to reduce hepatotoxicity. Furthermore, to increase the specificity to cancer cells, deletions in the E1b, the VA-RNA and the E3 genes were introduced (AdΔE1bΔVA). As this virus was found to replicate poorly in HEK293 packaging cells in the absence of the VA-RNA genes, Hep3B hepatocellular carcinoma cells were used to amplify AdΔE1bΔVA. Similar to its activity in HEK293 cells, the virus was found to replicate poorly in a panel of normal and cancer cells. However, Hep3B and HepG2 hepatocellular carcinoma cells were found to support the replication of the virus. Furthermore, 2'AP was found to specifically increase AdΔE1bΔVA growth in HCC cells as well as HCC cell lysis. Surprisingly, 2'AP treatment was found to compensate for the loss of E1b-55K rather than the loss of VA-RNA. Furthermore, the specific E1b-55K domain compensated by 2'AP treatment had previously been found to be essential for the inhibition of the DNA damage response. Results presented here suggest that co-administration of 2'AP derivatives that block host DNA damage response may increase the oncolytic activity of AdΔE1bΔVA without reducing its selectivity for HCC cells indicating a possible new strategy for treatment of HCC.

Chapter 2 - Construction, Amplification and Characterization of a Triple-Targeted Oncolytic Adenovirus

2.1 INTRODUCTION

The E1b-55K-deleted oncolytic adenovirus (Ad), Ad-dl1520 (Onyx-015) (353), has been extensively studied as a potential therapeutic agent for many cancer types, including hepatocellular carcinoma (HCC). One of the major roles of E1b-55K is thought to be inhibition of E1a-mediated p53 activation and induction of apoptosis. Therefore, it was originally believed that the cancer specificity of Ad-dl1520 was due to the loss of p53 activity in many cancers (204; 211; 250; 354-357). However, it was later found that the ability of cancer cells to replicate the virus was independent of p53 status, and therefore, the cancer-specificity of Ad-dl1520 might be due to a compensation of other roles of E1b-55K in cancer cells, such as promotion of late virus mRNA export as well as inhibition of host protein synthesis and DNA damage response (213; 229-239; 358-361). Furthermore, studies have shown that E1b-55K has a role in the inhibition of host antiviral immune response by blocking the induction of interferon-stimulated gene expression, as well as inhibition of PKR activation (251-253; 255).

While preclinical studies showed that Ad-dl1520 efficiently replicated in hepatocellular carcinoma cells as well as induced an anti-tumour response in mouse models, results from clinical trials found that the virus treatment was not sufficient to induce a strong therapeutic effect (353; 362; 364; 379). Interestingly, in vitro studies have shown that Ads containing deletions that block the expression of both E1b-19K and E1b-55K induced higher HCC cell killing without dramatically attenuating virus growth in comparison to E1b-55K-deleted

Ads (389-391). Furthermore, an Ad with deletion of the E3 region in addition to E1b-55K was found to induce a stronger cell death response in comparison to an E3-positive E1b-55K-deleted virus (384). Therefore, Ads with deletions of E1b-19K and E3 genes in addition to E1b-55K may have greater anti-tumour efficacy.

Although E1b-55K-deleted Ad replication is relatively selective for cancer cells, low levels of replication were observed in normal hepatocytes (360; 416). We propose that additional deletions of essential genes may increase the specificity of E1b-55K-deleted Ads. Similar to E1b-55K, VA-RNAs have been shown to be important in inhibiting the activation of PKR, as well as other members of the cellular antiviral pathways (267; 270; 280; 403; 417-420). Due to the overlapping roles of E1b-55K and VA-RNAs, deletion of both genes may decrease virus production in normal cells, providing an increase in safety for clinical applications.

We have designed a VA-RNA- and an E1b-deleted oncolytic Ad containing a deletion of the E3 region. To further increase virus-mediated cell death, the E1a gene was placed under the control of the strong immediate early murine cytomegalovirus (mCMV) promoter (AdΔE1bΔVA). As a VA-RNA-positive control, we also constructed an E1b- and E3-deleted Ad (AdΔE1b). To reduce E1a expression in normal hepatocytes, two miR-122-targeted sites were introduced into the 3'UTR of the E1a gene in both AdΔE1b and AdΔE1bΔVA. This strategy was based on previous studies showing that insertion of a sequence targeted by the liver-specific miR-122 (miR-122T) into the E1a gene inhibited virus replication in normal human hepatocytes, without affecting virus replication

in miR-122-negative HCC cells, thus reducing Ad-induced toxicity (344; 346-349).

Similar to previous reports (279; 280), we found that the deletion of the VA-RNA genes dramatically attenuated AdΔE1bΔVA virus mediated cell death and virus production in the packaging cell line HEK293 cells. Interestingly, we found that AdΔE1bΔVA was efficiently amplified to high titers in the HCC cell line Hep3B. We demonstrated that the HCC cell line HepG2 also supported AdΔE1bΔVA replication although AdΔE1bΔVA replicated poorly in other cancer and normal cells. Furthermore, we found that E1b-55K activities other than p53 inhibition could further enhance AdΔE1bΔVA production in HepG2 cells. Identification of the specific E1b-55K roles which allow enhanced AdΔE1bΔVA production in HepG2 cells could help in future design of improved HCC-targeted oncolytic Ads.

2.2 MATERIAL AND METHODS

2.2.1 Cell Culture

All cell lines in this study were maintained in growth conditions described in section A.2.1.4.

HEK293, HepG2, Hep3B and HuH7.5 cells were maintained as described in sections A.2.1.1 and A.2.1.2. HepG2 2.15 (421) and HepAD38 (422) HCC cell lines (gifts from Dr. Lorne Tyrell; University of Alberta, Canada) were previously established from HepG2 cells through stable transfection of the hepatitis B virus (HBV) genome. While HBV is constitutively produced in HepG2 2.15, the production of HBV in HepAD38 is under the control of tetracycline (422). HepG2 2.15 cells were maintained in DMEM. HepAD38 cells were maintained in DMEM supplemented with tetracycline-free 10 % FBS, 2 mM L-glutamine and 1X antibiotic-antimycotic solution. Experiments were performed with addition of 10 µg/mL tetracycline.

The human breast cancer cell lines, MDA-MB-231 (ATCC, HTB-26) (423), MDA-MB-468 (ATCC, HTB-132) (424) and ZR75.1 (ATCC, CRL-1500) (425), were maintained in RPMI-1640 (Gibco, Cat # 31800-022), whereas, SKBR-3 (ATCC, HTB-30) (426) was maintained in McCoy's 5α medium.

The human pancreatic cancer cell lines PL-45 (ATCC, CRL-2558) (427) and Panc-1 (ATCC, CRL-1469) (428), and the Syrian hamster pancreatic cancer cell line SHPC-6 (429) [a gift from Dr. Karoly Toth (Saint Louis University School of Medicine, MO, USA], were maintained in DMEM. The human

pancreatic cancer cell line, AsPC-1 (ATCC, CRL-1682) (430), was maintained in RPMI-1640.

The human bladder cancer cell lines UMUC3 (ATCC, CRL-1749) (431) and T24 (ATCC, HTB-4) (432) were maintained in RPMI-1640, whereas MGHU3 (433) and HT1376 (ATCC, CRL-1472) (434), were maintained in DMEM.

The human lung cancer cell line, A549 (ATCC, CCL-185) (435) and normal lung fibroblast cell line WI-38 (ATCC, CCL-75) (436) were maintained in DMEM. The normal lung fibroblast cell line MRC5 (ATCC, CCL-171) (437) was maintained in MEM.

2.2.2 Plasmid Construction

2.2.2.1 Previously Constructed Plasmids Obtained From Other Labs

The plasmid expression vector encoding the E1a gene (pXC1) was a kind gift from Dr. Frank Graham. The plasmid expression vector encoding adenoviral sequences spanning the wild-type VA-RNAs (pVA+) and a similar plasmid expression vector containing deletions in the promoter regions, which block the expression of both VA-RNAs (pVA-) (270), were gifts from Dr. Michael Schümann (Klinikum der Philipps-Universität Marburg, Germany). The plasmid expression vectors encoding the wild-type E1b-55K (pE1b-WT) and a mutant E1b-55K unable to inhibit host DNA damage response (pE1b-Mut; C454S/C456S) (231) were a gift from Dr. Thomas Dobner (Heinrich-Pette Institute, Germany). The plasmid expression vector encoding the HBV X protein

(pHBx) was a kind gift from Dr. Christopher Richardson (Dalhousie University, Canada).

The plasmid expression vectors encoding a microRNA-adapted shRNA targeting the p53 gene (pSMP-p53) as well as a microRNA-adapted scrambled shRNA (pSMP-Neg) were gifts from Dr. Andrew Shaw (University of Alberta, Canada). The plasmid encoding the luciferase reporter gene under the transcriptional control of p53 (Panomics, Cat # LR0057) as well as the control vector that does not contain transcriptional regulatory elements (Panomics, Cat # LR0000) were gifts from Dr. Raymond Lai (University of Alberta, Canada). The plasmid encoding the luciferase reporter gene under the transcriptional control of NFkB (Promega, Cat # E8491) was a gift from Dr. Andrew Shaw (University of Alberta, Canada).

2.2.2.2 Construction of Plasmids Encoding the E1a Gene Under the Control of the mCMV Promoter (Figure 2.1)

2.2.2.2.1 PCR Amplification of E1a Gene

In order to obtain the E1a gene (not including the E1a promoter) flanked by appropriate restriction enzyme sites, the E1a sequence was amplified from pXC1 (nucleotides 542-1564) through polymerase chain reaction (PCR) using Vent DNA Polymerase (NEB, Cat # M0254S). Sense primer (DS119; 5' A**CCATGG**CGACACCGGGACTGAA 3') containing the NcoI restriction site (shown in bold) and antisense primer (DS120; 5' **GCGGCCGC**AATCACAGGTTTACA 3') containing the NotI restriction site (shown in bold) were synthesized by Integrated DNA Technologies. The PCR

reaction solution included 0.1 ng of DNA, 1 unit of Vent polymerase (NEB, Cat # M0254S), 1x ThermoPol Reaction Buffer (NEB, Cat # B9004S), 1 µM of each primer and 0.4 µM deoxynucleotide (dNTP) solution set (Fermentas, Cat # R0192). The PCR conditions contained an initial denaturation step at 95°C for 3 min followed by 5 cycles of denaturation at 95°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 2 min. The initial cycles were followed by 25 additional cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 2 min. A final extension step at 72°C for 15 min was applied. The PCR product was identified using a 1 % agarose gel and purified using the GFX PCR DNA and Gel Band Purification Kit. The purified PCR product containing the E1a gene was then inserted into the pJET1/blunt plasmid using the GeneJET PCR Cloning Kit (Fermentas, Cat #, K1221) following the manufacturer's protocol, resulting in the plasmid pJET-E1a.

2.2.2.2.2 Construction of Plasmid Expression Vectors Encoding E1a Under the Control of the mCMV Promoter (Figure 2.1)

In order to construct a plasmid encoding the E1a gene containing two miR-122T sites, the E1a gene from pJET-E1a was inserted into pAD5-Bax-122Tx2 (Section A.2.2) to produce pAD5-E1a-miR122Tx2. The E1a gene within this plasmid was sequence-verified using sequencing primers obtained from IDT which flank the 5' and the 3' end of the E1a gene (DS121; 5' GCGGCTTCCTTGATCCTTGCC 3' and DS42; 5' ATCTTCGATGCTAGACGATCCAGACA 3', respectively).

The mCMV promoter was isolated from a previously constructed plasmid containing the mCMV promoter with the internal PacI site collapsed (pSJ4-DSMCS-SK5), and inserted in pAD5-E1a-miR122Tx2 to produce pmCMV-E1a-miR-122Tx2. In order to remove the miR-122T sites, pmCMV-E1a-miR-122Tx2 was cleaved with PstI and the mCMV-E1a fragment was inserted in the same plasmid at the PstI and NsiI sites to produce pmCMV-E1a-miR-122Tx0.

2.2.3 Adenovirus Construction, Amplification and Purification

2.2.3.1 Previously Constructed Viruses Obtained From Other Labs (Figure 2.2)

Ad-dl309, which was used as a positive control, and Ad-dl309ΔVA, which was used as an E1b-positive and VA-RNA-negative control, were gifts from Dr. Matthias Dobbelstein (Klinikum der Philipps-Universität Marburg, Germany) (270; 438). Furthermore, the non-replicating AdControl, which was used as an EGFP-positive and E1- and E3- negative control, was also a kind gift from Dr. Matthias Dobbelstein. Ad-dl1520, which was used as a VA-RNA-positive and E1b-55K-negative control (353), was a gift from Dr. Philip Branton (McGill University, Quebec, Canada). Ad-dl327, which was used as a VA-RNA-positive, E1b-55K-positive and E3-negative control, was a gift from Dr. David Ornelles (Wake Forest Baptist Medical Center, NC, USA).

2.2.3.2 Construction of AdΔE1b and AdΔE1bΔVA Oncolytic Adenoviruses

AdΔE1b and AdΔE1bΔVA were constructed as described in Figures 2.1 and 2.3 using the AdEasy-1 System (439). The pAdEasy-1 plasmid contains a deletion in the E3 region spanning from 28130-30820 bp. The pAd-Track plasmid has an E1 deletion spanning from 480-3533 bp, in which the EGFP gene has been

inserted under the control of the human cytomegalovirus immediate early (hCMV) promoter (439). While recombinant viruses were rescued in HEK293 cells (as described in section A.2.3.2), due to the low AdΔE1bΔVA replication efficiency in HEK293 cells, both viruses, as well as Ad-dl309, Ad-dl1520 andAd-dl309ΔVA, were amplified in Hep3B cells (as described in section A.2.3.3).

2.2.3.3 Determination of Adenovirus Titers

Virus titers in this study are expressed as VP/mL as well as PFU/mL (as described in section A.2.3.4). However, due to the high attenuation of AdΔE1bΔVA in HEK293 cells, plaque formation was highly reduced in these cells. Therefore, in order to determine the yields of AdΔE1b and AdΔE1bΔVA in infectious units, we used a limiting dilution assay. Hep3B cells were seeded in 96 well plates a day prior to infection. The next day, Hep3B cells were infected with 0.02 mL serially diluted AdΔE1b and AdΔE1bΔVA viruses in duplicate. Three days post-infection, GFP-positive Hep3B cells were counted using a digital fluorescent microscope (Advanced Microscopy Group EVOS fl). The concentrations of the undiluted virus samples in green fluorescence units (GFU)/mL were calculated from this assay.

2.2.4 Transfection of Plasmid DNA and miRNA Mimics

Plasmid DNA transfections were performed as described in section A.2.5. Miridian microRNA mimic negative control # 1 (Cat # CN-001000-01-05) and Miridian Human hsa-miR-122 mimic (Cat # C-300591-05) were obtained from Dharmacon (Fisher Scientific. Transfections were performed in 24 well plates

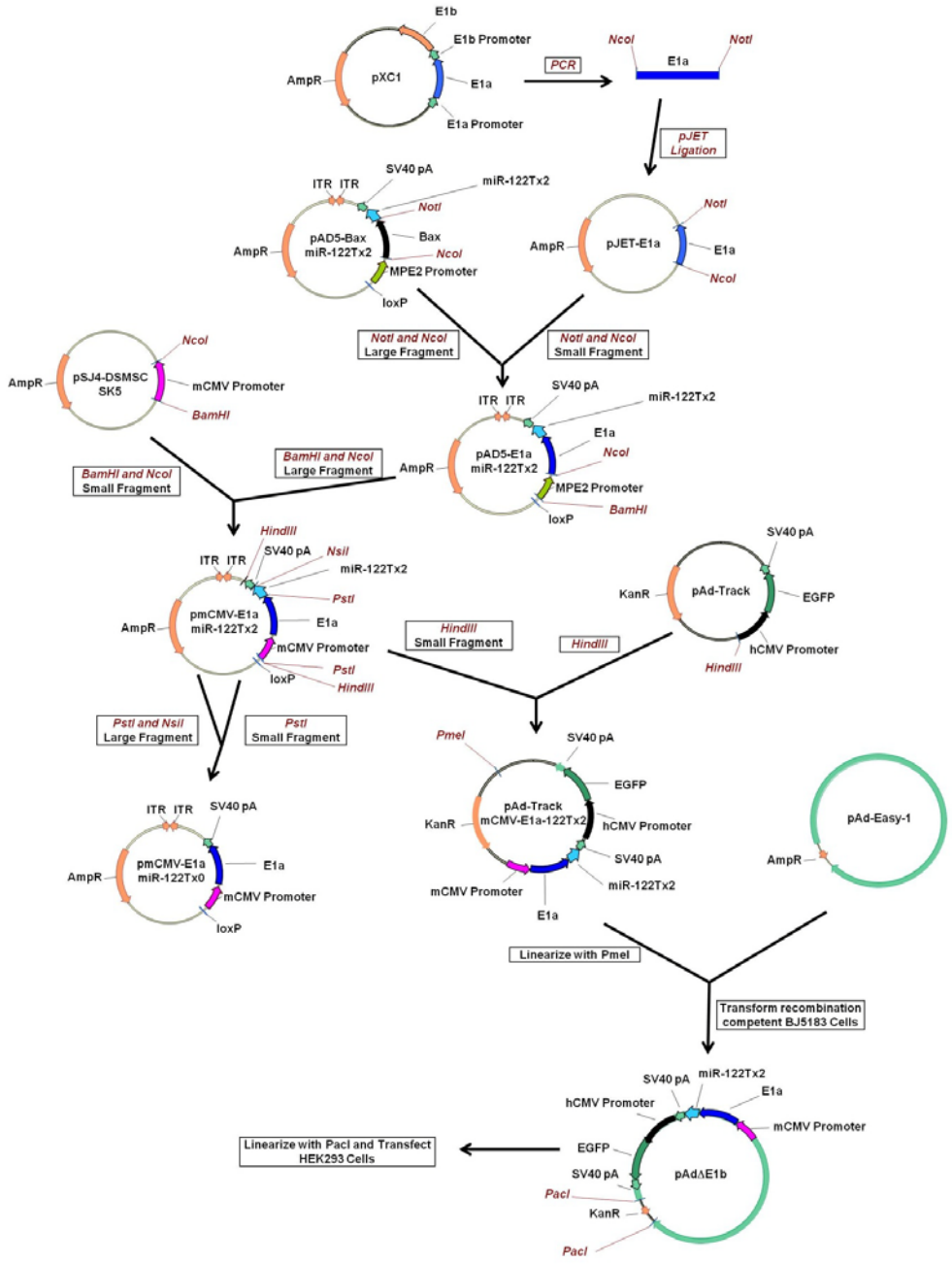

E1b
E1b Promoter
AmpR
pXC1
E1a
E1a Promoter
PCR
NcoI
E1a
NotI
pJET Ligation
ITR ITR
SV40 pA
miR-122Tx2
NotI
pAD5-Bax miR-122Tx2
Bax
NcoI
MPE2 Promoter
loxP
AmpR
pJET-E1a
E1a
NcoI
NotI and NcoI Large Fragment
NotI and NcoI Small Fragment
NcoI
pSJ4-DSMSC SK5
mCMV Promoter
BamHI
BamHI and NcoI Small Fragment
BamHI and NcoI Large Fragment
ITR ITR
SV40 pA
miR-122Tx2
E1a
NcoI
pAD5-E1a miR-122Tx2
MPE2 Promoter
BamHI
loxP
HindIII
ITR ITR
SV40 pA
NsiI
miR-122Tx2
PstI
pmCMV-E1a miR-122Tx2
E1a
mCMV Promoter
PstI
HindIII
loxP
HindIII Small Fragment
HindIII
SV40 pA
KanR
pAd-Track
EGFP
hCMV Promoter
HindIII
PstI and NsiI Large Fragment
PstI Small Fragment
ITR ITR
SV40 pA
pmCMV-E1a miR-122Tx0
E1a
mCMV Promoter
loxP
PmeI
SV40 pA
EGFP
KanR
pAd-Track mCMV-E1a-122Tx2
hCMV Promoter
SV40 pA
mCMV Promoter
miR-122Tx2
E1a
pAd-Easy-1
AmpR
Linearize with PmeI
Transform recombination competent BJ5183 Cells
SV40 pA
miR-122Tx2
E1a
hCMV Promoter
mCMV Promoter
EGFP
SV40 pA
pAdΔE1b
PacI
KanR
PacI
Linearize with PacI and Transfect HEK293 Cells

Figure 2.1: Cloning strategy for the construction of AdΔE1b.

The E1 shuttle plasmid pAd-Track-mCMV-E1a-122Tx2 was constructed as described in Figure 2.2. pAdΔE1b plasmid, which was constructed using AdEasy-1 System (439) by transformation of BJ5183 bacteria with pAd-Track-mCMV-E1a-122Tx2 and pAd-Easy-1 plasmids, was cleaved by PacI and transfected in HEK293 cells to construct AdΔE1b. PCR – polymerase chain reaction; miR-122T – miR-122 target sites; mCMV promoter – murine cytomegalovirus immediate early promoter; hCMV promoter – human cytomegalovirus immediate early promoter; SV40 pA – Simian virus 40 polyadenylation signal; ITR – adenoviral inverted terminal repeat; AmpR – ampicillin resistance gene; KanR – kanamycin resistance gene.

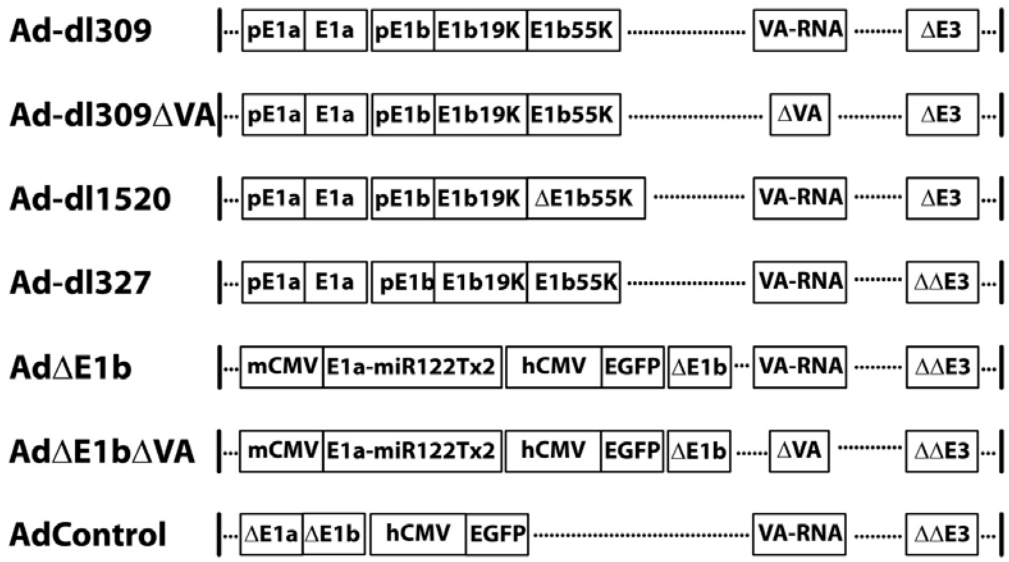


Figure 2.2: Schematic representation of adenoviruses used in this study.

Ad-dl309 encodes both E1a and E1b genes including their respective Ad promoters (pE1a and pE1b, respectively) but contains a small deletion in the E3 region (ΔE3). Ad-dl309ΔVA, Ad-dl1520 and Ad-dl327 have similar sequences as Ad-dl309 with additional mutations and/or deletions in the VA-RNA genes (ΔVA), the E1b-55K gene (ΔE1b-55K) or the E3 region (Δ ΔE3), respectively. AdΔE1b, AdΔE1bΔVA and AdControl (ΔE1aΔE1b), which contain a similar E3 deletion as Ad-dl327, encode the EGFP gene under the control of the human cytomegalovirus immediate early (hCMV) promoter. Furthermore, AdΔE1b and AdΔE1bΔVA also encode the E1a gene, with contains two miR-122T sites (E1a-miR122Tx2) in the 3'untranslated region, under the control of the murine cytomegalovirus immediate early (mCMV) promoter.

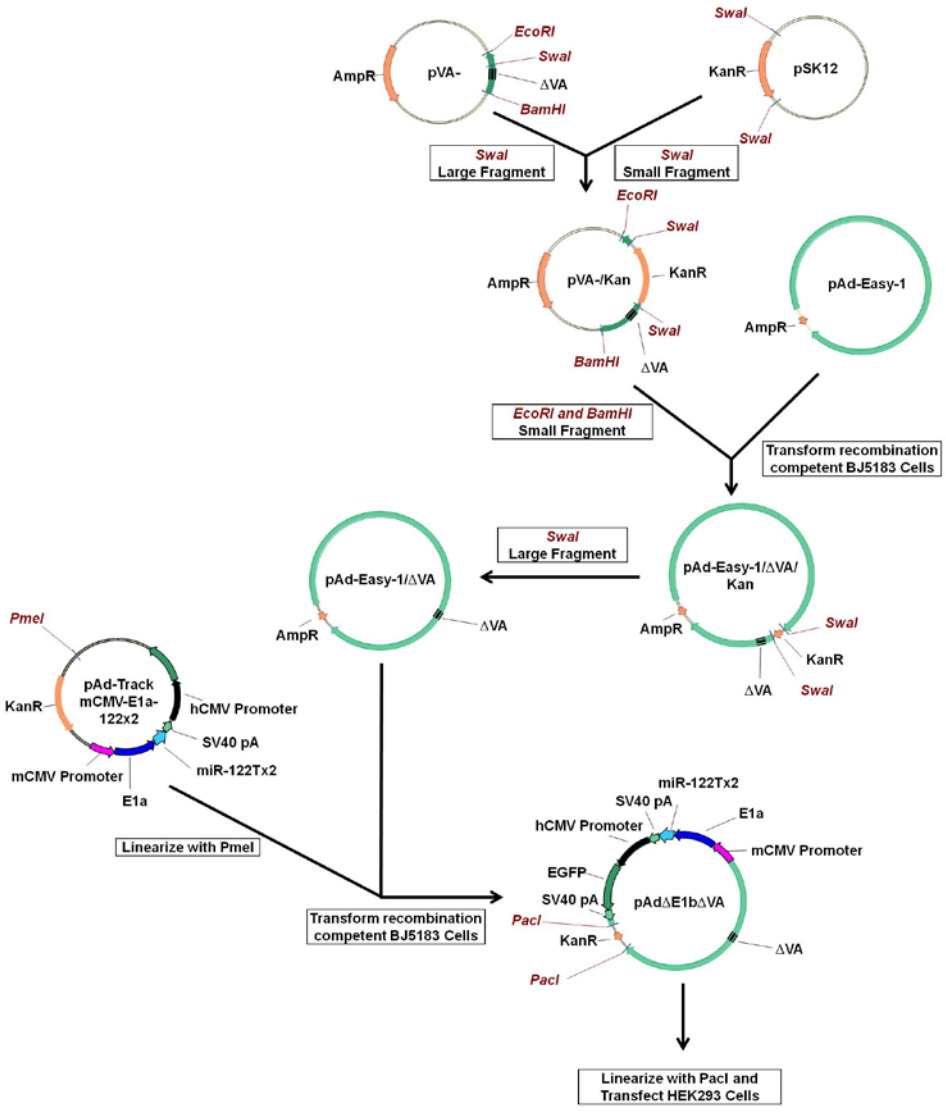


Figure 2.3: Cloning strategy for the construction of AdΔE1bΔVA.

pAdΔE1bΔVA plasmid, which was constructed using AdEasy-1 System (439), was cleaved by PacI and transfected in HEK293 cells to construct AdΔE1bΔVA. miR-122T – miR-122 target sites; mCMV promoter – murine cytomegalovirus immediate early promoter; hCMV promoter – human cytomegalovirus immediate early promoter; SV40 pA – Simian virus 40 polyadenylation signal; AmpR – ampicillin resistance gene; KanR – kanamycin resistance gene.

using DharmaFECT Transfection Reagent (Thermo Scientific, Cat # T-2004-01) according to the manufacturer's protocol at a ratio of 1 µl reagent: 2.5 µl of 5 µM microRNA mimic in 100 µl OptiMEM.

2.2.5 Virus Infections and Treatment with Drug Inhibitors

Virus infections were performed as described in section A.2.7. Briefly, cells were seeded in 24 well plates 1 day prior to infection with the indicated viruses at the indicated multiplicity of infections (MOIs). For drug treatment of infected cells, 1 hour after infection, cells were treated with medium alone or medium with 125 ng/mL geldanamycin (Invivogen, Cat # 30562-34-6), 12.5 µM mirin (Cayman, Cat # 13208), 2.5 mM 2-aminopurine (2'AP; Invivogen, Cat # 452-06-2), 1.25 µM C-16 (Sigma, Cat # I9785-5MG) or 1.25 µM C-16 negative control (EMD, Cat # 527455). Infected cells were harvested at the times indicated.

2.2.6 Analysis of Protein Expression

2.2.6.1 Western Blot Analysis

Cells were harvested at the indicated times following transfection or infection and western blot analysis was performed as described in section A.2.4 using mouse antibody against E1a (NeoMarkers, Cat # M73; 1/1000 dilution in 5 % milk in PBST) or rabbit antibody against hexon (Abcam, Cat # ab24240; 1/2000 dilution in 5 % milk in PBST).

2.2.6.2 Luciferase Reporter Assay

Cells were harvested at the indicated times following transfection or infection and luciferase expression was determined as described in section A.2.8.

2.2.7 Cell Survival Assay

2.2.7.1 Alamar Blue Assay

Cells were seeded in 96 well plates at 5000 cells/well leaving the last row without cells for use as a blank. The following day, the medium was removed and cells were uninfected (PBS) or infected in 20 µl PBS with the indicated virus at increasing MOIs of VP/cell in quadruplicate. One hour later, 100 µl of fresh medium was added to each well. Six days post-infection, Alamar Blue (Resazurin; Sigma, Cat # R7017-1G) was added to a final concentration of 44 uM. Fluorescence of each well was measured using the FLUOstar Optima or FLUOstar Omega plate reader with excitation at 544 nm and emission at 590 nm. The measurements of the blank well were subtracted from all wells. Measurements were represented as relative fluorescence units (RFU) or as normalized to uninfected control wells.

2.2.7.2 MTS Assay

Cells were seeded and infected as in 2.2.8.1. Six days post-infection, cell survival was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Cat # G5430) using the manufacturer's protocol. Absorbance of each well was measured using the FLUOstar Optima plate reader emission at 492 nm. After subtracting the blank, the measurements of infected wells were normalized to the measurements of uninfected wells.

2.2.7.3 Crystal Violet Assay

Cells were seeded and infected as in 2.2.8.1. Five days post-infection, the media was aspirated and the wells were washed with PBS, prior to addition of 100

µl methanol. The plates were then incubated at – 20C overnight. The next day, the methanol was removed and wells were incubated with 100 µl of 0.5 % crystal violet solution [5 g crystal violet (Fisher, Cat # C581-100), 250 mL methanol, 750 mL ddH2O] for four hours t room temperature. Next, the crystal violet solution was removed and plates were washed with water. The plates were air dried overnight, prior to solubilization of the crystal violet stain with 100 µl 0.01N hydrochloric acid (HCl; Fisher, SA48B-1). Absorbance of each well was measured using the FLUOstar Optima plate reader emission at 620 nm. After subtracting the blank, the measurements of infected wells were normalized to the measurements of uninfected wells.

2.3 RESULTS

2.3.1 Replicative Properties of Ads Deleted Only in E1b-55K or VA-RNA Genes in HEK293 and Hep3B Cells

VA-RNA deletion was previously found to attenuate Ads in HEK293 packaging cells (279-281). Similarly, we found that the loss of VA-RNA reduced the ability of AdΔE1bΔVA to grow well in HEK293 cells, even though the recombinant was initially rescued in these cells. Therefore, an alternative packaging cell line was sought that did not require VA-RNA and E1b functions to support production. As Hep3B cells were previously shown to efficiently replicate E1b-deleted Ads (386; 440), VA-RNA-deleted Ad production was examined in these cells.

Hep3B and HEK293 cells were infected with a VA-RNA-deleted Ad (Ad-dl309ΔVA), an E1b-55K-deleted Ad (Ad-dl1520) or Ad-dl309, as an E1b-55K-positive, VA-RNA-positive control. Virus production was determined four days later (Figure 2.4). Similar to previous reports, we found that the deletion of E1b-55K (Ad-dl1520) did not affect virus production in either cell line. While Ad-dl309ΔVA was attenuated in both cell lines, amplification of VA-RNA-deleted Ad was greater in Hep3B cells than in HEK293 cells.

2.3.2 Replicative Properties of E3-deleted Ads in HEK293 and Hep3B cells

The E3 region is not thought to be essential for virus replication *in vitro*. However, previous studies have shown that deletion of the E3 adenovirus death protein (ADP) gene reduces virus replication in certain cells (169-171). As AdΔE1b and AdΔE1bΔVA do not encode ADP, it was important to determine the

effect of ADP deletion on virus production in Hep3B cells. Cells were infected with ADP-deleted Ad-dl327 and Ad-dl309 as an ADP-positive control, and virus titers were determined four days later (Figure 2.5A). ADP deletion was found to slightly attenuate Ad-dl327 production relative to Ad-dl309 (by approximately 1 log) in both HEK293 and Hep3B cells.

Next, the effect of the combined deletions of E1b and E3 on virus production was determined in Hep3B cells. Cells were infected with AdΔE1b (E1b/ADP-deleted), Ad-dl309 (E1b/ADP-positive), as a positive control, and AdControl (E1a/E1b/ADP deleted) as a negative control (Figure 2.5B). Similar to Ad-dl327, AdΔE1b replication was attenuated by 1 log in HEK293 cells. Surprisingly, while the deletion of E1b-55K in Ad-dl1520 did not affect virus production levels in Hep3B cells (Figure 2.4), AdΔE1b was strongly attenuated in these cells. Since ADP deletion was found to slightly reduce virus production in Hep3B cells (Figure 2.5A), the loss of E1b-19K, in addition to ADP, may have further attenuated virus production in Hep3B cells.

2.3.3 Comparison of Replication Properties of AdΔE1b and AdΔE1bΔVA in HEK293 and Hep3B cells

For these experiments, Hep3B cells were used to amplify AdΔE1bΔVA to high titers since this was not possible in HEK293 cells. To control for possible differences in viral properties resulting from Ad propagation in different host cell lines other viruses used in this study, AdΔE1b, Ad-dl309, Ad-dl309ΔVA and Ad-dl1520, were also propagated in Hep3B cells. Additionally, due to the inability of AdΔE1bΔVA to produce plaques in HEK293 cells, AdΔE1b and AdΔE1bΔVA

infectious units were determined by titration and counting of GFP-positive Hep3B cells.

The efficiencies of AdΔE1b and AdΔE1bΔVA production in Hep3B and HEK293 cells were compared in one step growth curve assays (Figure 2.6A). Interestingly, while the VA-RNA deletion in AdΔE1bΔVA resulted in a strong attenuation in HEK293 cells compared to AdΔE1b, VA-RNA deletion did not affect the production level of AdΔE1bΔVA in Hep3B cells.

In order to verify that the decrease in AdΔE1bΔVA production in HEK293 cells was due to the lack of VA-RNA function, HEK293 and Hep3B cells were transfected with a plasmid encoding the VA-RNA genes (pVA+) prior to infection. As negative controls, cells were mock transfected (No DNA) or transfected with a VA-RNA-encoding plasmid containing deletions in the promoter region of both genes (pVA-) (Figure 2.6B). While transient expression of VA-RNAs had no effect on AdΔE1bΔVA production in Hep3B cells, VA-RNA expression strongly increased AdΔE1bΔVA production in HEK293 cells.

Since late virus gene expression occurs following DNA replication, we examined the expression of hexon, a late virus protein, in VA-RNA-deleted virus infected cells two days pos-infection to determine whether the attenuation in HEK293 cells occurred prior to DNA replication (Figure 2.6C). While hexon expression was detected in Hep3B cells infected with all the replicating viruses, hexon expression was only detected in HEK293 cells infected with VA-RNA-positive Ads. Furthermore, hexon expression in Hep3B cells infected with

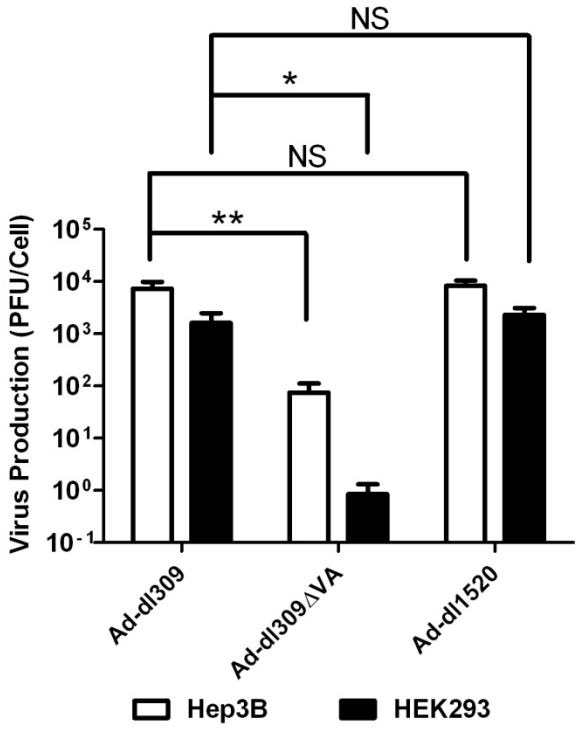


Figure 2.4: Efficiency of Ad-dl309ΔVA and Ad-dl1520 production in Hep3B and HEK293 cells.

Cells were infected with the indicated viruses at an MOI of 1 PFU/cell. Four days post-infection, cells were harvested and virus titers were determined by plaque assays in HEK293 cells. Error bars correspond to +/-SD of duplicate infections (NS – not significant, * - $p < 0.05$, ** - $p < 0.01$; One-way ANOVA).

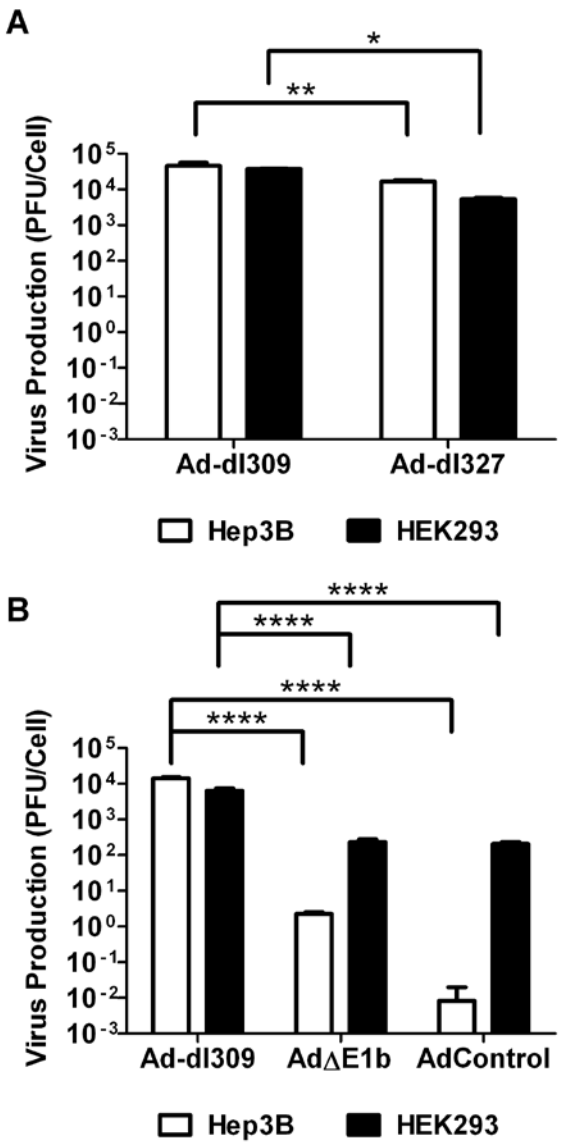


Figure 2.5: Efficiency of Ad-dl327 and AdΔE1b production in Hep3B and HEK293 cells.

(A and B) Cells were infected with the indicated viruses at an MOI of 1 PFU/cell. Four days post-infection, cells were harvested and virus titers were determined by plaque assays in HEK293 cells. Error bars correspond to +/-SD of duplicate infections (* - $p < 0.05$, ** - $p < 0.01$, **** - $p < 0.0001$; One-way ANOVA).

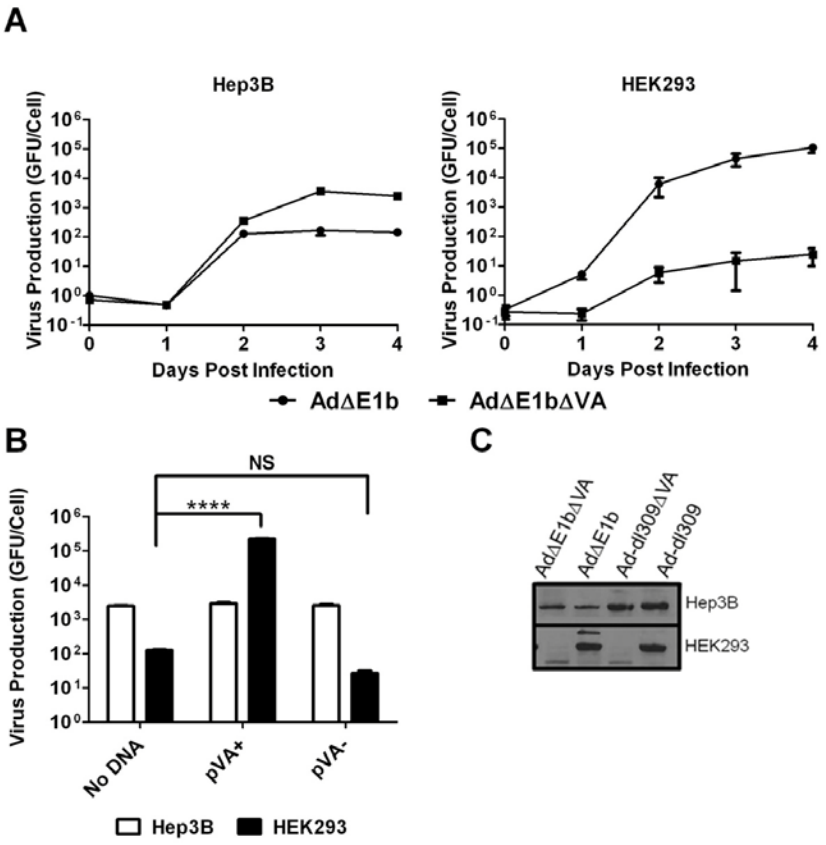


Figure 2.6: Deletion of VA-RNA genes in AdΔE1bΔVA reduced virus production and hexon expression in HEK293 cells but not Hep3B cells.

(A) Cells were infected with AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell. Infected cells were harvested at 1 hour (day 0) as well as at days 1, 2, 3 and 4 post-infection and the lysates titered on Hep3B cells. Error bars correspond to +/- SD of duplicate infections. (B) Cells were mock transfected or transfected with pVA+ and pVA- one day prior to infection with AdΔE1bΔVA. Two days post-infection, virus titers were determined. Error bars correspond to +/-SD of duplicate infections. (C) Cells were infected with the indicated virus for two days, then washed and lysed with RIPA. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed with a hexon-specific antibody.

AdΔE1b and AdΔE1bΔVA was lower than in infections with Ad-dl309 and Ad-dl309ΔVA, consistent with the attenuation of the full E1b-deleted viruses in this cell line (Figure 2.5B).

2.3.4 AdΔE1bΔVA-Mediated Cell Death in Hep3B and HEK293 Cells

Adenovirus-mediated cell death is important for mature virus release and spread to surrounding cells. Therefore, to determine whether the reduced production of AdΔE1bΔVA in HEK293 cells was associated with reduced virus-mediated cell death, cells were seeded in 96 well plates one day prior to infection with AdΔE1bΔVA. Cells were also infected with the VA-RNA-positive AdΔE1b and the nonreplicating AdControl as controls. The resulting survival curve is shown in Figure 2.7. As expected, AdΔE1b and AdΔE1bΔVA induced similar levels of Hep3B cell death. Interestingly, AdControl, which can replicate in HEK293 cells due to the endogenous expression of E1 gene products, induced similar levels of HEK293 cell death as AdΔE1b. However, the level of AdΔE1bΔVA-mediated HEK293 cell death was much lower than that induced by AdControl and AdΔE1b. Together with results from section 2.3.3, these results suggest that while VA-RNA expression is dispensable for virus replication in Hep3B cells, VA-RNA expression is important for virus production and lysis of HEK293 cells.

2.3.5 Regulation of Gene Expression by MiR-122

Due to the activity of the mCMV promoter, E1a expression in normal hepatocytes following infection with either AdΔE1b or AdΔE1bΔVA may result in liver toxicity even without virus replication. Therefore, we assessed whether

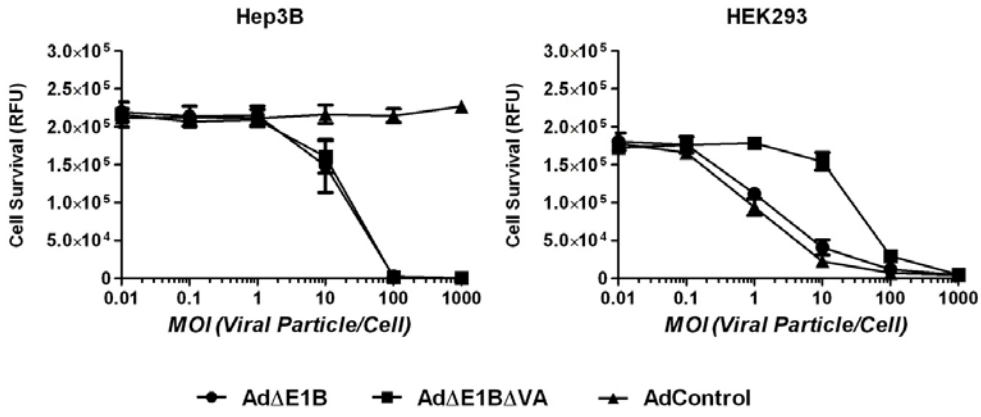


Figure 2.7: Deletion of VA-RNA genes in AdΔE1bΔVA reduced lysis of HEK293 Cells but not of Hep3B cells.

Cells were infected with AdΔE1b, AdΔE1bΔVA or AdControl at increasing MOIs (VP/cell) then incubated for 6 days. Cell survival was determined by Alamar Blue fluorescence measurements expressed as relative fluorescence units (RFU). Error bars correspond to +/-SD of quadruplicate wells.

E1a expression could be post-transcriptionally inhibited using miR-122T sites. We have previously found that miR-122 specifically inhibited the expression of plasmid- and FGAd vector-encoded transgenes containing six miR-122T sites in the 3'UTR (results in Appendix). To reduce the likelihood of loss of repeated sequences due to homologous recombination during adenoviral DNA replication (441), we examined whether 2 miR-122T sites were sufficient to inhibit transgene expression in miR-122-postive cells. MiR-122-positive and miR-122-negative HCC cells were transfected with luciferase expression vectors with or without the insertion of two miR-122T sites (pmCMV-Luc-miR122Tx2 and pmCMV-Luc-miR122Tx0, respectively). Two days post-transfection, luciferase expression was measured (Figure 2.8A). Luciferase expression was approximately 10 fold lower in HuH7.5 cells transfected with pmCMV-Luc-miR122Tx2 than in transfections with pmCMV-Luc-miR122Tx0. In contrast, miR-122T sites had no impact on luciferase expression levels in miR-122-negative cells.

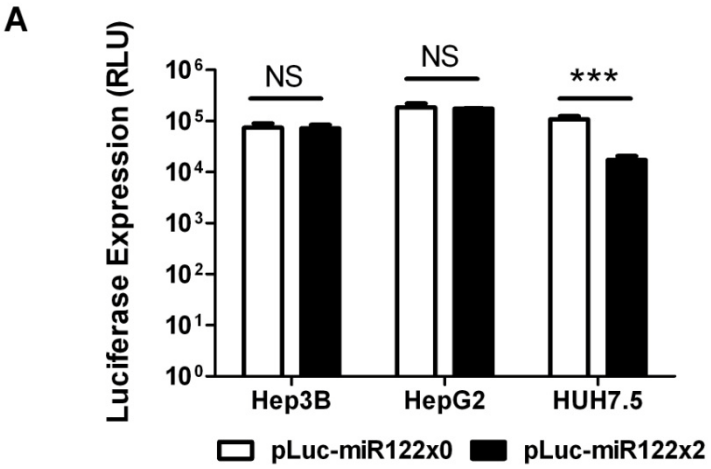
To verify that inhibition was due to miR-122 sequences, we examined the inhibition of luciferase expression in Hep3B cells (miR-122-neg) following transfection with a miR-122 mimic. As a miR-122-negative control, Hep3B cells were transfected with an miRNA mimic that does not target any known mammalian genes (miR-Neg). Furthermore, luciferase transfected HuH7.5 cells, which were not transfected with an miRNA mimic, were used as a positive miR-122 control (Figure 2.8B). Similar to the results in Figure 2.8A, strong miR-122-mediated luciferase inhibition was found in HuH7.5 cells. As predicted, transient transfection with miR-122 induced a strong miR-122-mediated luciferase

inhibition in Hep3B cells. Interestingly, these data also suggest that miR-122 in transiently transfected Hep3B cells was more active than endogenous miR-122 levels in HuH7.5.

Since the insertion of miR-122T sites reduced luciferase gene expression in miR-122-positive cells, two miR-122T sites were also inserted in the 3'UTR of the E1a gene. To verify that the miR-122T insertion reduced E1a expression, HuH7.5 cells were mock transfected (No DNA) or transfected with plasmids encoding the E1a gene with or without the two miR-122T sites (pmCMV-E1a-miR122Tx2 and pmCMV-E1a-miR122Tx0, respectively). Hep3B and HepG2 cells were used as miR-122-negative controls. Two days post-transfection, E1a expression levels were determined by western blot analysis (Figure 2.9). Higher E1a expression was found in HuH7.5 cells transfected with pmCMV-E1a-miR122Tx0 compared to pmCMV-E1a-miR122Tx2, as expected. Furthermore, in the miR-122-negative cell lines, E1a expression was unchanged by the addition of miR-122T sites. Taken together, these results suggest that insertion of two miR-122T sites was sufficient to inhibit expression of exogenous genes. Therefore, pmCMV-E1a-miR122Tx2 was used to construct the E1b-deleted oncolytic viruses encoding the miR-122-targeted E1a with or without the VA-RNA genes (AdΔE1b and AdΔE1bΔVA, respectively).

2.3.6 Retention of Regulation of E1a Expression by MiR-122 in AdΔE1b and AdΔE1bΔVA Infected HCC Cells

In order to verify that that miR-122-mediated inhibition of E1a expression was retained in cells infected with AdΔE1b and AdΔE1bΔVA, E1a expression


A
Luciferase Expression (RLU)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
NS
NS
***
Hep3B
HepG2
HUH7.5
pLuc-miR122x0
pLuc-miR122x2

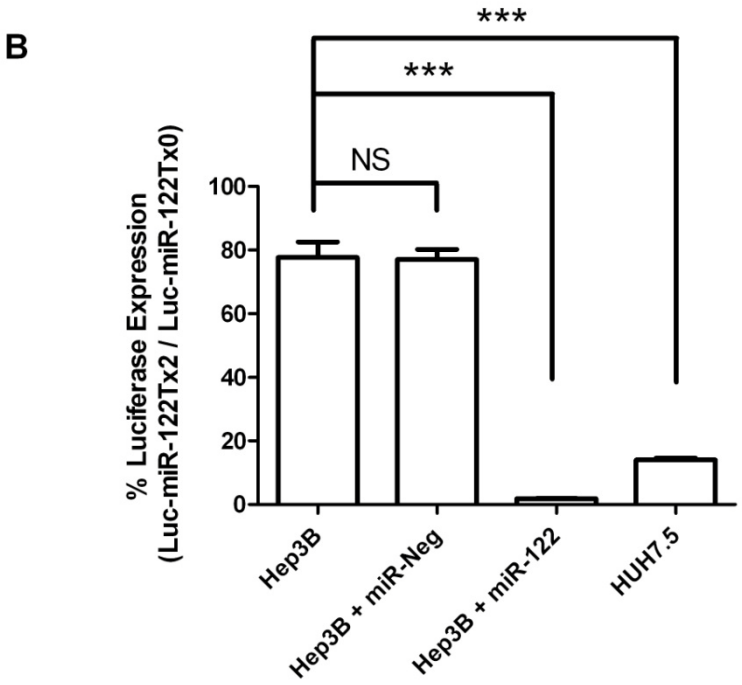

B
***
***
NS
% Luciferase Expression
(Luc-miR-122Tx2 / Luc-miR-122Tx0)
100
80
60
40
20
0
Hep3B
Hep3B + miR-Neg
Hep3B + miR-122
HUH7.5

Figure 2.8: Insertion of miR-122T sites reduced luciferase expression in miR-122-positive HCC cells.

(A) Cells were transfected with a luciferase expression vector either containing or not containing an insertion of 2 miR-122T sites in the 3'UTR (pmCMV-Luc-miR-122Tx2 and pmCMV-Luc-miR-122Tx0, respectively). Two days post-transfection, cells were harvested and luciferase expression was determined. Error bars correspond to +/-SD of triplicate wells (NS – not significant, *** - $p < 0.001$; t-test). (B) Cells were mock transfected or transfected with a miR-122 mimic or a nontargeted miRNA mimic (miR-Neg). The next day, cells were transfected with an expression vector either containing or not containing 2 miR-122T sites in the 3'UTR of the luciferase gene. Two days post-transfection, cells were harvested and luciferase expression was determined. % luciferase expression was determined by normalization of luciferase values obtained from cells transfected with pmCMV-Luc-miR-122Tx2 to that from cells transfected with the control pmCMV-Luc-miR-122Tx0. Error bars correspond to +/-SD of triplicate transfections (NS – not significant, *** - $p < 0.001$; One-way ANOVA).

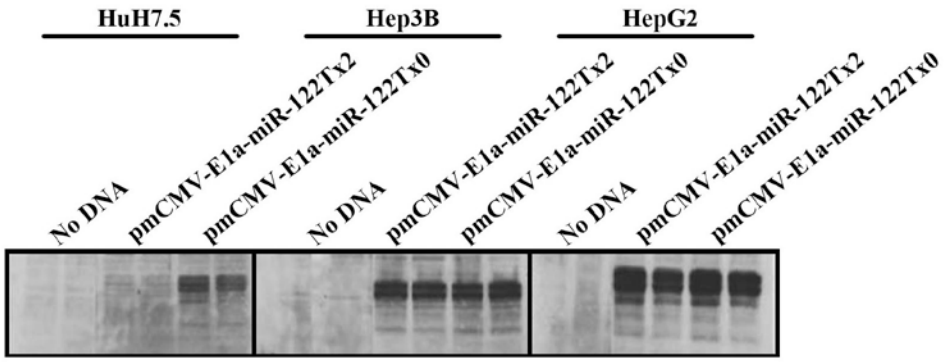


Figure 2.9: Insertion of miR-122T sites reduced E1a expression in miR-122-positive HCC cells.

Cells were untransfected or transfected in duplicate with E1a expression vectors either containing or not containing 2 miR-122T sites. Two days post-transfection, cells were washed and lysed with RIPA. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed with an E1a-specific antibody.

was detected two days following infection of Hep3B cells transiently transfected with the miR-122 mimic. As non-silencing controls, Hep3B cells were also transfected with the negative miRNA mimic, or infected with Ad-dl309 and Ad-dl309ΔVA, which lack the targeted sequences, as well as the E1a-negative AdControl (Figure 2.10). As expected, the expression of E1a was high in Hep3B cells infected with the controls Ad-dl309 and Ad-dl309ΔVA, regardless of miR-122 activity level. In contrast, miR-122 expression strongly reduced E1a levels in infections with AdΔE1b and AdΔE1bΔVA, confirming that miR-122 targeting of E1a expression was retained in the oncolytic viruses.

2.3.7 Characterization of AdΔE1bΔVA Growth Properties in Non-HCC Cells

The growth properties of AdΔE1bΔVA as well as virus-mediated cell death were compared to AdΔE1b in a panel of cancer cell lines originating from different tissues.

2.3.7.1 Determination of AdΔE1bΔVA Activity in a Panel of Breast Cancer Cell Lines

Previous studies have found that E1b- and E3-deleted Ads, such as AdΔE1b, were highly cytotoxic to a number of breast cancer cell lines (392; 393). Furthermore, one of the studies suggested that in addition to E1a-mediated apoptosis, high cell death was also due to E1a-mediated transcriptional inhibition of oncogenic tyrosine kinase receptors, such as HER2 and EGFR (392). However, the study did not report whether the virus were replicating in the breast cancer cells. To address this question, we determined the replication efficiency of AdΔE1b and AdΔE1bΔVA in MDA-MB-231, MDA-MB-468, SKBR-3 and ZR-

75-1 human breast cancer cell lines (Figure 2.11A). Production levels of both viruses were extremely low in the tested cell lines, suggesting that the deletions within the AdΔE1b, as well as the high E1a expression, resulted in reduced adenoviral replication in these cells.

Since the breast cancer cells were unable to support AdΔE1b and AdΔE1bΔVA replication, we next determined whether hexon expression, a marker for virus DNA replication, was also reduced in the fully E1b-deleted virus-infected breast cancer cells. Cells were infected with AdΔE1b and AdΔE1bΔVA and hexon expression was analyzed two days later. Cells were also infected with Ad-dl309 as a positive control and Ad-dl309ΔVA as a VA-RNA-negative control. Furthermore, cells were also mock infected or infected with AdControl as negative controls (Figure 2.11B). While hexon expression was detected in all cells infected with Ad-dl309, no hexon was detected in cells infected with AdΔE1b or AdΔE1bΔVA, suggesting that DNA replication of the two viruses had not occurred in these cells. Furthermore, hexon expression was not detected in MDA-MB-468 cells infected with the E1b-positive Ad-dl309ΔVA, suggesting that VA-RNA expression might be essential for adenovirus replication in these cells, similar to HEK293 cells.

The levels of virus-mediated cell death were also determined in MDA-MB-468, SKBR-3 and ZR-75-1 cells infected with AdΔE1b, AdΔE1bΔVA and control viruses (Figure 2.12). Surprisingly, while hexon expression was not detected in MDA-MB-468 cells infected with Ad-dl309ΔVA, AdΔE1b or AdΔE1bΔVA, these viruses, as well as Ad-dl309 induced high cell death even at

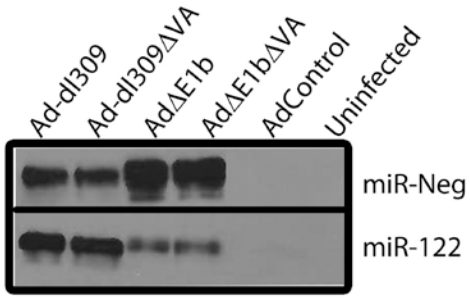


Figure 2.10: MiR-122T insertion sites reduced E1a expression in AdΔE1b and AdΔE1bΔVA-infected Hep3B cells transiently transfected with miR-122. Cells were transfected with miR-122 mimic or miR-Neg a day prior to infection with the indicated viruses at an MOI of 100 VP/cell. Two days post-infection, cells were washed and lysed with RIPA. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed with an E1a-specific antibody.

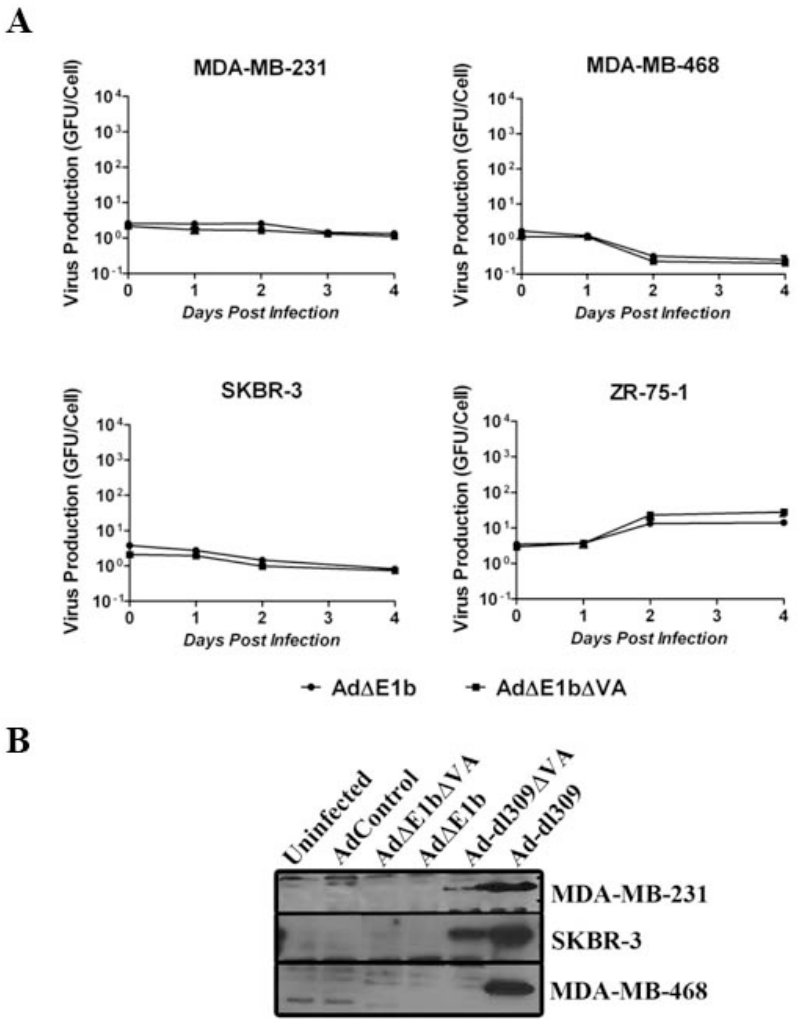


Figure 2.11: AdΔE1b and AdΔE1bΔVA production and hexon expression in human breast cancer cell lines.

(A) Cells were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections. (B) Cells were infected with the indicated viruses at an MOI of 100 VP/cell. Two days post infection, cells were harvested. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed using anti-hexon antibody.

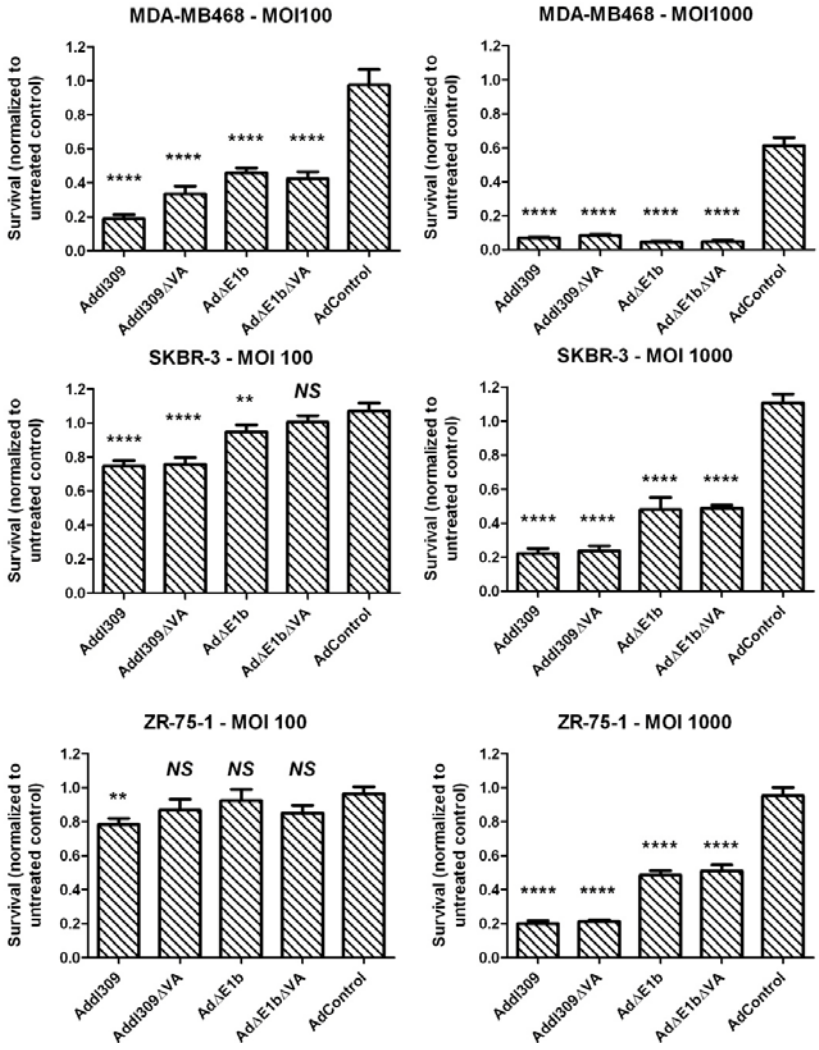


Figure 2.12: Virus-mediated cell death in human breast cancer cell lines.

Cells were infected with the indicated viruses at an MOI of 100 or 1000 VP/cell for 1 hour followed by addition of fresh medium. Five days post-infection, cell survival was determined by crystal violet solubilization followed by normalization to uninfected cells. Error bars correspond to +/-SD of quadruplicate wells. Measurements from infected wells were compared to AdControl measurements to determine significant differences. (NS – not significant, ** - $p < 0.01$, **** - $p < 0.0001$; One-way ANOVA).

the lower MOI. Furthermore, high cell death was observed with all replicating viruses at the higher MOI in all three cell lines. These results suggest that infections with AdΔE1b and AdΔE1bΔVA resulted in premature cell death and reduced virus replication, due to the high E1a expression and the lack of E1b proteins (205; 206).

2.3.7.2 Determination of AdΔE1b and AdΔE1bΔVA Activity in a Panel of Pancreatic Cancer Cell Lines

Previous studies have shown that pancreatic cancer cells support the replication of VA-RNA-deleted Ads, E1b-19K-deleted Ads and E1b-55K-deleted Ads *in vitro* and *in vivo* (280; 403; 442; 443). Therefore, the replication properties of AdΔE1b and AdΔE1bΔVA were tested in a panel of pancreatic cancer cell lines (Figure 2.13A). While PL-45, AsPC-1 and Panc-1 human pancreatic cancer cells were found to support AdΔE1b production, the deletion of the VA-RNA resulted in complete attenuation of AdΔE1bΔVA production in these cells. Furthermore, production of both AdΔE1b and AdΔE1bΔVA was inhibited in the Syrian hamster SHPC-6 cell line.

To determine whether DNA replication was also attenuated in AdΔE1bΔVA-infected pancreatic cancer cells, hexon expression was analyzed in Panc-1, AsPC-1 and SHPC-6 two days following infection with the indicated viruses (Figure 2.13B). High hexon expression levels were detected in Panc-1 cells infected with Ad-dl309, Ad-dl309ΔVA and AdΔE1b. In contrast, hexon was undetectable in cells infected with AdΔE1bΔVA, suggesting that VA-RNA deletion had a stronger effect on AdΔE1bΔVA when compared to Ad-dl309ΔVA.

Surprisingly, hexon expression was detected in SHPC-6 cells infected AdΔE1b, even though virus production was undetectable in this cell line. This result suggests the inhibition of virus production in SHPC-6 might be downstream of viral DNA replication. Interestingly, while AsPC-1 cells were found to support AdΔE1b replication, hexon expression was undetectable at two days post infection. However, even with Ad-dl309 infection, hexon expression in AsPC-1 cells was very low.

AdΔE1b and AdΔE1bΔVA-mediated cell death was also determined in pancreatic cancer cell lines, PL-45, AsPC-1, Panc-1 and SHPC-6 cells (Figure 2.14). AdΔE1bΔVA, which was unable to replicate in the tested cell lines, did not induce significant cell death in the tested cell lines, suggesting that the pancreatic cancer cells were more resistant than the breast cancer cells to replication-independent lysis mediated by AdΔE1bΔVA. In contrast, AdΔE1b induced high cell death in Panc-1 and PL-45 cells at the higher MOI. Furthermore, AdΔE1b induced strong PL-45 cell death at the lower MOI. Interestingly, while SHPC-6 did not support AdΔE1b replication, AdΔE1b-mediated SHPC-6 death was stronger than Ad-dl309 and Ad-dl309ΔVA. In contrast, while AsPC-1 was found to support AdΔE1b replication, the virus did not induce a strong virus-mediated cell death of AsPC-1 cells.

2.3.7.3 Determination of AdΔE1bΔVA Activity in Human Bladder Cancer Cell Lines

The efficiency of AdΔE1b and AdΔE1bΔVA production was determined in a panel of human bladder cancer cell lines (Figure 2.15A). AdΔE1b and

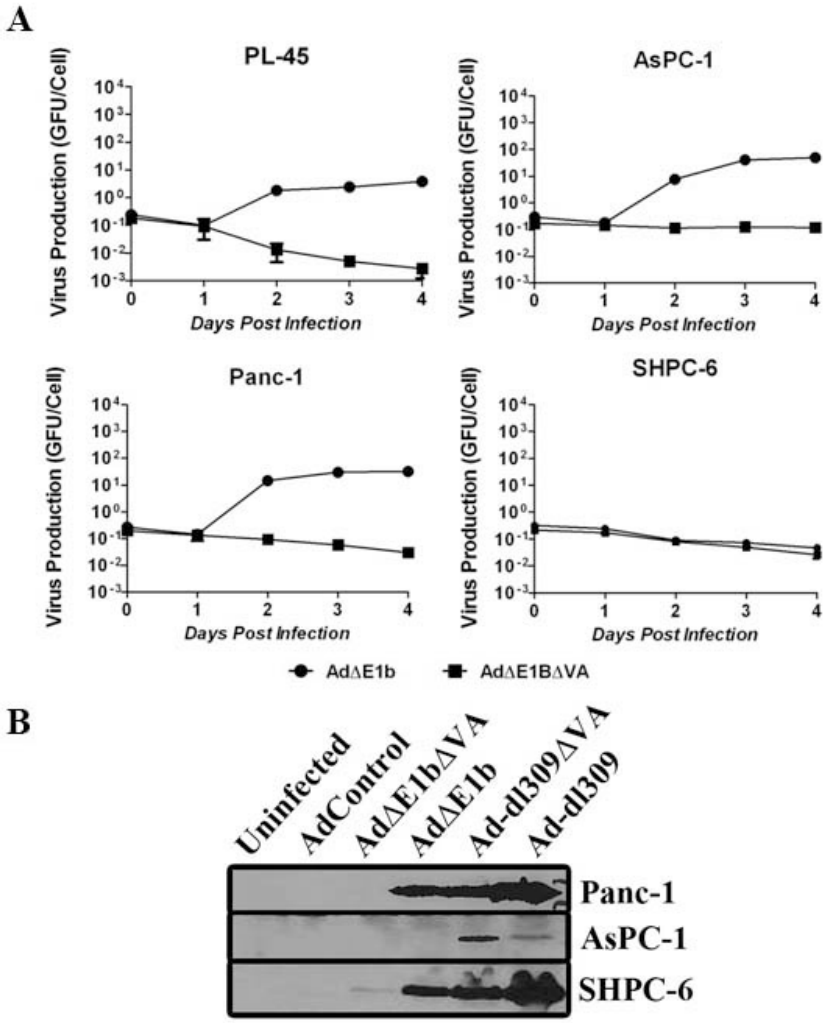


Figure 2.13: AdΔE1b and AdΔE1bΔVA production and hexon expression in human and Syrian hamster pancreatic cancer cell lines.

(A) Cells were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections. (B) Cells were infected with the indicated viruses at an MOI of 100 VP/cell. Two days post infection, cells were harvested. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed using anti-hexon antibody.

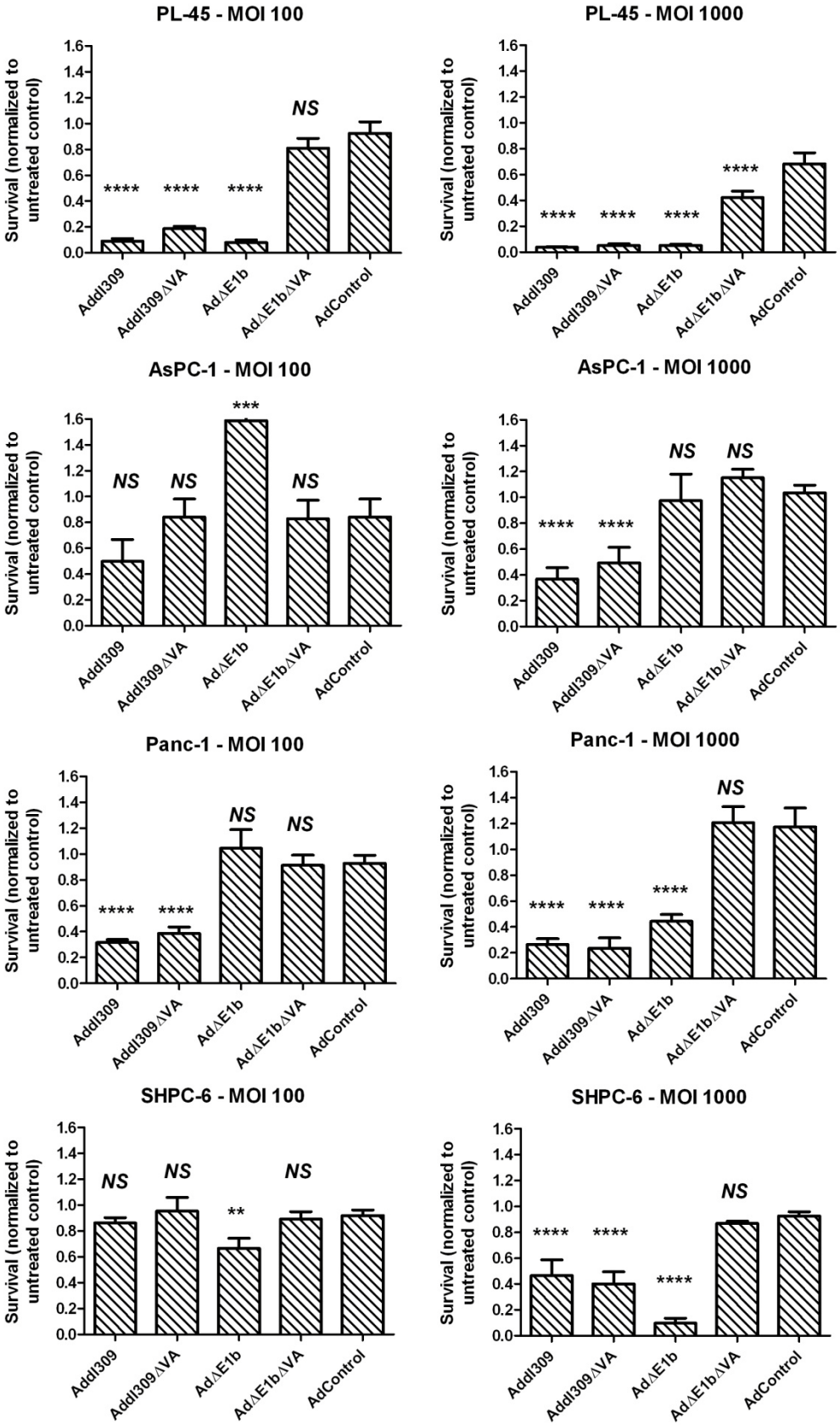

PL-45 - MOI 100
PL-45 - MOI 1000
AsPC-1 - MOI 100
AsPC-1 - MOI 1000
Panc-1 - MOI 100
Panc-1 - MOI 1000
SHPC-6 - MOI 100
SHPC-6 - MOI 1000
Survival (normalized to untreated control)
Addl309
Addl309ΔVA
AdΔE1b
AdΔE1bΔVA
AdControl
NS
****
***
**

Figure 2.14: Virus-mediated cell death in pancreatic cancer cell lines.

Cells were infected with the indicated viruses at an MOI of 100 or 1000 VP/cell for 1 hour followed by addition of fresh medium. Six days post-infection, cell survival was determined by MTS assay followed by normalization to uninfected cells. Error bars correspond to +/-SD of quadruplicate wells. Measurements from infected wells were compared to AdControl measurements to determine significant differences (NS – not significant, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$; One-way ANOVA).

AdΔE1bΔVA production was inhibited in MGHU3, T24 and HT1376 cells, but UMUC3 supported production of both viruses.

Hexon expression levels in UMUC3 cells were tested to confirm a correlation with virus production (Figure 2.15B). As a control, hexon expression was also determined in virus-infected MGHU3 cells that did not support AdΔE1b and AdΔE1bΔVA replication. High levels of hexon were detected in both cell lines infected with Ad-dl309 and Ad-dl309ΔVA, suggesting that VA-RNA-deletion alone did not prevent viral DNA replication in either cell line. Furthermore, consistent with virus production, hexon was detected UMUC3 cells, but not MGHU3 cells, infected with AdΔE1b or AdΔE1bΔVA.

E1a expression was also determined in both cell lines. E1a expression in MGHU3 cells infected with AdΔE1b with AdΔE1bΔVA was slightly higher than with Ad-dl309 and Ad-dl309ΔVA. This result may be due to the differences between the viruses in the promoter controlling E1a expression. Similar to hexon levels, E1a levels in UMUC3 cells infected with AdΔE1b with AdΔE1bΔVA were much lower than with Ad-dl309 and Ad-dl309ΔVA. This result may be due to higher viral DNA replication in Ad-dl309- and Ad-dl309ΔVA-infected UMUC3 cells, which would result in increased template DNA.

Survival of infected UMUC3 and MGHU3 was also determined (Figure 2.16). Ad-dl309 and Ad-dl309ΔVA induced cell death in both cell lines. Surprisingly, both AdΔE1b and AdΔE1bΔVA induced high cell death in both cell lines at the higher MOI even though MGHU3 did not support replication of either virus. Thus, similar to the breast cell lines, MGHU3 was sensitive to the

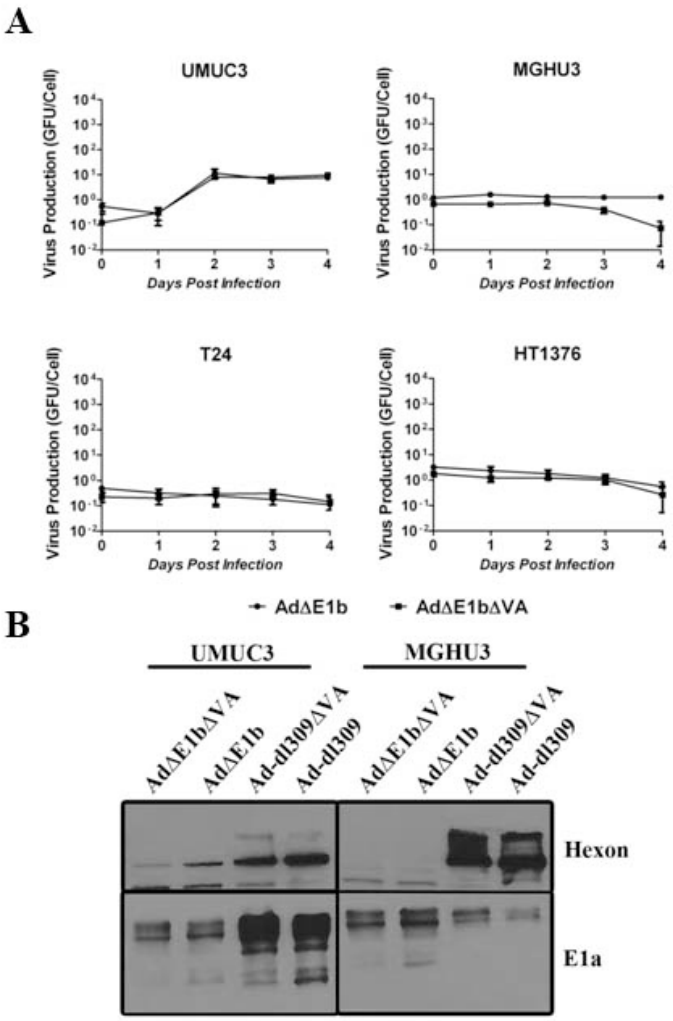


Figure 2.15: AdΔE1b and AdΔE1bΔVA production and Ad protein expression in bladder cancer cell lines.

(A) Cells were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections. (B) Cells were infected with the indicated viruses at an MOI 100 of VP/cell. Two days post infection, cells were harvested. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed using anti-hexon or anti-E1a antibody.

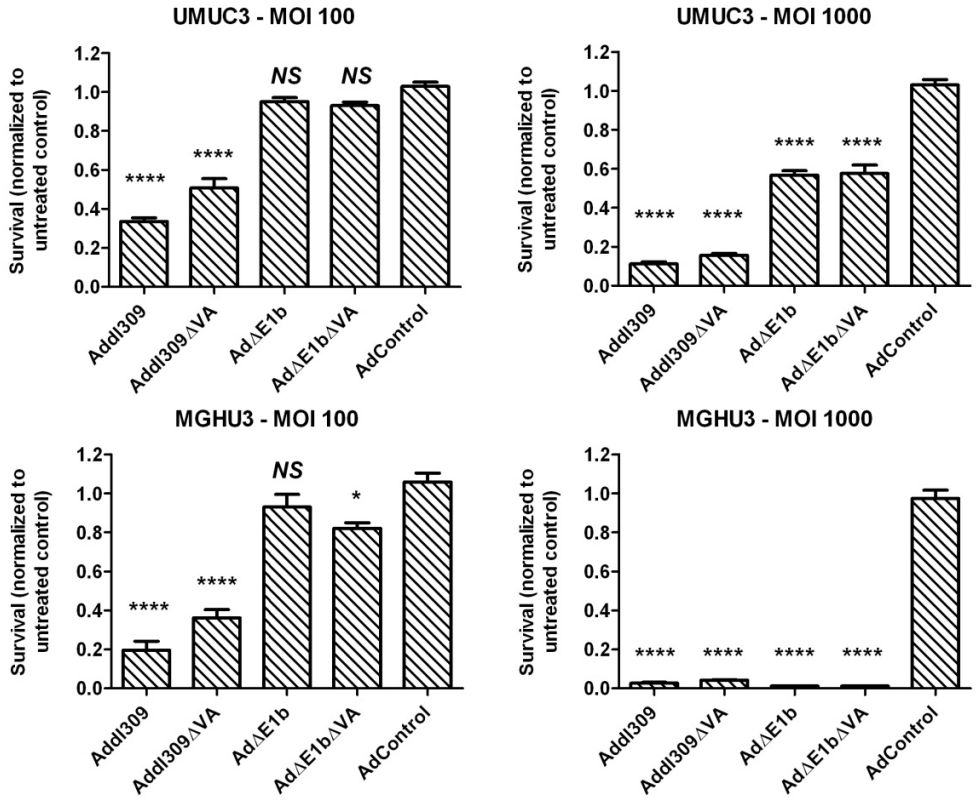


Figure 2.16: AdΔE1b and AdΔE1bΔVA-mediated cell death in bladder cancer cell lines.

Cells were infected with the indicated viruses at an MOI of 100 or 1000 VP/cell for 1 hour followed by addition of fresh medium. Six days post-infection, cell survival was determined by Alamar Blue assay followed by normalization to uninfected cells. Error bars correspond to +/-SD of quadruplicate wells. Measurements from infected wells were compared to AdControl measurements (NS – not significant, * - $p < 0.05$, **** - $p < 0.0001$; One-way ANOVA).

replication-independent cytolytic activity of AdΔE1b and AdΔE1bΔVA.

2.3.7.4 Determination of AdΔE1bΔVA Activity in Lung Cancer and Fibroblast Cell Lines

The replication efficiencies of AdΔE1b and AdΔE1bΔVA were determined in A549 lung cancer cells, as well as the normal lung fibroblasts, MRC5 (Figure 2.17A). Interestingly, both A549 and MRC5 cells were found to support AdΔE1b replication, suggesting that the mutations within AdΔE1b were not sufficient to block replication in normal cells. In contrast, the deletion of VA-RNA genes completely inhibited AdΔE1bΔVA replication in MRC5 cells and only partially in A549 cells. Therefore, although VA-RNA expression was important for AdΔE1b replication in A549 cells, it was not as essential as in MRC5 cells. Analysis of hexon expression in infected MRC5 cells showed high levels with Ad-dl309 and AdΔE1b, but undetectable levels with the VA-RNA-deleted viruses, Ad-dl309ΔVA and AdΔE1bΔVA (Figure 2.17B). In contrast, hexon expression was more dependent on E1b expression in infected A549 cells with the lowest level of hexon expression in A549 cells infected with the doubly-deleted AdΔE1bΔVA virus.

Virus-mediated death of A549 and MRC5 cells (Figure 2.18) largely correlated with virus production (Figure 2.17A).

2.3.8 Determination of AdΔE1b and AdΔE1bΔVA Activity in HepG2 Cell Line

2.3.8.1 AdΔE1b and AdΔE1bΔVA-Mediated HepG2 and Hep3B Cell Death

The results presented in section 2.3.7 show that many tumour cell lines do

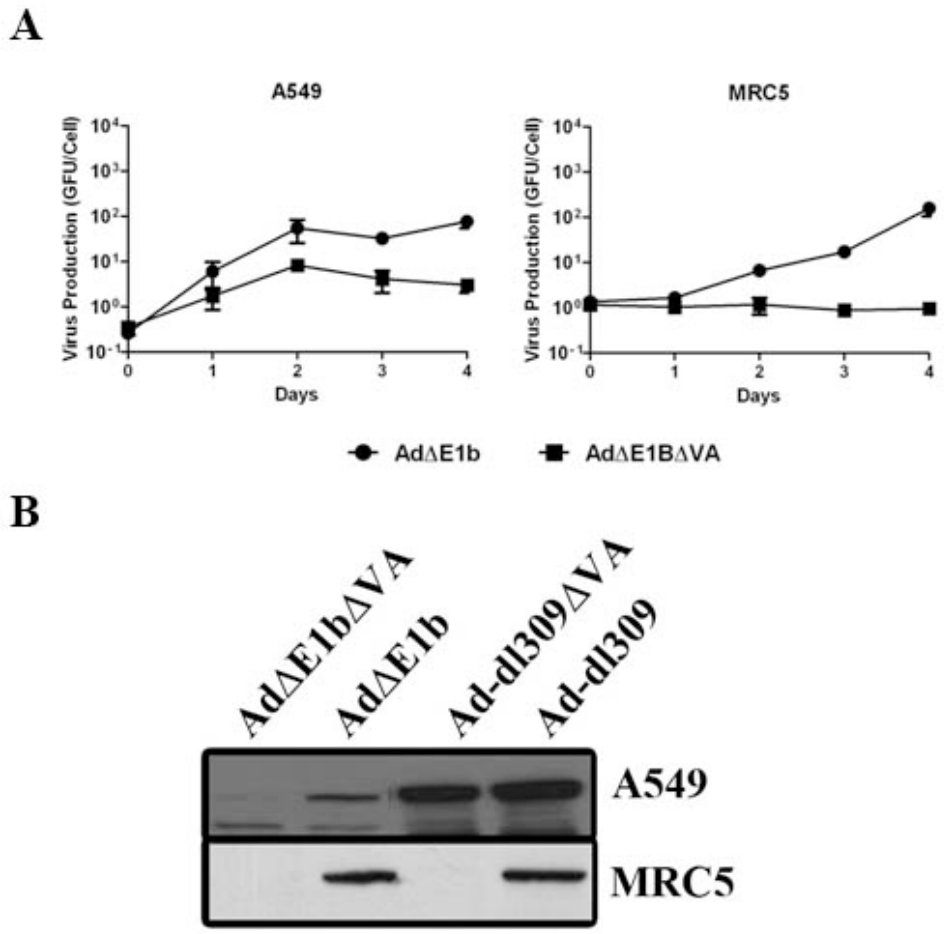


Figure 2.17: AdΔE1b and AdΔE1bΔVA production and hexon expression in A549 lung cancer cells and MRC5 normal lung fibroblasts.

(A) Cells were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections

(B) Cells were infected with the indicated viruses at an MOI of 100 VP/cell. Two days post infection, cells were harvested and 10 µg protein was separated by SDS-PAGE and transferred to a western blot. Hexon levels were determined using anti-hexon antibody.

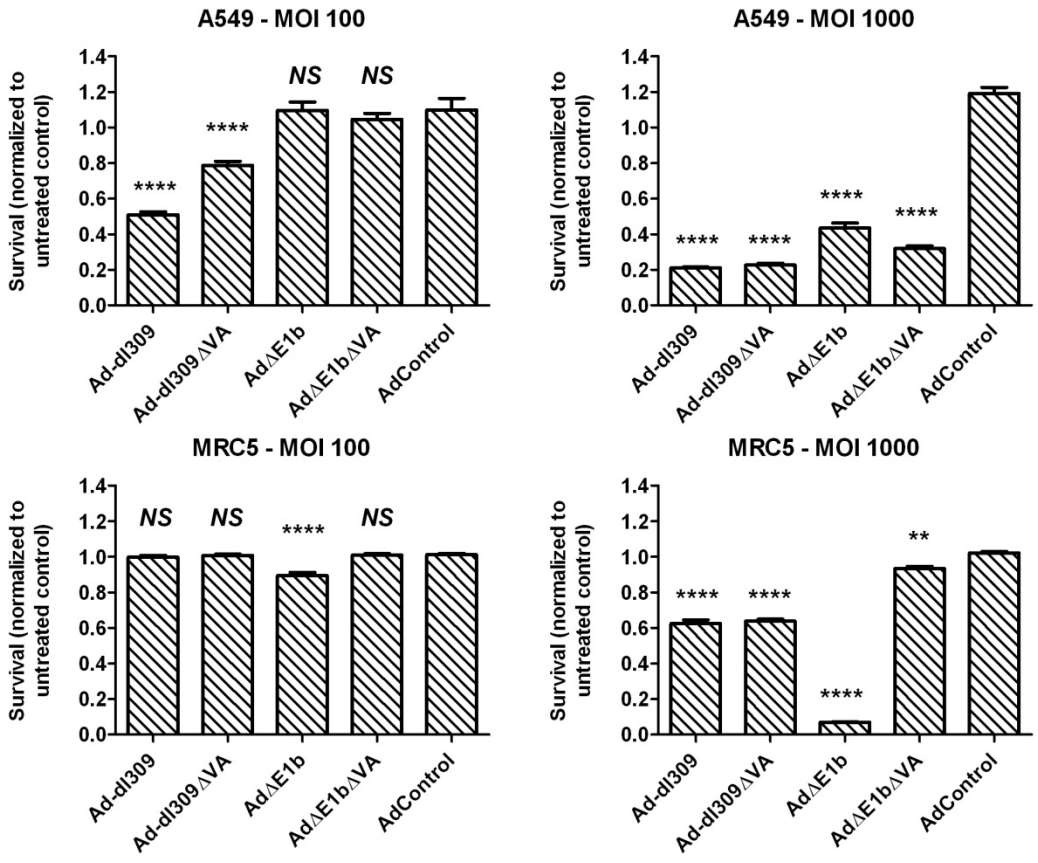


Figure 2.18: Virus-Mediated cell death in A549 lung cancer cells and MRC5 normal lung fibroblasts.

Cells were infected with the indicated viruses at an MOI of 100 or 1000 VP/cell for 1 hour followed by addition of fresh medium. Six days post-infection, cell survival was determined by Alamar Blue assay followed by normalization to uninfected cells. Error bars correspond to +/-SD of quadruplicate wells. Measurements from infected wells were compared to AdControl measurements (NS – not significant, ** - $p < 0.01$, **** - $p < 0.0001$; One-way ANOVA).

not support robust AdΔE1bΔVA replication. Since Hep3B cells supported the replication of AdΔE1bΔVA (Figure 2.6A), we next examined whether this observation would extend to an additional HCC cell line, HepG2 (Figure 2.19). Similar to Hep3B cells, HepG2 cells supported the replication of both AdΔE1b and AdΔE1bΔVA.

AdΔE1b- and AdΔE1bΔVA-mediated cell death was also examined in these two cell lines (Figure 2.20). Consistent with our previous observations, Hep3B cells were highly sensitive to lysis by both viruses. In contrast, HepG2 cells were relatively resistant to virus-mediated cell death.

2.3.8.2 Effect of Transient E1b-55K Expression on AdΔE1b and AdΔE1bΔVA Production in HepG2 and MRC5 Cell Lines

To determine whether E1b-55K expression would complement the defect in AdΔE1b and AdΔE1bΔVA production in HepG2 cells, cells were transfected with an E1b-55K expression vector or a mutant E1b-55K expression vector (C454S/C456S), which has lost the ability to block the host DNA damage response (231). As a control, MRC5 cells, that are unable to support AdΔE1bΔVA replication, were transfected with the expression vectors. One day post-transfection, cells were infected with the viruses and four days post-infection, virus titers were determined (Figure 2.21). E1b-55K expression enhanced AdΔE1b and AdΔE1bΔVA production in HepG2. In contrast, E1b-55K enhanced only AdΔE1b production in MRC5 cells, suggesting that these cells were missing additional functions needed to complement the VA-RNA deletion.

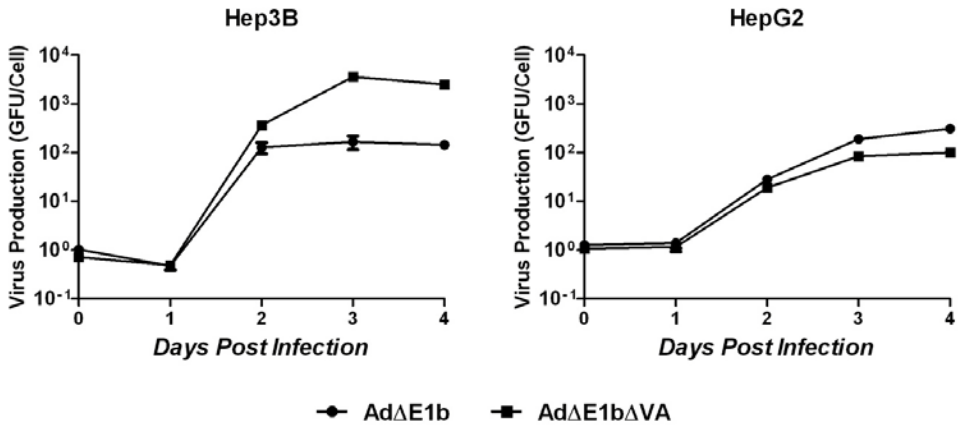


Figure 2.19: AdΔE1b and AdΔE1bΔVA production in HepG2 and Hep3B liver cancer cell lines.

Cells were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/- SD of duplicate infections.

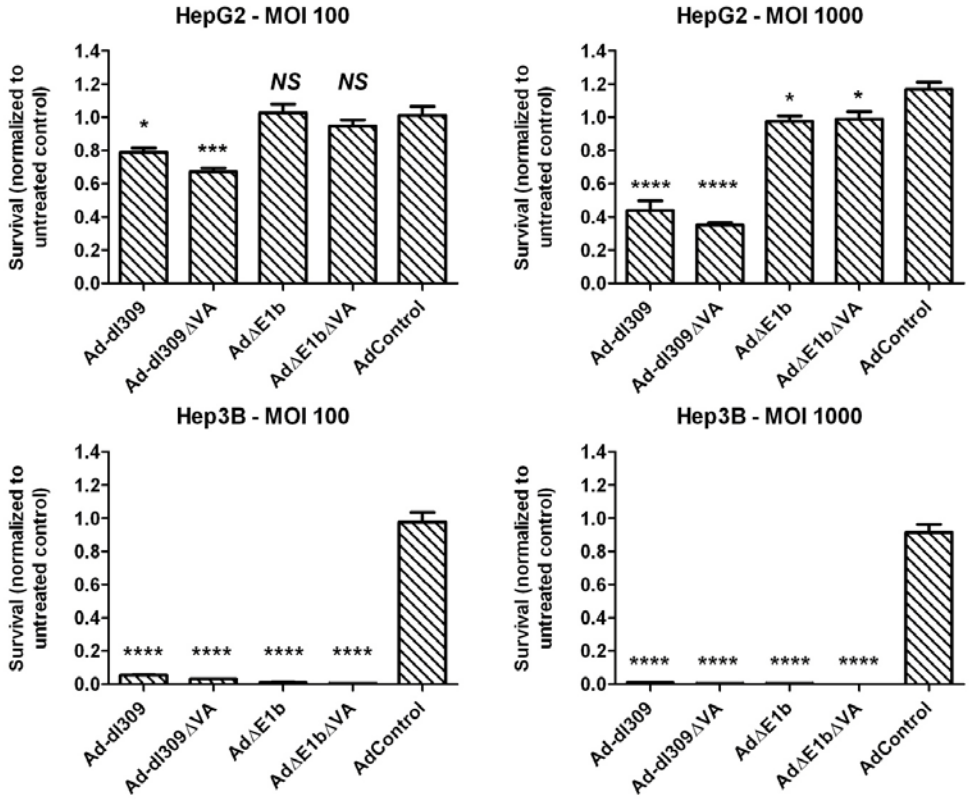


Figure 2.20: Virus-Mediated cell death in HepG2 and Hep3B HCC cell lines.

Cells were infected with the indicated viruses at an MOI of 100 or 1000 VP/cell for 1 hour followed by addition of fresh medium. Six days post-infection, cell survival was determined by Alamar Blue assay followed by normalization to uninfected cells. Error bars correspond to +/-SD of quadruplicate wells. Measurements from infected wells were compared to AdControl measurements to determine significant differences (NS – not significant, * - $p < 0.05$, *** - $p < 0.001$, **** - $p < 0.0001$; One-way ANOVA).

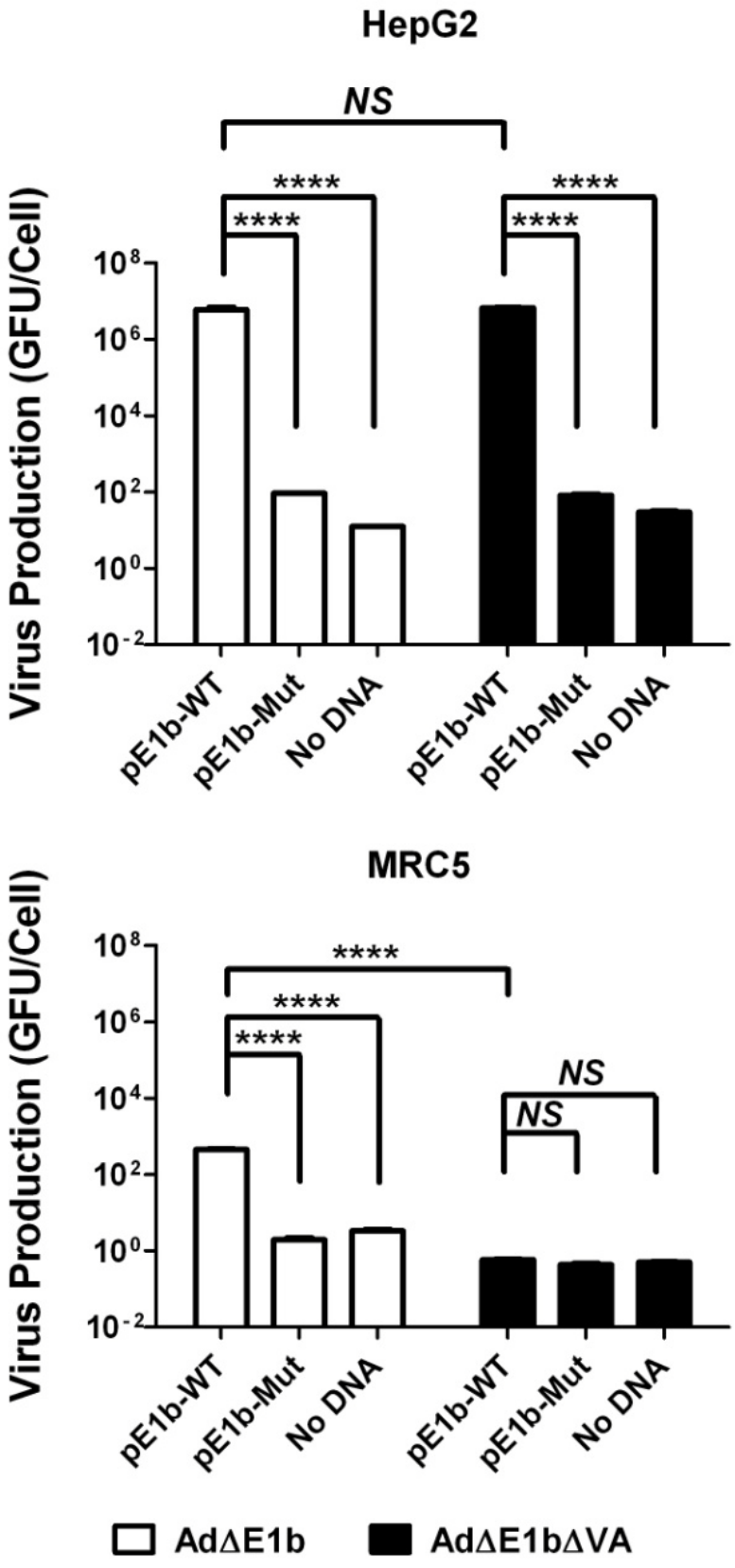

HepG2
NS
****
****
****
****
Virus Production (GFU/Cell)
10^8
10^6
10^4
10^2
10^0
10^-2
pE1b-WT
pE1b-Mut
No DNA
pE1b-WT
pE1b-Mut
No DNA
MRC5
****
****
****
NS
NS
Virus Production (GFU/Cell)
10^8
10^6
10^4
10^2
10^0
10^-2
pE1b-WT
pE1b-Mut
No DNA
pE1b-WT
pE1b-Mut
No DNA
AdΔE1b
AdΔE1bΔVA

Figure 2.21: Transient E1b-55K expression increased AdΔE1b and AdΔE1bΔVA production levels in HepG2 cells.

Cells were transfected with a wild-type or mutant (C454S/C456S) E1b-55K expression vector one day prior to infection with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections (NS – not significant, **** - $p < 0.0001$; One-way ANOVA).

Furthermore, mutant E1b-55K had no effect on virus production in either cell line, suggesting that activities of E1b-55K modulated by residues C454 and C456 were required for the enhancement of AdΔE1b production.

2.3.8.3 p53 Inhibition Does Not Increase AdΔE1b or AdΔE1bΔVA Production in HepG2 Cells

High E1a expression in the absence of E1b proteins in AdΔE1b infections was expected to induce high p53 activation in HepG2 cells (250; 362; 444; 445), which could have had a detrimental effect on virus production. Therefore, we tested whether p53 inhibition would increase AdΔE1b production in this cell line. First, to confirm that p53 activity was increased in HepG2 cells infected with AdΔE1b, cells were co-transfected with a firefly luciferase reporter plasmid containing a p53 response element in the promoter sequences and a *Renilla* luciferase reporter plasmid, as a transfection control. The p53-negative control cell line Hep3B was also transfected with the plasmids. The next day cells were infected with AdΔE1b or control viruses. Luciferase expression was determined two days later (Figure 2.22). As expected, p53 activity was undetectable in the p53-negative Hep3B cells. Furthermore, while p53 activity levels in HepG2 cells infected with the E1b-postive Ad-dl309 and the E1-negative AdControl viruses were similar to that in uninfected cells, p53 activity was increased 5 fold in AdΔE1b-infected HepG2 cells.

Next, we determined whether p53 activity could be inhibited in AdΔE1b-infected HepG2 cells by either wild-type E1b-55K, mutant E1b-55K (C454S/C456S) or shRNA targeting p53, using a scrambled shRNA and mock

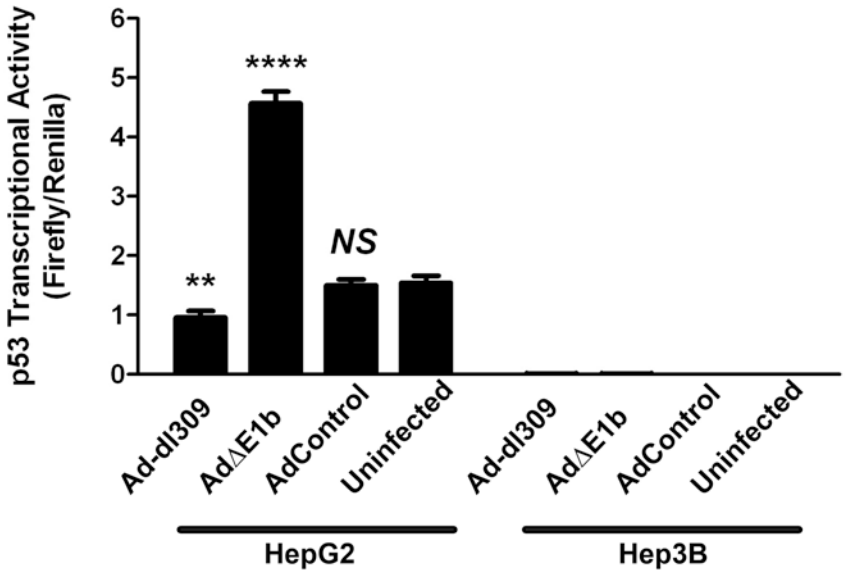


Figure 2.22: Activation of p53 in AdΔE1b-infected HepG2 cells.

Cells were co-transfected with a p53-responsive firefly luciferase reporter plasmid and a *Renilla* luciferase reporter plasmid, as a transfection control. The next day, cells were mock infected or infected with Ad-dl309, AdΔE1b or AdControl, at an MOI of 100 VP/cell. Luciferase expression was determined two days post-infection. Error bars correspond to +/-SD of triplicate infections. Measurements from infected wells were compared to the measurements of uninfected wells to determine significant differences (NS – not significant, **** - $p < 0.0001$; One-way ANOVA).

transfections as controls (Figure 2.23). As expected, the activity of p53 was inhibited in HepG2 cells expressing wild-type and mutant E1b-55K as well as cells expressing p53-shRNA.

Finally, to determine whether p53 inhibition would enhance AdΔE1b production, HepG2 cells were transfected with either wild-type E1b-55K, mutant E1b-55K (C454S/C456S), shRNA targeting p53 or a scrambled shRNA, then infected with AdΔE1b or AdΔE1bΔVA, and virus production determined 4 days later (Figure 2.24). Interestingly, while AdΔE1b and AdΔE1bΔVA production was enhanced in HepG2 cells expressing wild-type E1b-55K, no enhancement was found in virus production in cells expressing the mutant E1b-55K or the p53 shRNA. Therefore, p53 inhibition alone had no effect on production of these viruses. Furthermore, similar to Figure 2.21, the p53-independent activities associated with residues C454 and C456 of E1b-55K were important in enhancing virus production.

2.3.8.4 HBx Expression and HBV Genome Does Not Increase AdΔE1b and AdΔE1bΔVA Production in HepG2 Cells

The p53 status is one of many differences between Hep3B and HepG2 cells. Unlike HepG2 cells, Hep3B cells were previously found to contain the HBV genome (378). Since HBV is a strong risk factor for HCC development, we wanted to test whether the observed higher sensitivity of Hep3B cells to AdΔE1b and AdΔE1bΔVA in comparison to HepG2 cells was due to HBV proteins or sequences in Hep3B cells. Similar to E1b-55K, the HBV protein HBx was previously shown to block proteins involved in the DNA damage response, such

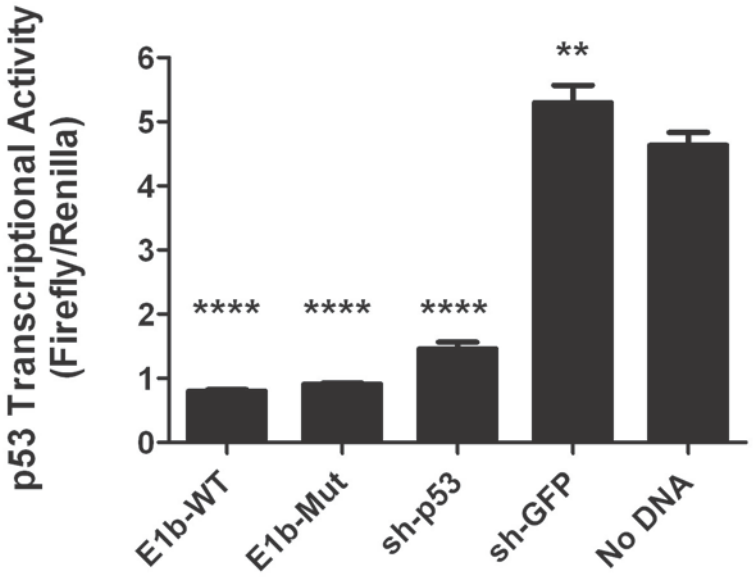


Figure 2.23: Inhibition of p53 activation in AdΔE1b-infected HepG2 cells through transient expression of p53 inhibitors.

Cells were co-transfected with a p53-responsive firefly luciferase reporter plasmid and a *Renilla* luciferase reporter plasmid as a transfection control, as well as the indicated plasmid vectors or no plasmid (No DNA), as a negative control. The next day, cells were infected with AdΔE1b at an MOI of 100 VP/cell. Luciferase expression was determined two days post-infection. Error bars correspond to +/- SD of triplicate infections. (NS – not significant (** - $p < 0.01$, **** - $p < 0.0001$; One-way ANOVA).

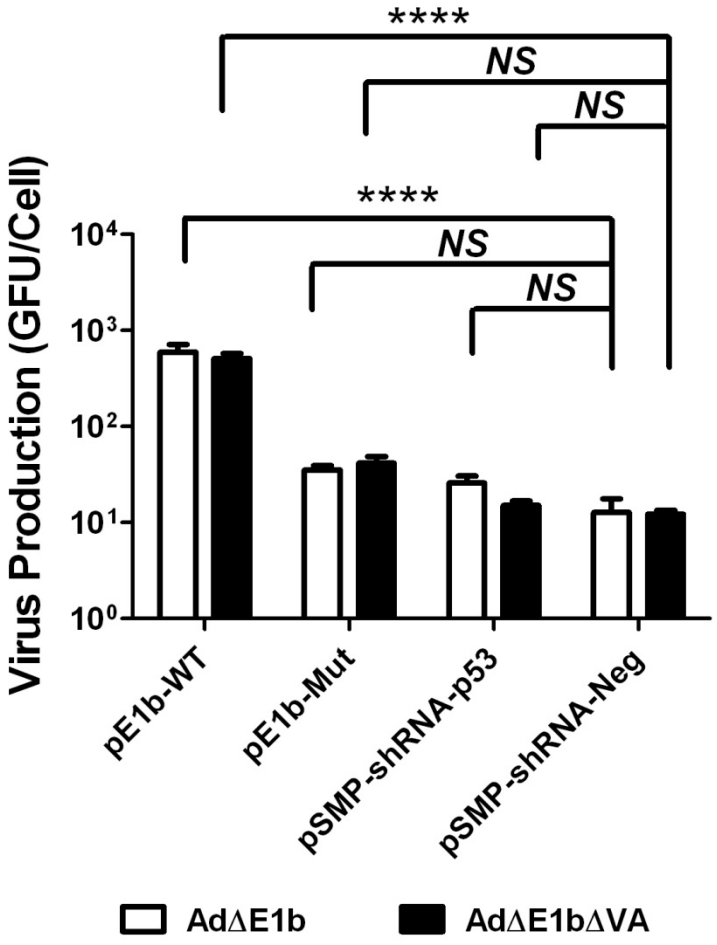


Figure 2.24: Inhibition of p53 does not increase AdΔE1b or AdΔE1bΔVA production levels in HepG2 cells.

Cells were transfected with the indicated plasmid 1 day prior to infection with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections (NS – not significant, **** - $p < 0.0001$; One-way ANOVA).

as p53 (371; 446; 447), as well as proteins mediating the interferon response, such as PKR (375). Interestingly, overexpression of HBx was also found to increase E1b-55K-deleted-virus-mediated cell death (377). Due to the similar activities between HBx and E1b-55K, the effect of HBx expression on AdΔE1b and AdΔE1bΔVA was tested. HepG2 cells were transfected with an HBx expression vector a day prior to infection with AdΔE1b and AdΔE1bΔVA. Cells were also transfected with the wild-type E1b-55K expression vector as a positive control. Four days post-infection, virus production was determined (Figure 2.25). While E1b-55K expression was found to increase AdΔE1b and AdΔE1bΔVA production, expression of HBx had no effect on virus production in HepG2 cells. Therefore, while HBx has similar targets as E1b-55K, expression of HBx in *trans* was not sufficient to complement the lack of E1b-55K during AdΔE1b and AdΔE1bΔVA replication in HepG2 cells.

Interestingly, unlike E1b-55K, HBx can also induce NFkB activation, which may have resulted in activation of pro-survival genes (369). This activation might have affected Ad replication or lysis of infected cell (448; 449). Previous studies have shown that Ad proteins E1a and E1b-19K could block NFkB activation (247; 450), however, the lack of E1b-19K in AdΔE1b infections might have resulted in the inability of the virus to block HBx-mediated NFkB activation.

First, to examine whether HBx-mediated activation of NFkB could be blocked by E1a or E1b-19K, HepG2 cells were co-transfected with the NFkB-responsive luciferase expression vector and E1a or E1b-19K expression vectors

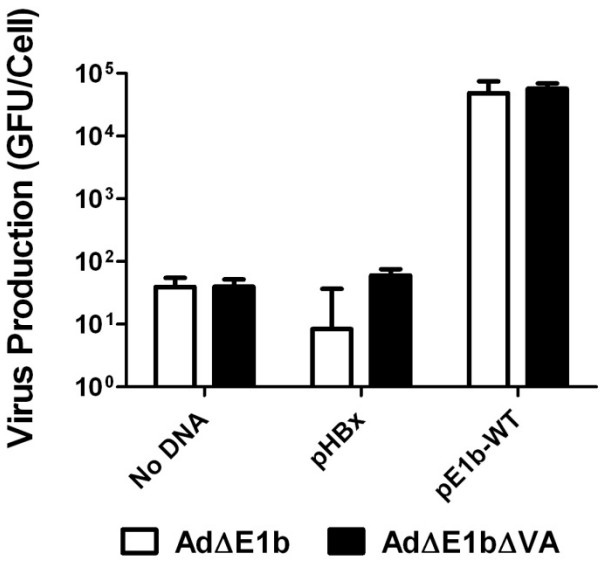


Figure 2.25: HBx expression does not increase AdΔE1b and AdΔE1bΔVA production levels in HepG2 cells.

Cells were mock transfected or transfected with the indicated plasmid 1 day prior to infection with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections.

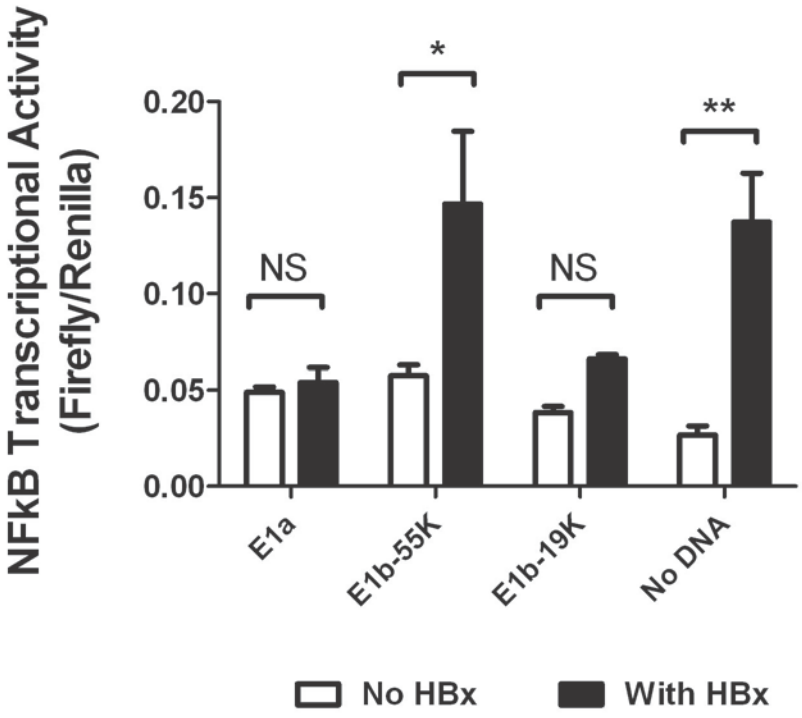


Figure 2.26: E1a and E1b-19K block HBx-mediated activation of NFkB.

Cells were co-transfected with an NFkB-responsive firefly luciferase reporter plasmid, a *Renilla* luciferase reporter plasmid as a transfection control, and the indicated plasmids, with or without an HBx expression vector. Luciferase expression was determined two days post-infection. Error bars correspond to +/- SD of triplicate infections (NS – not significant, * - $p < 0.05$; One-way ANOVA).

with or without an HBx expression vector. As a negative control, an E1b-55K expression vector, which was not found to affect NFkB activity (450), was co-transfected with the NFkB-responsive luciferase expression vector. Luciferase expression was determined two days post-transfection (Figure 2.26). As expected, while E1b-55K had no effect on HBx-mediated activation of NFkB, NFkB activation was inhibited by E1a and E1b-19K.

Next we tested whether E1a produced in infections with AdΔE1b was sufficient to block HBx-mediated activation of NFkB. HepG2 cells were transfected with the NFkB-responsive luciferase expression vector with or without the HBx expression vector a day prior to infection with AdΔE1b. HepG2 cells were also infected with Ad-dl309 or Ad-dl1520, as E1a/E1b-19K-positive controls, and mock infected or infected with AdControl, as E1a/E1b-19K-negative controls (Figure 2.27). Surprisingly, HBx-mediated NFkB activation was not inhibited in HepG2 cells infected with any of the viruses. Therefore, Ad infection appeared to reduce the ability of E1a and/or E1b-19K to block HBx-mediated NFkB activation. While HBx might have similar targets as E1b-55K, the activation of NFkB might have reduced the ability of HBx to compensate for the loss of E1b-55K in HepG2 cells infected with AdΔE1b and AdΔE1bΔVA. Furthermore, since HBx-mediated NFkB activation was not inhibited in HepG2 cells infected with Ad-dl309, HBx overexpression might have also reduced the ability of HepG2 cells to replicate Ad-dl309. Nevertheless, further experiments are required to determine whether HBx-mediated NFkB activation inhibited adenovirus replication, for example through co-treatment with NFkB inhibitors.

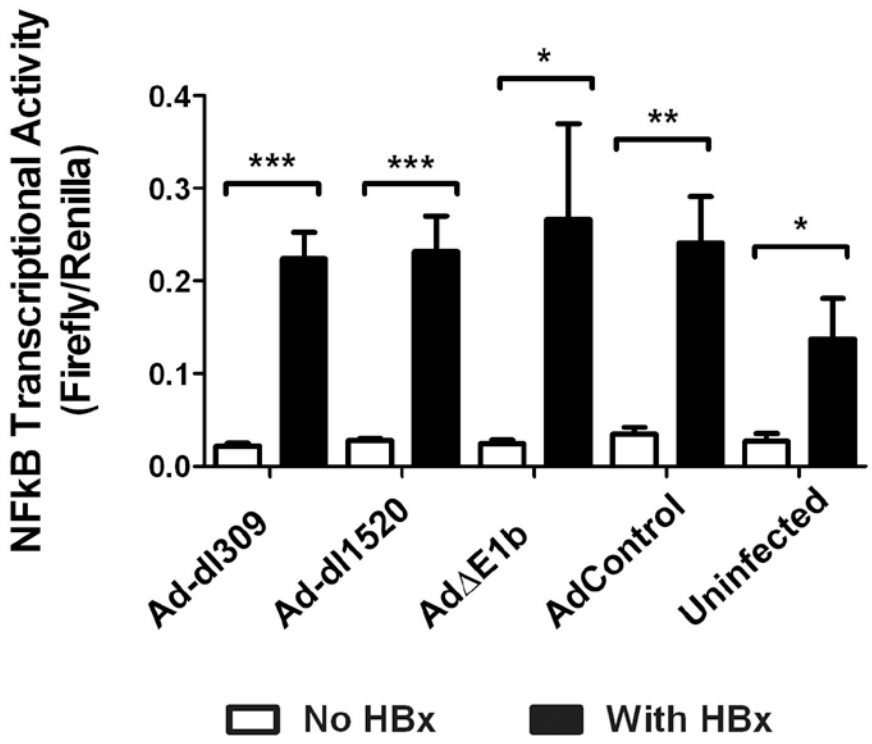


Figure 2.27: Adenovirus infection does not block HBx-mediated NFkB activation.

Cells were transfected with an NFkB-responsive firefly luciferase reporter plasmid and a *Renilla* luciferase reporter plasmid as a transfection control, with or without the HBx expression vector. The next day, cells were mock infected or infected with the indicated viruses at an MOI of 100 VP/cell. Luciferase expression was determined two days post-infection. Error bars correspond to +/- SD of triplicate infections (NS – not significant, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$; t-test).

In addition to HBx overexpression studies, we also tested whether HBV sequences may increase AdΔE1b production and cell lysis. We obtained HBV-positive HepG2-derived cell lines, HepG2 2.15 and HepAD38. HepG2 2.15 cells continuously produce HBV viruses, while production of HBV viruses in HepAD38 cells is activated by tetracycline treatment. Although AdΔE1b or AdΔE1bΔVA production was detected in HepG2 2.15 and in tetracycline-treated HepAD38 cells (Figure 2.28), production was not substantially enhanced when compared to HepG2 cells (Figure 2.19).

To determine whether HBV sequences would affect adenovirus-mediated cell death, we carried out a viability assay on HepG2 2.15 and HepAD38 cells after infection with AdΔE1b, AdΔE1bΔVA and AdControl (Figure 2.29). Similar to HepG2 cells (Figure 2.20), AdΔE1b and AdΔE1bΔVA only induced slight cell death at an MOI of 1000 VP/cell, suggesting that HBV did not increase the sensitivity of HepG2 cells to virus-mediated cell death.

2.3.8.5 Effect on HSP90, Mre11 and PKR Inhibition on AdΔE1b and AdΔE1bΔVA Production in HepG2 Cells

Previous studies have shown that E1b-55K-deleted Ad production could be enhanced by co-treatment with drugs that target pathways similar to those targeted by E1b-55K. Treatment with geldanamycin, an HSP90 inhibitor, was found to increase E1b-55K-deleted virus production through promotion of late mRNA export (366). Furthermore, E1b-55K has a role in the PKR and Mre11 inhibition (231; 232; 251; 255). To determine whether inhibition of HSP90, PKR or Mre11would enhance AdΔE1b and AdΔE1bΔVA production, HepG2-infected

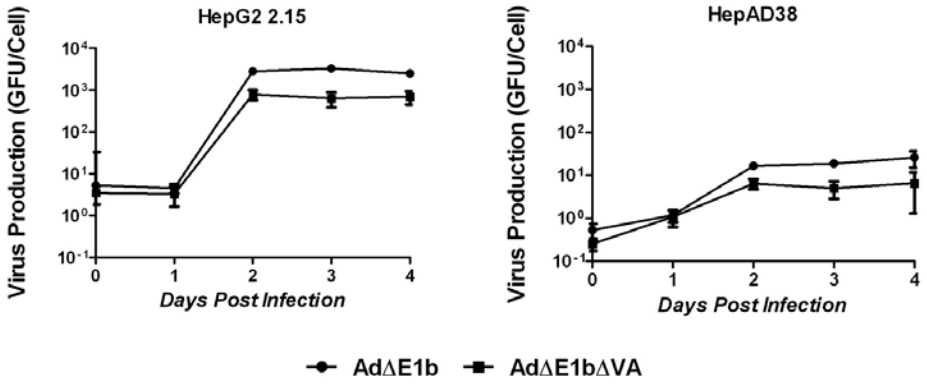


Figure 2.28: AdΔE1b and AdΔE1bΔVA production in HBV-positive HepG2 cells.

HepG2 2.15 or HepAD38 cells pretreated for 1 day with 10 µg/mL tetracycline were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell and harvested 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections.

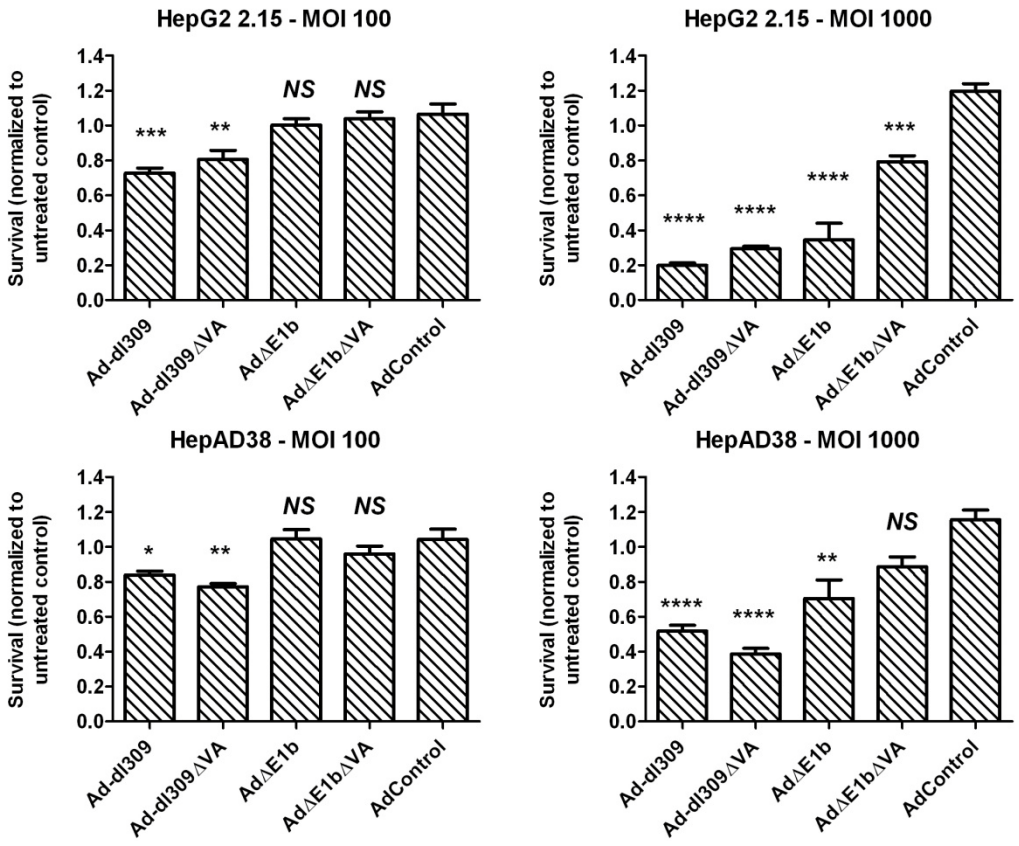


Figure 2.29: Virus-mediated cell death in HBV-positive HepG2 cells.

HepG2 2.15 or HepAD38 cells pretreated for 1 day with 10 µg/mL tetracycline were infected with the indicated viruses at an MOI of 100 or 1000 VP/cell for 1 hour followed by addition of fresh medium or medium containing 10 µg/mL tetracycline, respectively. Six days post-infection, cell survival was determined by Alamar Blue assay followed by normalization to uninfected cells. Error bars correspond to +/-SD of quadruplicate wells. Measurements from infected wells were compared to AdControl measurements to determine whether statistical differences (NS – not significant, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$; One-way ANOVA).

cells were treated with geldanamycin, the PKR inhibitors C-16 and 2'AP, or the Mre11 inhibitor mirin, at non-toxic concentrations. As a control, infected cells were treated with PKRi Neg, which is similar in structure to C-16 but is unable to inhibit PKR. Virus production was determined 4 days post-infection (Figure 2.30). Geldanamycin and mirin treatment did not increase virus production in HepG2 cells, suggesting that the E1b-55K activities compensated by these drugs were not sufficient to increase AdΔE1b and AdΔE1bΔVA production. Interestingly, while 2'AP treatment dramatically increased production of both AdΔE1bΔVA and AdΔE1b in HepG2 cells, C-16 treatment reduced production of both viruses when compared to treatment with the negative control. As C-16 is thought to be a more specific PKR inhibitor than 2'AP, the lack of increased virus production following C-16 treatment suggests that activities of 2'AP separate from PKR inhibition may be required in HepG2 cells for compensation of the E1b deletion in AdΔE1b and AdΔE1bΔVA.

We next examined whether hexon expression correlated with the increased virus production observed in 2'AP-treated HepG2 cells. As controls, infected HepG2 cells were untreated or treated with geldanamycin or mirin (Figure 2.31). Hexon expression in Ad-dl309 and Ad-dl309ΔVA infections was unaffected by any of the treatments. Consistent with virus production assays (Figure 2.30), geldanamycin treatment had little effect on hexon expression in AdΔE1b and AdΔE1bΔVA-infected cells, and mirin treatment resulted in reduction of hexon expression. Surprisingly, 2'AP treatment resulted in only slight increase in hexon expression in HepG2 cells infected with AdΔE1b or AdΔE1bΔVA. Since hexon is

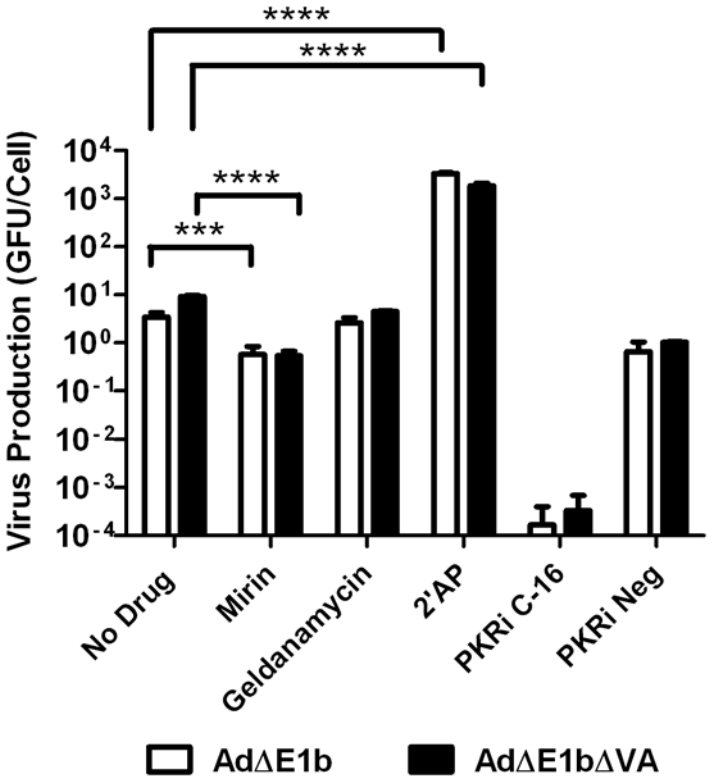


Figure 2.30: AdΔE1b and AdΔE1bΔVA production in drug-treated HepG2 cells.

Cells were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell for 1 hour prior to addition of untreated medium or medium containing 12.5 µM mirin, 125 ng/mL geldanamycin, 2.5 mM 2'AP, 12.5 µM of C-16 or 12.5 µM PKRi negative control. Four days post-infection, virus yields were determined by titrations in Hep3B cells. Error bars correspond to +/-SD of duplicate infections (*** - $p < 0.001$, **** - $p < 0.0001$; t-test).

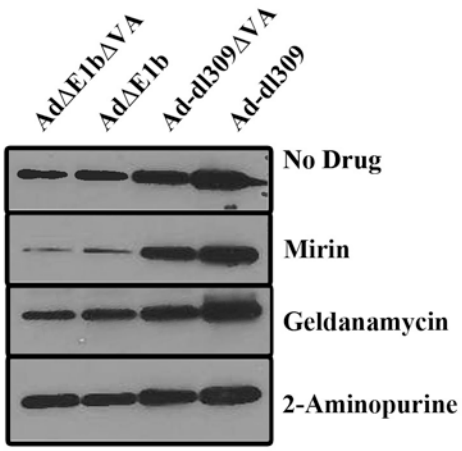


Figure 2.31: Hexon expression in virus-infected HepG2 cells treated with mirin, geldanamycin or 2'AP.

Cells were infected with indicated viruses at an MOI of 100 VP/cell for 1 hour prior to addition of untreated medium or medium containing 12.5 μM mirin, 125 ng/mL geldanamycin or 2.5 mM 2'AP. Two days post-infection, cells were harvested. 10 μg protein was separated by SDS-PAGE gel, transferred to a western blot and hexon levels were determined using anti-hexon antibody.

a late protein, this result suggests that 2'AP-mediated enhancement of virus production in these cells may have been due to compensation of an E1b-55K function required after initiation of DNA replication.

To verify that 2'AP-mediated AdΔE1b production was due to complementation of E1b-55K functions, HepG2 cells were infected with the E1b-positive Ad-dl309, the E1b-55K-negative Ad-dl1520, or the fully E1b-negative AdΔE1b, then treated with 2'AP, geldanamycin or mirin. Virus production was determined 4 days later by plaque assays in HEK293 cells (Figure 2.32). While mirin treatment had no effect on virus production of Ad-dl309 and Ad-dl1520, the treatment slightly reduced AdΔE1b production. Also, geldanamycin treatment of HepG2 infected cells had little effect on production of any virus. Interestingly, while 2'AP treatment had no effect on Ad-dl309 production, the treatment significantly increased Ad-dl1520 and AdΔE1b production in HepG2 cells, suggesting that 2'AP may compensate for the E1b-55K deletion. Chapter 3 of the thesis describes our efforts to identify cellular pathways affected by 2'AP treatment which allow for increased AdΔE1b production.

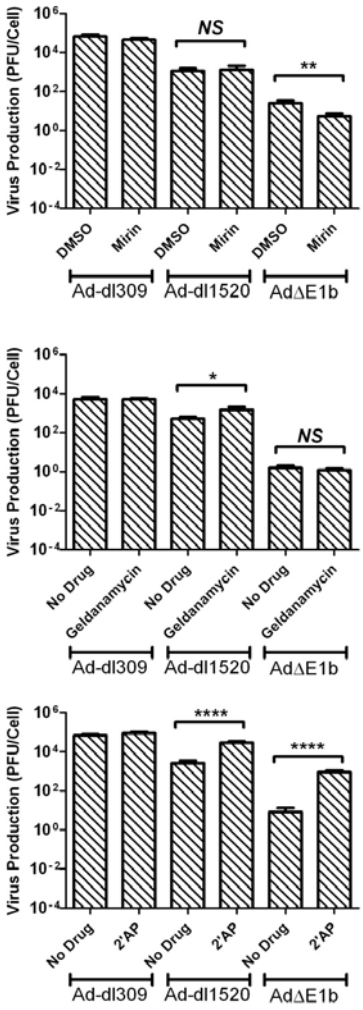


Figure 2.32: Ad-dl309, Ad-dl309 and AdΔE1b production in drug-treated HepG2 cells.

Cells were infected with the indicated viruses at an MOI of 1 PFU/cell for 1 hour prior to addition of medium containing 12.5 uM mirin, 6.25 ng/mL geldanamycin or 2.5 mM 2'AP. Four days post-infection, cells were harvested, and virus yields were determined by plaque assays in HEK293 cells. Error bars correspond to +/- SD of duplicate infections (NS – not significant, * - $p < 0.05$, ** - $p < 0.01$, **** - $p < 0.0001$; One-way ANOVA).

2.4 DISCUSSION

E1b-55K-deleted oncolytic Ads, such as Ad-dl1520 and H101, have long been studied to determine their therapeutic potential for many cancers, including HCC (353; 379; 381; 383; 451). While pre-clinical studies showed great promise as a potential therapy, Ad-dl1520 did not demonstrate strong anti-tumour activity in clinical trials with HCC patients (362; 363; 379). More recent studies have shown that infection with Ads containing a deletion of E3 or E1b-19K genes, in addition to E1b-55K gene, results in stronger cell lysis when compared to Ads with an E1b-55K deletion alone (389-391). Therefore, in order to increase virus-mediated cell death, we constructed the oncolytic virus AdΔE1b, which contained deletions of the full E1b and the E3 genes. Furthermore, to reduce potential hepatotoxicity induced by AdΔE1b, the VA-RNA genes were deleted, and two miR-122T sites were inserted in the 3'UTR of the E1a gene.

Similar to previous reports (280; 406), we found that the deletion of VA-RNA genes in AdΔE1bΔVA highly attenuated virus production in HEK293 cells when compared to the VA-RNA-positive, AdΔE1b. Since these HEK293 cells are unable to act as packaging cells for the amplification of VA-RNA-deleted viruses, we searched for an alternative cell line. As the VA-RNA deletion in Ad-dl309ΔVA had a lower inhibitory effect on virus production in Hep3B cells when compared to HEK293 cells, we tested the efficiency of AdΔE1bΔVA production in Hep3B cells and found that the VA-RNA deletion did not attenuate AdΔE1bΔVA production in comparison to AdΔE1b. Furthermore, we showed that transient expression of VA-RNAs increased AdΔE1bΔVA production in

HEK293 cells, but not in Hep3B cells, suggesting that Hep3B cells are not dependent on VA-RNA expression for replication of this virus.

Surprisingly, AdΔE1b production was attenuated in comparison to Ad-dl309. This is in contrast to a previous study, which showed that full E1b deletion had no effect on virus production in Hep3B cells (389). However, since that study was performed with a virus encoding the complete E3 region, expression of the E3 proteins might enhance the production of E1b-deleted Ads in Hep3B cells.

To assess the potential of AdΔE1b and AdΔE1bΔVA as oncolytic viruses, virus replication and cell death mediated by both viruses were determined in a panel of cancer cell lines. As pancreatic cancer cell lines were previously shown to support the replication of Ads with deletions of VA-RNA (280; 403), we expected that the pancreatic cancer cells would support the replication of AdΔE1bΔVA. However, AdΔE1bΔVA replication, hexon expression and virus-mediated cell death were attenuated in the tested human pancreatic cancer cell lines.

Interestingly, while the Syrian hamster pancreatic cancer cell line SHPC-6 did not support AdΔE1b replication, hexon expression was detected in these cells, suggesting that the attenuation of AdΔE1b production may occur late during the infection cycle following DNA replication but prior to encapsidation. Furthermore, since AdΔE1b-mediated SHPC-6 cell death was high yet virus production was low, AdΔE1b-mediated cell death might have been due to early induction of apoptosis mediated by the high E1a expression in AdΔE1b-infected cells. In contrast, human breast and bladder cancer cell lines that did not support

AdΔE1b replication were found to be highly sensitive to the lytic activity of this virus. The lack of hexon expression in these infected cells, as well as the sensitivity to AdΔE1b-mediated cell death, suggest that the attenuation of AdΔE1b replication occurred at an even earlier step within the virus lifecycle in breast and bladder cancer cells compared to SHPC-6 cells. Similar to AdΔE1b Ad-dl309 and Ad-dl309ΔVA were found to induce lysis of breast cancer cells as well as many of the other tested cancer cells. However, it seems likely that these cancer cells supported the replication of both Ad-dl309 and Ad-dl309ΔVA since hexon was highly expressed in cells infected with both viruses. Therefore, in contrast to AdΔE1b, Ad-dl309- and Ad-dl309ΔVA-mediated cell death might have occurred late during the replication cycle to allow viral release. Further studies are required to verify that these cancer cells support the replication of Ad-dl309 and Ad-dl309ΔVA.

While AdΔE1bΔVA production was found to be highly attenuated in most non-HCC cell lines, the HCC cell lines HepG2 and Hep3B were found to support AdΔE1bΔVA production and hexon expression. Furthermore, transient expression of E1b-55K was found to increase AdΔE1bΔVA and AdΔE1b production in HepG2 cells, suggesting that certain roles of E1b-55K are important for the efficient replication of the E1b-deleted Ads in these cells. However, as the inhibition of p53 activity had no effect on virus production in these cells, it appears that E1b-55K roles other than p53 inhibition are required to increase virus replication in these cells.

E1b-55K is known to be important in the inhibition of the DNA damage response, a function also identified for the HBV protein HBx (371-374). However, neither HBx expression nor HBV genome replication was able to complement the loss of E1b-55K in AdΔE1b-infected cells.

HBx has many targets besides the DNA damage response. To specifically inhibit activation of the DNA damage response, AdΔE1b-infected HepG2 cells were treated with the Mre11 inhibitor, mirin (452). Surprisingly, we found that mirin treatment reduced AdΔE1b production, as well as hexon expression, in HepG2 cells, indicating that direct Mre11 inhibition could not compensate for the lack of E1b-55K in these cells.

Since E1b-55K is important in the inhibition of PKR during virus infection, AdΔE1b-infected HepG2 cells were treated with PKR inhibitors C-16 (453) and 2'AP (281). C-16 treatment was found to reduce AdΔE1b production, suggesting that PKR inhibition was not the limiting factor in the growth of AdΔE1b. Interestingly, 2'AP treatment was found to increase AdΔE1b production, however, 2'AP is reported to have targets in addition to PKR, such as enzymes within the DNA damage response pathway (454-456). Therefore, 2'AP activities other than PKR inhibition might have compensated for the deletion of E1b-55K in AdΔE1b-infected HepG2 cells.

In contrast to HCC cells, AdΔE1bΔVA production was highly attenuated in normal fibroblasts when compared to AdΔE1b. Furthermore, while transient expression of E1b-55K increased AdΔE1b production in MRC5 cells, E1b-55K expression had no effect on AdΔE1bΔVA production, suggesting that the VA-

RNAs are required for virus replication in normal cells. In addition to VA-RNA deletion, insertion of miR-122T sites in AdΔE1bΔVA may have the potential to further reduce the hepatotoxicity associated with oncolytic Ads without affecting the specificity of the virus for HCC cells.

Chapter 3 - 2-Aminopurine Enhances the Oncolytic Activity of an E1b-Deleted Adenovirus in Hepatocellular Carcinoma Cells*

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* Mary M. Hitt and David Sharon wrote the manuscript as well as designed the experiments and figures. David Sharon performed all the experiments.

3.1 ABSTRACT

Adenoviruses with deletions of viral genes have been extensively studied as potential cancer therapeutics. Although a high degree of cancer selectivity has been demonstrated with these conditionally replicating adenoviruses, low levels of virus replication can be detected in normal cells. Furthermore, these mutations were also found to reduce the activity of the replicating viruses in certain cancer cells. Recent studies have shown that co-administration of chemotherapeutic drugs may increase the activity of these viruses without affecting their specificity. We constructed an adenovirus with deletions of both the E1b and the VA-RNA genes and found that replication of this virus was selective for human hepatocellular carcinoma (HCC) cell lines when compared to normal cell lines. Furthermore, we show that 2-aminopurine (2'AP) treatment selectively enhanced virus replication and virus-mediated death of HCC cells. 2'AP did not compensate for the loss of VA-RNA activities, but rather the loss of an E1b-55K activity, such as the DNA damage response, suggesting that co-administration of 2'AP derivatives that block host DNA damage response, may increase the oncolytic activity of AdΔE1bΔVA without reducing its selectivity for HCC cells.

Keywords: Adenovirus, E1b-55K, 2-aminopurine, VA-RNA, hepatocellular carcinoma.

3.2 INTRODUCTION

Adenoviruses with deletions or mutations within the early region 1b (E1b) gene have been shown to replicate selectively in cancer cells. The most commonly studied cancer selective oncolytic adenovirus is Ad-dl1520 (Onyx-015) (353). Due to the role of the E1b-55K protein in the inhibition of p53 (250; 444; 445), selectivity was first thought to be due primarily to inactivating mutations or deletions of the p53 gene in cancer cells, thus relieving the requirement for E1b-55K in virus replication (354; 358; 457; 458). However, the cancer selectivity was later found to be independent of p53 and it is currently thought that loss of other functions of E1b-55K may confer viral selectivity to cancer cells (360; 366; 459; 460). One of these functions is to inhibit the DNA damage response. Sensing the linear viral DNA genome as double-stranded (ds) DNA breaks activates the DNA damage response pathway, which in turn, activates checkpoint proteins that block further DNA replication of both host and viral DNA (212; 213). Furthermore, in an attempt to repair the damage, host proteins can induce concatemerization of viral genomes, which produces DNA sequences larger than the packaging limit (213-215). Several viral proteins have been shown to block activation of the DNA damage response, such as E1a, E4orf3 and E1b-55K in association with E4orf6 (231; 232; 461-463). In particular, two cysteine residues of E1b-55K (C454 and C456) are important in the inhibition of the DNA damage response through inhibition of at least 2 key proteins within the pathway, Mre11 and DNA ligase IV (231; 232).

In addition to E1b-55K deletion, adenoviruses with deletions of the E1b-19K gene were also shown to be oncolytic (390; 442). Similar to E1b-55K, E1b-19K has a role in the inhibition of premature virus-mediated cell death, therefore, E1b-19K deletion is thought to increase virus-mediated killing. Furthermore, adenoviruses with deletions of both E1b-19K and E1b-55K were found to have increased selectivity for cancer cells when compared to adenoviruses with a single deletion of either E1b-19K or E1b-55K (391; 464).

In addition to the E1b deletions, deletions of other adenoviral genes were shown to allow selective virus production in cancer cells, such as the deletion of the virus-associated RNA (VA-RNA) genes (270; 280; 403). These genes express two non-coding RNA molecules (VA1 and VA2). Although the role of VA2 in virus replication is largely unknown, VA1 is thought to be important for inhibiting the activation of the interferon response, an important cellular antiviral response (267; 417). This inhibition occurs through direct binding and inactivation of RNA sensors that activate the interferon response, such as PKR (418-420). Activated PKR can inhibit both viral and cellular protein synthesis through phosphorylation of eIF2α, as well as induce premature cell death during virus infection (256; 465; 466). Activating ras mutations, which are found in many cancer cells, block PKR phosphorylation of eIF2α. Therefore, cancer cells with activating ras mutations have been hypothesized to support VA-RNA deleted adenovirus replication (280; 405).

The adenine analog 2-aminopurine (2'AP) alters a number of pathways that are important in adenoviral infection. It was shown to block PKR activity,

thus blocking shutdown of protein synthesis (281). 2'AP was also shown to inhibit interferon-stimulated gene expression, which is a downstream effect of the interferon response (467; 468). Several studies have shown that 2'AP can also inhibit ATM and ATR, proteins within the DNA damage response, which are activated by Mre11 (454-456). Furthermore, the expression and activity of p53 following DNA damage were found to decrease in cells treated with 2'AP. Interestingly, PKR directly interacts with and activates p53 following DNA damage (257; 258), and p53 induces PKR expression (469), suggesting a possible convergence between the interferon response and the DNA damage response. Recent reports showed that, in addition to the VA-RNAs, E1b-55K can also inhibit PKR and eIF2α phosphorylation as well as inhibit the activation of interferon-stimulated genes following adenoviral infection (251; 255). Due to the ability of 2'AP to rescue the replication of a VA-RNA-deleted adenovirus in certain cells, the drug may also be able to rescue the replication of adenoviruses deleted of both VA-RNA and E1b-55K sequences.

We report here the construction and assessment of E1b-deleted adenoviruses with or without additional deletion of the VA-RNA genes (AdΔE1bΔVA and AdΔE1b, respectively). A previous study has shown that increased E1a expression levels in cancer cell lines can increase E1b-deleted virus replication (470). Therefore, the E1a gene in our construct was placed under the control of the strong immediate early murine cytomegalovirus (mCMV) promoter (471).

Through growth analysis of AdΔE1b and AdΔE1bΔVA in HCC and

normal cell lines we found that, while the deletion of VA-RNA attenuated virus replication in normal cells, VA-RNAs were dispensable for E1b-deleted virus replication in the HCC cell lines, Hep3B and HepG2. Due to the effect of 2'AP on both the interferon response and the DNA damage response, we examined whether this drug could rescue the replication of E1b-deleted adenoviruses either encoding or not encoding VA-RNAs. Surprisingly, although 2'AP treatment increased the replication of both AdΔE1b and AdΔE1bΔVA in normal MRC5 cells, the drug was unable to rescue AdΔE1bΔVA replication to the same level as AdΔE1b in these cells. In contrast, 2'AP treatment increased replication of both AdΔE1b and AdΔE1bΔVA to similar high levels in HepG2 cells. Furthermore, we found that the E1b-55K domain required for the inhibition of host DNA damage response was important for adenovirus replication in HepG2 cells, and that 2'AP compensates for the loss of this domain.

3.3 MATERIALS AND METHODS

3.3.1 Cell lines

Hep3B (provided by Dr. Roseline Godbout, University of Alberta) and HepG2 (ATCC HB-8065) hepatocellular carcinoma cell lines, as well as WI-38 lung fibroblasts (ATCC CCL-75), were grown in DMEM high glucose (Gibco). MRC5 lung fibroblasts (ATCC CCL-171) and HEK293 E1-transformed human embryonic kidney cells (313) (provided by Dr. Frank Graham, McMaster University) were grown in MEM (Gibco). All media were supplemented with 10 % FBS (Gibco), 2 mM L-glutamine (Gibco) and 1x antibiotic-antimycotic (100 units of penicillin, 100 μg streptomycin, 0.0085 % fungizone) (Gibco).

3.3.2 Viral constructs

Ad-dl309, Ad-dl309ΔVA (dVAs) and the non-replicating AdControl (rAd-gal-GFP) (270; 438) were kind gifts from Dr. Matthias Dobbelstein (Göttingen University). Ad-dl1520 (353) was a kind gift from Dr. Philip Branton (McGill University). AdΔE1b and AdΔE1bΔVA were constructed using the AdEasy-1 system (439). Briefly, Ad5 E1a gene (excluding viral E1a promoter) was PCR amplified from the plasmid pXC1 (nucleotides 542-1564; a gift from Dr. Frank Graham). This fragment was added to an expression vector encoding a PacI-deleted murine cytomegalovirus immediate early (mCMV) promoter. The expression cassette was then transferred into the pAd-Track shuttle vector. This pAd-Track plasmid was then recombined in BJ5183 bacterial cells with the pAd-Easy-1 plasmid or a derivative lacking the VA-RNA genes (270). The resulting recombinant plasmids were cleaved with PacI to release the viral genome and

used to transfect HEK293. Recombinant viruses were isolated and amplified in Hep3B cells due to the reduced growth of VA-RNA deleted viruses in HEK293 cells. Ad-dl309 and Ad-dl309ΔVA were also amplified in Hep3B cells, while the replication-deficient AdControl was amplified in HEK293 cells. All viruses (Figure 3.1) were purified by CsCl gradient sedimentation (472) and their genomes were isolated and verified by restriction enzyme digestion. The concentration of virus particles was determined spectrophotometrically (472). The concentration of infectious virus was determined through plaque assays performed on HEK293 cells as previously described (472). Due to the inability of AdΔE1bΔVA to produce plaques in HEK293 cells, the concentration of infectious units of AdΔE1bΔVA as well as AdΔE1b were determined from the number of GFP-positive Hep3B cells observed 3 days after infection with limiting dilutions of virus (designated green fluorescent units, GFU). The ratios of virus particle to infectious units of AdΔE1b and AdΔE1bΔVA were approximately 70, similar to the particle to PFU ratio for the other viruses.

3.3.3 Virus replication assays

Confluent cells in a 24 well plate were infected with Ad-dl309, Ad-dl309ΔVA, Ad-dl1520 or AdΔE1b at an MOI of 1 PFU/cell (or 1 GFU/cell where indicated) in 0.2 mL PBS in duplicate. One hour post-infection fresh medium was added either with or without 2.5 mM 2'AP (Invivogen, San Diego, CA, USA). Infected cells and media were harvested at the indicated times, and subjected to 3 rounds of freeze-thaw cycles. For the virus release assay, the media from infected wells were also harvested and centrifuged at 1000 rpm for 5 min prior to 3 freeze-

thaw cycles. Virus concentrations were determined by plaque assay (in duplicate) in HEK293 cells, or where indicated, by limiting dilution assay on Hep3B cells (in triplicate) as described above.

3.3.4. E1b complementation assay

In order to determine the effect of the E1b-55K expression vector on viral replication, HepG2 cells were stably transfected with wild-type pE1b-55K or pE1b-55K-Mut(C454S/C456S) plasmid DNA (231) (a kind gift from Dr. Thomas Dobner, Heinrich-Pette Institute) using selection medium containing G418 at the concentration of 1 μg/mL. The two E1b-55K expressing cell lines (HepG2-E1b-WT and HepG2-E1b-Mut) as well as parental HepG2 cells were infected with Ad-dl309, Ad-dl1520 or AdΔE1b. One hour post-infection fresh medium was added either with or without 2.5 mM 2'AP. Infected cells and media were harvested at the indicated times and subjected to 3 freeze-thaw cycles. Infectious units were determined as either PFUs or GFUs as indicated.

3.3.5 Cell survival assay

Cells were transferred into 96 well plates at 5000 cells/well. The following day, the medium was removed and cells were infected at the indicated concentrations in quadruplicate. One hour later, fresh medium was added with or without supplementation of 2.5 mM 2'AP. Six days post-infection, Alamar Blue (Resazurin; Sigma, St Louis, MO, USA) was added to a final concentration of 44 μM. Fluorescence of each well was measured (excitation at 544 nm; emission at 590 nm) using the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany).

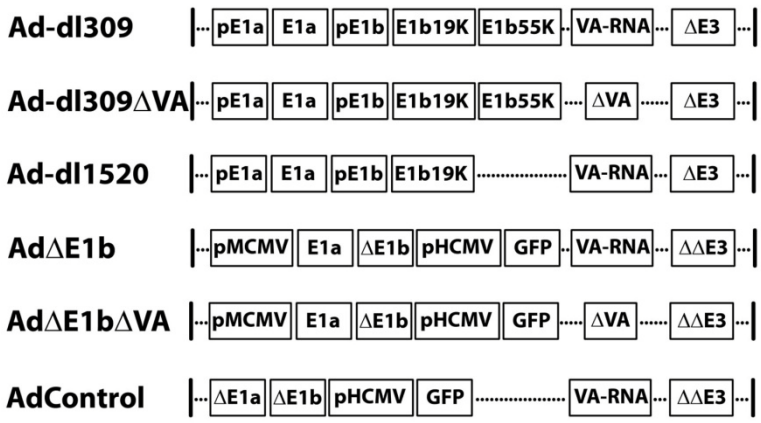

Figure 3.1: Schematic representation of the adenoviral genomes used in this study.

Ad-dl309 encodes both E1a and E1b genes including their respective Ad promoters (pE1a and pE1b, respectively). The virus has a deletion in the E3 region spanning 30005-30750 bp (473). Ad-dl309ΔVA and Ad-dl1520 have additional deletions in the VA-RNA genes (10667-10702 bp as well as 10929-10943 bp) or in the E1b-55K gene, respectively. AdΔE1b, AdΔE1bΔVA and AdControl (ΔE1aΔE1b) were constructed using the AdEasy-1 system and the pAdTrack shuttle plasmid. The pAd-Easy-1 plasmid has a larger deletion in the E3 region spanning 28130-30820 bp. The pAd-Track plasmid has E1 sequences spanning 480-3533 bp replaced with the EGFP gene under the control of the human cytomegalovirus immediate early (hCMV) promoter (439). The E1a gene under the control of the murine cytomegalovirus immediate early (mCMV) promoter was introduced back into the genomes of AdΔE1b and AdΔE1bΔVA. Additionally, AdΔE1bΔVA has the same VA-RNA deletions as Ad-dl309ΔVA.

3.3.6 Western blot analysis

Cells were infected at an MOI of 100 virus particles per cell (VP/cell). Two and four days post-infection, cells were harvested and lysed with RIPA buffer supplemented with 1 mM PMSF (Sigma) and 1x Protease Inhibitor Cocktail (Sigma). 10 μg protein samples were electrophoresed on an SDS polyacrylamide gel then transferred to a nitrocellulose membrane (Biorad, Mississauga, ON, CA). Membranes were blocked with 5 % powdered skim milk in PBST (PBS with 0.5 % Tween-20) for 1 hour and incubated overnight at 4^{o}C with antibodies against E1a (M73, NeoMarkers, Fremont, CA, USA), fiber (RDI-Adenov2Abm, Research Diagnostics Inc, Flanders, NJ, USA), β-actin (MA5-15739, Thermo Fisher Scientific Inc, Rockford, IL, USA) or p53 (Pab 1801, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed in PBST and incubated for 1 hour in horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

3.3.7 p53-activated luciferase reporter assay

HepG2 and Hep3B cells in a 24 well plate were transfected with 1 μg of p53-reporter expression vector (Panomics, Fremont, CA, USA) using Lipofectamine 2000. The following day, cells were infected with Ad-dl309, Ad-dl1520 or AdΔE1b at an MOI of 100 VP/cell for 1 hour in PBS, followed by addition of fresh medium or medium containing 2.5 mM 2'AP. Two days post-infection, cells were washed with PBS and lysed, and firefly luciferase levels were measured using the Dual Luciferase Reporter Assay System (Promega) with the FLUOstar Omega plate reader (BMG).

3.3.8 GFP fluorescence intensity measurements

Cells were transferred into 96 well plates at a concentration of 5000 cells per well. The following day, the medium was removed and cells were infected at an MOI of 100 VP/cell in quadruplicate. One hour later, fresh medium or medium with 2.5 mM 2'AP was added to the infected cells. GFP fluorescence was determined with the FLUOstar omega plate reader (BMG) at the indicated time-points post-infection.

3.4 RESULTS

3.4.1 Replication properties of VA-RNA- or E1b-55K-deleted adenoviruses in HCC and normal cell lines

In order to determine the effect of deletions of VA-RNA genes and/or E1b-55K on adenovirus growth, we generated a series of time-courses of virus production in a panel of hepatocellular carcinoma (HCC) and normal cell lines (for clarity, these data are presented in Figures 3.2 and 3.3). Ad-dl309 was used as a VA-RNA and E1b positive control (Figure 3.1), and therefore, its production time-course was plotted in both figures 3.2 and 3.3.

First, replication of viruses deleted in the VA RNA genes alone, or the E1b-55K coding sequence alone, were compared to Ad-dl309 (Figure 3.2A and 3.2B, respectively). Although the deletion of VA-RNA in Ad-dl309ΔVA was found to reduce adenovirus growth in both HCC and normal cells, the virus was more strongly attenuated in MRC5 and WI-38 normal cells when compared to HepG2 and Hep3B cells (4 and 2 orders of magnitude, respectively). Furthermore, Ad-dl309ΔVA was attenuated in HEK293 cells, which express

proteins encoded by the E1 region of Ad5 (313). Previously, 2'AP was shown to rescue the replication of a VA-RNA-deleted adenovirus (280), therefore, the panel of infected cells were also treated with 2.5mM 2'AP. Surprisingly, 2'AP treatment did not have a significant effect on Ad-dl309ΔVA production in any of the tested cell lines.

Similar to the VA-RNA deletion (Figure 3.2A), the E1b-55K deletion in Ad-dl1520 also reduced virus growth in MRC5 and WI-38 normal cells when compared to Ad-dl309 (Figure 3.2B). However, the VA-RNA deletion had a stronger inhibitory effect on virus growth than the E1b-55K deletion in normal cells (4 and 2 orders of magnitude, respectively). Furthermore, while E1b-55K deletion had no effect on adenovirus production in Hep3B cells, the level of virus production was reduced in HepG2 cells. In addition, it should be noted that, although 2'AP treatment had no effect on Ad-dl309ΔVA production, the treatment appeared to slightly increase Ad-dl1520 production in HepG2, MRC5 and WI-38, suggesting that 2'AP could be partially compensating for lack of E1b-55K activities.

Taken together, these results suggest that Ad-dl309ΔVA may have higher selectivity for

HCC cells than Ad-dl1520 does.

3.4.2 Replication properties of E1b-fully-deleted adenoviruses in HCC and normal cells

Previous studies have shown that E1b-19K deletion may increase cancer-selectivity of E1b-55K deleted adenoviruses (391; 464). Therefore, In order to

increase the selectivity of E1b-55K-deleted adenoviruses for HCC cells compared to normal cells, we constructed an E1b-deleted adenovirus (AdΔE1b), which does not encode either E1b-19K or E1b-55K (Figure 3.1). Furthermore, due to a previous report showing that increasing E1a levels may increase the activity of E1b-55K-deleted-adenoviruses (470), we placed the E1a gene under the control of the strong immediate early murine cytomegalovirus (mCMV) promoter.

To examine the effect of these modifications on virus replication, we compared the growth properties of AdΔE1b to Ad-dl309 in the panel of HCC and normal cell lines (Figure 3.3A). We found that the deletions in AdΔE1b attenuated virus production in all the tested cell lines when compared to Ad-dl309. Similar to Ad-dl309ΔVA, AdΔE1b attenuation was stronger in MRC5 and WI-38 than in HepG2 and Hep3B. Interestingly, AdΔE1b production was also attenuated in the E1-positive HEK293 cells. This attenuation may be due to the larger deletion within the E3 region of AdΔE1b (encoding the adenovirus death protein gene) than that in both Ad-dl309 and Ad-dl1520 (Figure 3.1).

Because we observed an increase in Ad-dl1520 production following 2'AP treatment (Figure 3.2B), we next tested whether 2'AP treatment could also increase AdΔE1b production. Interestingly, we found that 2'AP strongly increased AdΔE1b production in HepG2 and Hep3B cells, with a somewhat smaller effect in normal cells (Figure 3.3A).

To further increase the selective production of E1b-deleted adenovirus in HCC cells relative to normal cells, we constructed an adenovirus deleted in both E1b and the VA-RNA genes (AdΔE1bΔVA, Figure 3.1). Due to the low

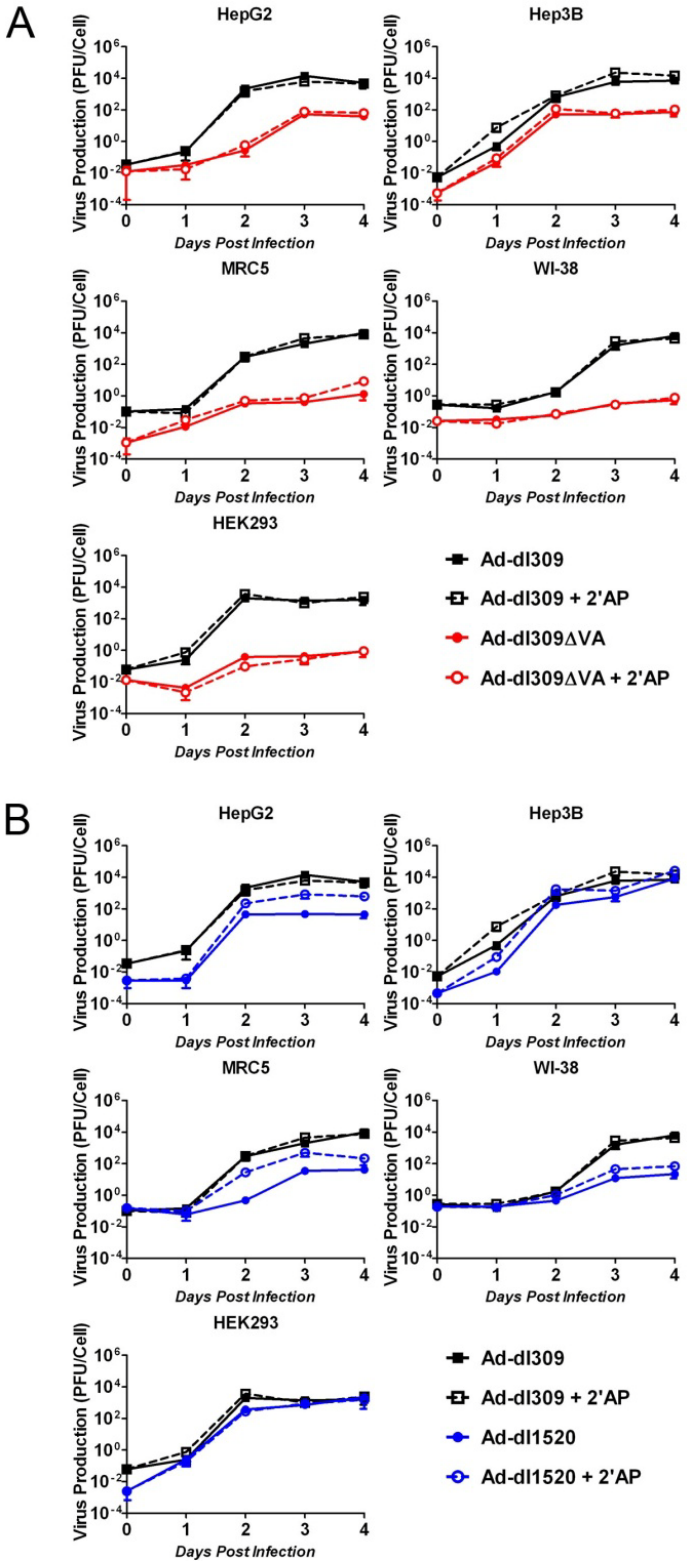
A
HepG2
Hep3B
MRC5
WI-38
HEK293
Virus Production (PFU/Cell)
Days Post Infection
Ad-dl309
Ad-dl309 + 2'AP
Ad-dl309ΔVA
Ad-dl309ΔVA + 2'AP
B
HepG2
Hep3B
MRC5
WI-38
HEK293
Virus Production (PFU/Cell)
Days Post Infection
Ad-dl309
Ad-dl309 + 2'AP
Ad-dl1520
Ad-dl1520 + 2'AP

Figure 3.2: 2'AP increases the replication of an adenovirus with an E1b-55K deletion but not with a VA-RNA deletion.

Cells were infected in duplicate with (A) Ad-dl309ΔVA, (B) Ad-dl1520, or (A and B) Ad-dl309 at a multiplicity of infection (MOI) of 1 plaque forming unit per cell (PFU/cell) 1 hour prior to treatment with medium containing no drug or 2.5 mM 2'AP. Cells and media were harvested at 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined using plaques assays on HEK293 cells. Error bars correspond to +/-SD.

replication efficiency of AdΔE1bΔVA in HEK293 cells as well as the inability of the virus to produce plaques in these cells, AdΔE1bΔVA, and a stock of AdΔE1b for comparison purposes, were amplified in Hep3B cells. Furthermore, viral titers were determined using Hep3B cells and are expressed as "GFP-positive-cell forming units" per mL (GFU/mL). In order to determine whether the deletion of VA-RNA further attenuated virus production in normal cells, we generated a time-course of AdΔE1b and AdΔE1bΔVA growth in the panel of cell lines (Figure 3.3B). Cells were infected at an MOI of 1 GFU/cell and harvested at the indicated time points following infection. In HepG2 and Hep3B cells, the yields and growth rates of both viruses were similar, showing that the VA-RNAs are dispensable for the growth of AdΔE1b in these cells. In contrast, AdΔE1bΔVA production was attenuated compared to AdΔE1b production in the normal cells, MRC5 and WI-38. As noted previously, AdΔE1bΔVA production was highly attenuated in the E1-positive HEK293 cells. These results show that similar to Ad-dl309ΔVA, AdΔE1bΔVA replication is selective for HCC cells relative to normal fibroblasts.

We next investigated whether 2'AP treatment of cells could compensate for the lack of both E1b and the VA RNAs during virus production (Figure 3.3B). The production of both AdΔE1b and AdΔE1bΔVA were increased following 2'AP treatment in HepG2, Hep3B, MRC5 and WI-38 cell lines, but not E1b-positive HEK293 cells. Interestingly, we also found that the treatment of MRC5 and WI-38 with 2'AP did not increase production of AdΔE1bΔVA to the same level as that of AdΔE1b, suggesting that 2'AP compensated for the activities lost

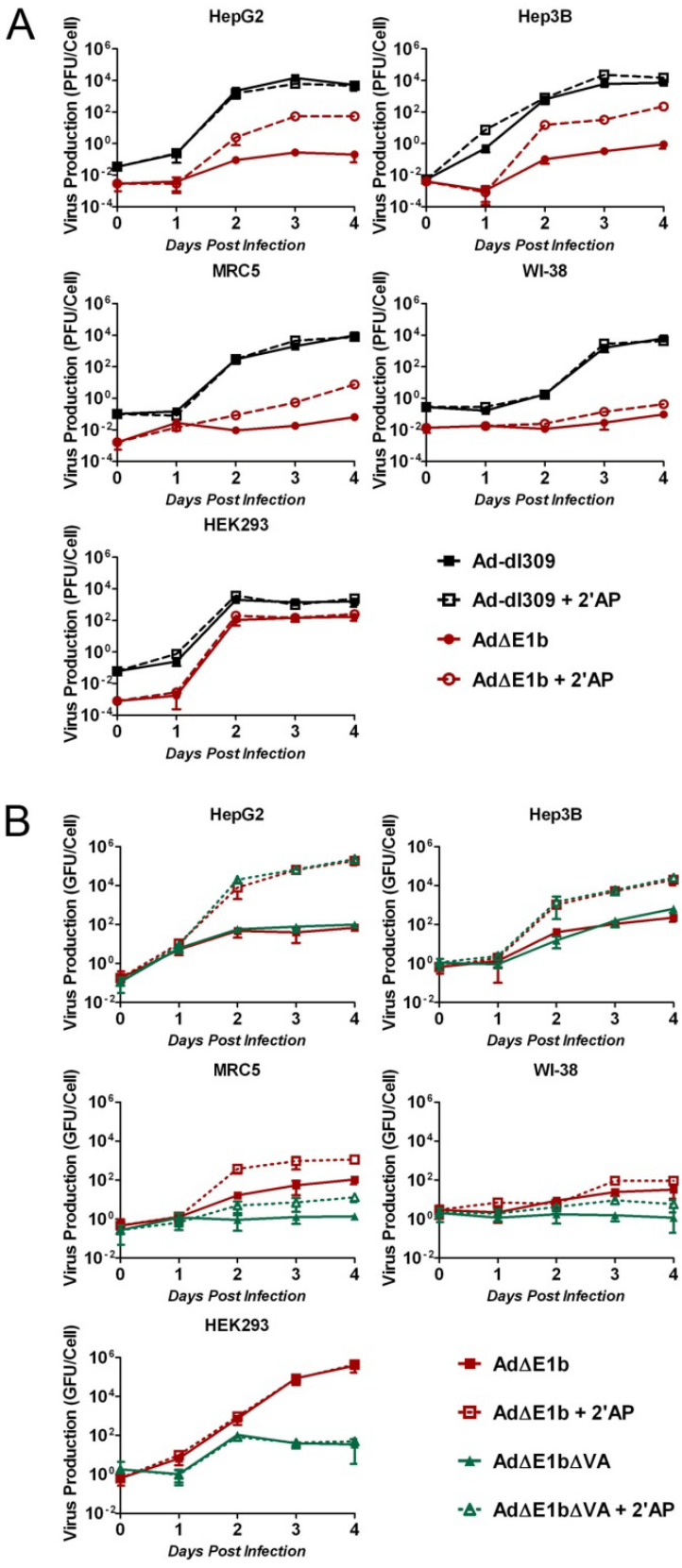
A
HepG2
Hep3B
MRC5
WI-38
HEK293
Virus Production (PFU/Cell)
Days Post Infection
Ad-dl309
Ad-dl309 + 2'AP
AdΔE1b
AdΔE1b + 2'AP
B
Virus Production (GFU/Cell)
AdΔE1b
AdΔE1b + 2'AP
AdΔE1bΔVA
AdΔE1bΔVA + 2'AP

Figure 3.3: 2'AP increases the replication of adenoviruses with an E1b deletion or with both E1b and VA-RNA deletions.

(A) Cells were infected in duplicate with Ad-dl309 and AdΔE1b at an MOI of 1 PFU/cell 1 hour prior to treatment with medium containing no drug or 2.5 mM 2'AP. Cells and media were harvested at 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined using plaques assays on HEK293 cells. (B) Cells were infected with AdΔE1b and AdΔE1bΔVA at an MOI of 1 GFU/cell 1 hour prior to addition of medium containing no drug or 2.5 mM 2'AP. Cells and media were harvested at 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titration in Hep3B cells. Error bars correspond to +/-SD.

by the E1b deletion rather than by the VA-RNA deletion in normal cells. Consistent with this hypothesis, 2'AP treatment did not have a significant effect on AdΔE1bΔVA production in HEK293 cells that supply E1b activities in *trans*.

To examine the reported ability of 2'AP to reduce adenovirus release from infected cells (241), virus concentrations were measured from both the cells and media (total virus produced) as well as only the media (released virus) in HepG2 and MRC5 cells (Figure 3.4). In agreement with data shown in Figure 3.3B, we found little difference between the replication properties of AdΔE1b and AdΔE1bΔVA in HepG2 cells at 4 days post-infection, whereas there was a significant difference between the two viruses in MRC5. Furthermore, 2'AP treatment of HepG2 increased production of both viruses by approximately 4 orders of magnitude. Production of both AdΔE1b and AdΔE1bΔVA was also increased in MRC5 cells treated with 2'AP, however, this increase (10-fold) was much lower than in HepG2. Similar to a previous report (241), while most of the infectious virus particles were released when no drug was added, only approximately 10 % of the virus was released from the HepG2 cells treated with 2'AP. Even though 90 % of the virus was retained in cells treated with 2'AP, more virus was released following drug treatment than produced in untreated cells.

3.4.3 2'AP treatment increased virally encoded protein expression in HepG2 cells infected with the E1b-fully-deleted viruses

Previous studies have shown that 2'AP can increase the translation of exogenous genes (474; 475), among its many other activities. Therefore, we

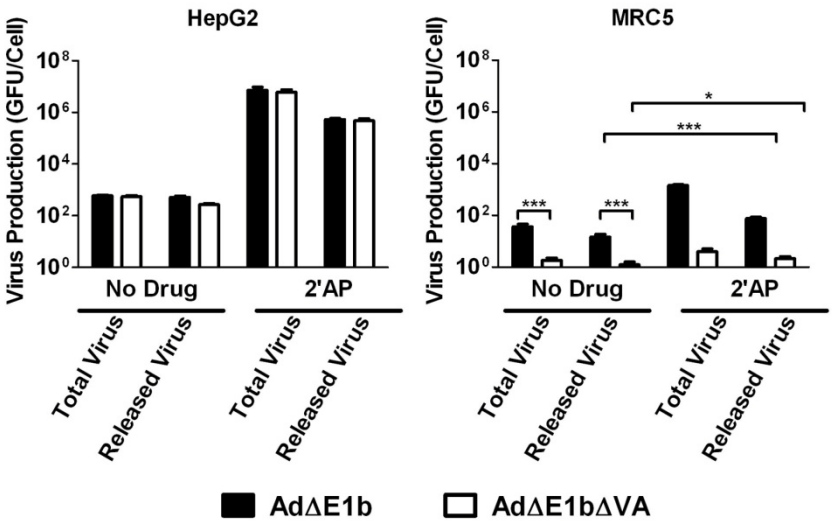

Figure 3.4: Treatment of HepG2 and MRC5 cells with 2.5 mM 2'AP inhibited AdΔE1b and AdΔE1bΔVA release.

Cells were infected with either AdΔE1b or AdΔE1bΔVA at an MOI of 1 GFU/cell for 1 hour prior to treatment with medium containing no drug or 2.5 mM 2'AP. Four days following infection, infected cells combined with the media (total virus) or the media alone (released virus) were harvested. Virus yields were determined by titration in Hep3B cells. Error bars correspond to +/-SD of quadruplicates (*$p<0.05$, ***$p<0.001$, one-way ANOVA).

examined E1a, fiber, and GFP expression in virus infected cells with and without 2'AP treatment. The objective was to assess whether an increase in E1a expression corresponded to expression of other virally encoded genes, as well as to virus production.

HepG2 and MRC5 cells were infected with AdΔE1b or AdΔE1bΔVA at an MOI of 100 virus particles (VP) per cell for 2 or 4 days, with or without treatment with 2'AP. The E1b- and VA-RNA-positive Ad-dl309 was used as a positive control, while Ad-dl309ΔVA and Ad-dl1520 were used as VA-RNA-deleted and E1b-55K-deleted controls, respectively. AdControl (non-replicating) as well as an uninfected sample were used as negative controls.

Two days after infection of untreated HepG2 cells with the E1b-fully-deleted viruses, E1a expression (under the control of the mCMV promoter) was similar to that in infections with Ad-dl309, Ad-dl309ΔVA and Ad-dl1520 viruses (under the control of the viral E1a promoter) (Figure 3.5A, left panel). By 4 days, much higher E1a expression levels were detected in HepG2 cells infected with the E1b-deleted viruses than in cells infected with the other replicating viruses (Figure 3.5A, right panel). This increase may be due to the loss of E1a autoregulation (476), as a consequence of replacement of the E1a promoter by the mCMV promoter, and/or due to activation of the mCMV promoter by E1a (477-479).

Treatment with 2'AP increased E1a levels in HepG2 cells infected with the E1b-deleted viruses at both 2 and 4 days post-infection. There could be a number of factors contributing to this observation. 2'AP may have increased E1a

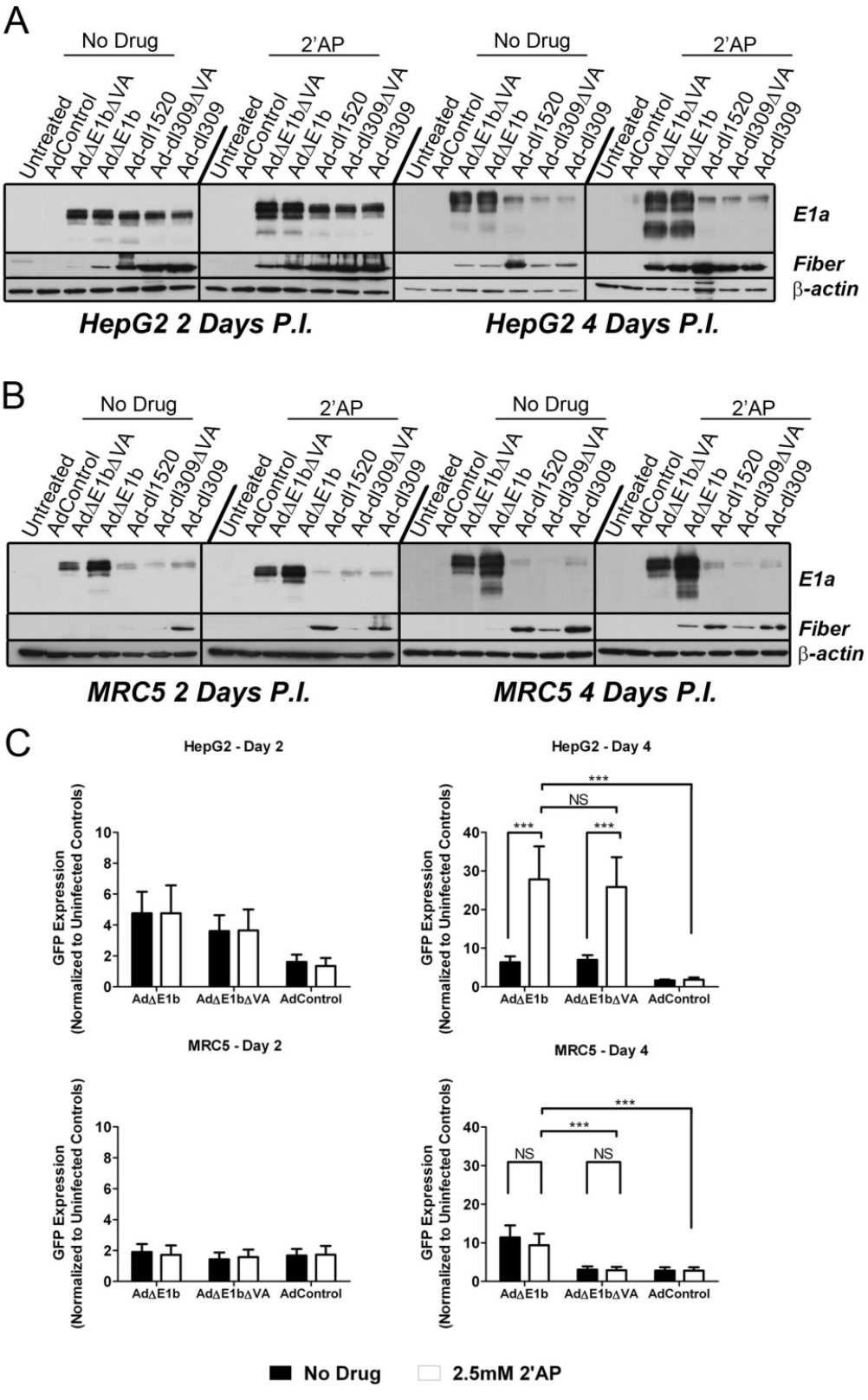
A
No Drug
2'AP
No Drug
2'AP
Untreated
AdControl
AdΔE1bΔVA
AdΔE1b
Ad-dl1520
Ad-dl309ΔVA
Ad-dl309
E1a
Fiber
β-actin
HepG2 2 Days P.I.
HepG2 4 Days P.I.
B
MRC5 2 Days P.I.
MRC5 4 Days P.I.
C
HepG2 - Day 2
HepG2 - Day 4
MRC5 - Day 2
MRC5 - Day 4
GFP Expression
(Normalized to Uninfected Controls)
AdΔE1b
AdΔE1bΔVA
AdControl
***
NS
No Drug
2.5mM 2'AP

Figure 3.5: Treatment of HepG2 cells with 2'AP increased expression of virally encoded E1a, fiber and GFP.

(A) HepG2 or (B) MRC5 cells were mock infected or infected with the indicated viruses at an MOI of 100 VP/cell and then incubated for 2 days or 4 days, with or without 2.5 mM 2'AP treatment. Cells were washed and lysed and western blot analysis was performed on 10 μg protein per lane using antibodies against E1a, fiber and β-actin. (C) HepG2 and MRC5 cells were infected with AdΔE1b, AdΔE1bΔVA or AdControl at an MOI of 100 VP/cell and then incubated in the absence or presence of 2.5 mM 2'AP. Green fluorescence intensity was measured 2 and 4 days post-infection and normalized to uninfected controls. Error bars correspond to +/-SD of quadruplicates (NS – Not Significant; ***$p<0.001$, one-way ANOVA).

levels either directly through modifying the activity of the mCMV promoter controlling E1A expression in the E1b-deleted viruses, or indirectly through increasing virus replication thus increasing template copy number. In addition, 2'AP may have mediated increased translation (474; 475) of E1a.

In contrast to HepG2 cells, E1a levels in untreated MCR5 cells infected with the E1b-fully-deleted viruses were higher even at 2 days post-infection compared to cells infected with the other replicating viruses, suggesting that mCMV promoter is much more active than the E1a promoter in MRC5 cells (Figure 3.5B). Interestingly, 2'AP had little effect on mCMV promoter activity in MRC5 cells. Furthermore, E1a levels in MRC5 cells infected with AdΔE1bΔVA were lower than in cells infected with AdΔE1b suggesting that the loss of VA-RNA genes reduced virus activity in these cells.

We also examined whether the expression of fiber, a late viral protein, corresponded to either E1a expression levels or to virus production in infected HepG2 cells. The absolute levels of E1a clearly did not correlate with fiber levels (Figure 3.5A). Nor did virus production correlate with absolute fiber levels, as all of the viruses except Ad-dl1520 had similar levels of fiber at day 4 in the absence of 2'AP, but replicated to very different levels at this time. However, if one considers relative changes induced by 2'AP, the increase in E1a expression mediated by 2'AP roughly correlated with an increase in fiber expression in infections of HepG2 cells with all of the replicating viruses (Figure 3.5A). With respect to virus production, however, the 2'AP-mediated increase in fiber levels

in these cells only corresponded to increased growth of Ad-dl1520 and the E1b-fully-deleted viruses, and not Ad-dl309 or Ad-dl309ΔVA.

Similar to our observations with HepG2 cells, absolute E1a and fiber levels did not correlate in normal MRC5 cells (Figure 3.5B). It is notable that less fiber was produced in MRC5 cells infected with the E1b-fully-deleted viruses than with the other replicating viruses, paralleling virus production in these cells. Furthermore, increased fiber levels mediated by 2'AP treatment corresponded to increased virus production following treatment. Therefore, in contrast to HepG2 cells, fiber expression correlated closely with virus production in MRC5 cells.

To evaluate the activity of another virally encoded gene under these conditions, GFP expression (under the control of the hCMV promoter) was measured in HepG2 and MRC5 cells 2 and 4 days after infection with the E1b-fully-deleted viruses or the non-replicating AdControl (Figure 3.5C). As expected, GFP expression was low in AdControl infections of HepG2 cells at both time points, since this virus is non-replicating. Interestingly, we did not detect a significant difference in GFP expression between 2'AP-treated and untreated HepG2 cells infected with AdControl, suggesting that 2'AP did not activate the hCMV promoter. In contrast, GFP expression 4 days after infection of HepG2 cells with the E1b-deleted viruses was significantly increased by 2'AP treatment, similar to the results of E1a analysis. Taken together, these results suggest that the 2'AP-mediated increase in GFP expression may be due to increased viral DNA template rather than to increased hCMV promoter activity.

Similar to results of E1a analysis, 2'AP treatment of infected MRC5 cells

did not have a significant effect on GFP expression. Also similar to E1a results, at 4 days post-infection, GFP expression was significantly lower in MRC5 cells infected with AdΔE1bΔVA compared to AdΔE1b. Therefore, in contrast to results with HepG2 cells, we have found that 2'AP was unable to increase virus production or the expression of virally encoded E1a, GFP or fiber in AdΔE1bΔVA-infected normal MRC5 cells to the same levels as in AdΔE1b-infected cells. This could have important implications for future therapies, as a drug similar to 2'AP might enhance activity of AdΔE1bΔVA in HCC cells relative to normal cells.

3.4.4 2'AP specifically increased virus-mediated HCC cell death

The ability of 2'AP treatment to increase virus-mediated cell death was evaluated using the HCC cell lines, Hep3B and HepG2, and the normal cell lines, MRC5 and WI-38 (Figure 3.6). In the absence of 2'AP, HepG2 and Hep3B cells were found to be sensitive to Ad-dl309- and Ad-dl309ΔVA-mediated cell killing at an MOI of 10 VP/cell. Furthermore, unlike HepG2 cells, Hep3B cells were also found to be sensitive to killing by the E1b-deleted viruses, although at a higher MOI. 2'AP treatment increased killing of both HepG2 and Hep3B cells by all of the viruses except Ad-dl1520. Interestingly, even in the presence of 2'AP, the normal cell lines were resistant to killing by all the replicating viruses, suggesting that the 2'AP increase in virus-mediated HCC cell death could be dependent on pathways specifically deregulated in HCC cells.

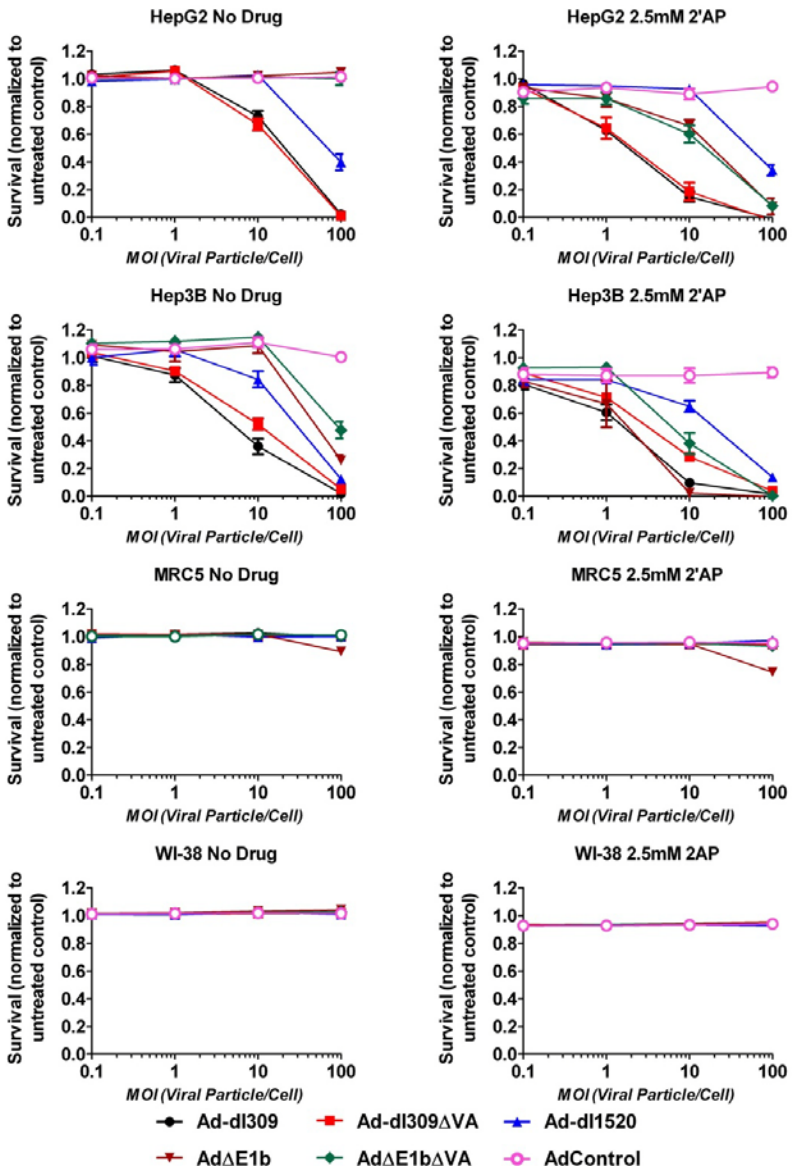

Figure 3.6: 2'AP increased virus-mediated death of HepG2 and Hep3B HCC cells but not normal fibroblasts.

HepG2 and Hep3B HCC cells as well as MRC5 and WI-38 normal cells were infected at the indicated MOIs and then incubated with or without 2.5 mM 2'AP. Six days post-infection, cell survival was measured by Alamar Blue fluorescence measurements and normalized to uninfected controls. Error bars correspond to +/- SD of quadruplicates.

3.4.5 2'AP-mediated virus replication and cell death was independent of p53 levels or activity

One of the main functions of E1b-55K in an infected cell is to block p53 pathways, either by inhibiting p53 activity or decreasing its stability (250; 444; 445). Here we examined the potential influence of p53 on replication of E1b-deleted virus in p53-positive HepG2 (362). In HepG2 cells, p53 was only detectable in infections with AdΔE1b (Figure 3.7A). This is consistent with E1a-mediated stabilization of p53 (190; 480-482). However, we did not detect p53 in Ad-dl1520-infected HepG2 cells which had levels of E1a comparable to AdΔE1b infected cells at this time point. As Ad-dl1520 has previously been shown to induce or stabilize p53 levels (360; 483; 484), it is not immediately apparent why this virus did not enhance p53 protein levels in our system.

It has been reported (456) that 2'AP, like E1b-55K, inhibits p53 stabilization. Therefore, we examined whether the increase in E1b-deleted virus replication in response to 2'AP could be a result of alterations in p53 levels or activity. Contrary to our expectations, 2'AP treatment did not reduce p53 levels in E1b-deleted virus infected HepG2 cells (Figure 3.7A).

To further investigate the potential ability of 2'AP to alter p53 activity in this system, HepG2 cells were transfected with a p53-responsive reporter vector one day prior to infection with Ad-dl309, Ad-dl1520 or AdΔE1b (Figure 3.7B), then assayed for reporter gene expression 2 days later. Hep3B cells, used as a p53-negative control (Figure 3.7A and (363)), were unable to activate the p53-responsive reporter, demonstrating the specificity of this assay. In agreement with

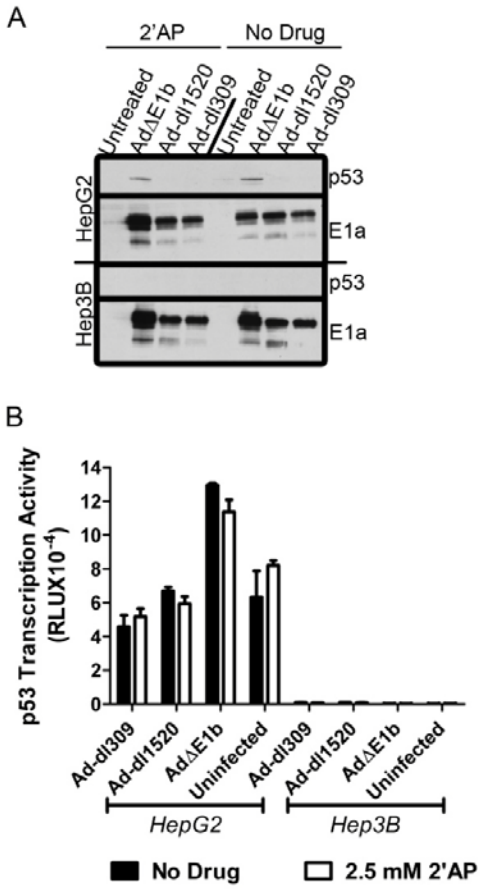

Figure 3.7: p53 expression and activity were not inhibited by 2'AP treatment of infected cells.

(A) HepG2 and Hep3B cells were infected with Ad-dl309, Ad-dl1520 or AdΔE1b at an MOI of 100 VP/cell and treated with no drug or 2.5 mM 2'AP for 2 days. p53 and E1a levels were detected by western blot analysis. (B) HepG2 and Hep3B cells were transfected with a p53-responsive firefly luciferase expression vector. The next day, transfected cells were mock infected or infected with Ad-dl309, Ad-dl1520 or AdΔE1b at an MOI of 100 VP/cell and treated with no drug or 2.5 mM 2'AP. Luciferase expression was measured 2 days post-infection. Both HepG2 and Hep3B have high transfection efficiencies. Error bars correspond to +/-SD of triplicates.

our analysis of p53 protein levels (Figure 3.7A), p53 transcriptional activity was higher in HepG2 cells infected with AdΔE1b than it was in infections with Ad-dl309 or Ad-dl1520. Furthermore, 2'AP treatment did not reduce the transcriptional activity of p53 in HepG2 cells infected with AdΔE1b. Taken together, these data suggest that the 2'AP-mediated increase in virus production was independent of p53 inhibition.

3.4.6 2'AP specifically rescued C454S/C456S substitution of E1b-55K in HepG2

Both 2'AP and E1b-55K have been shown to block the DNA damage response (212; 213; 257; 258). Furthermore, previous studies have found that one of the E1b-55K domains responsible for this inhibition includes the residues C454 and C456 (231; 232). Therefore, we tested whether inhibition of the DNA damage response could potentially be involved in E1b-deleted virus production in HepG2 cells treated with 2'AP. HepG2 cells were mock transfected or transfected with vectors encoding either wild-type E1b-55K or a mutated E1b-55K (C454S/C456S) one day prior to infection with AdΔE1b. Infected cells were then cultured with or without 2'AP treatment for 4 days prior to virus production determination (Figure 3.8). Similar to our previous results (Figures 2.21 and 2.24), we found that AdΔE1b production was increased in HepG2 cells transiently expressing wild-type E1b-55K. Furthermore, while transient expression of mutant E1b-55K was able to inhibit p53 activity in AdΔE1b-infected HepG2 cells (Figure 2.23), it was unable to increase AdΔE1b production in these cells. Interestingly, while 2'AP treatment strongly increased AdΔE1b production in HepG2 cells transiently expressing mutant E1b-55K, the same treatment had no effect on the

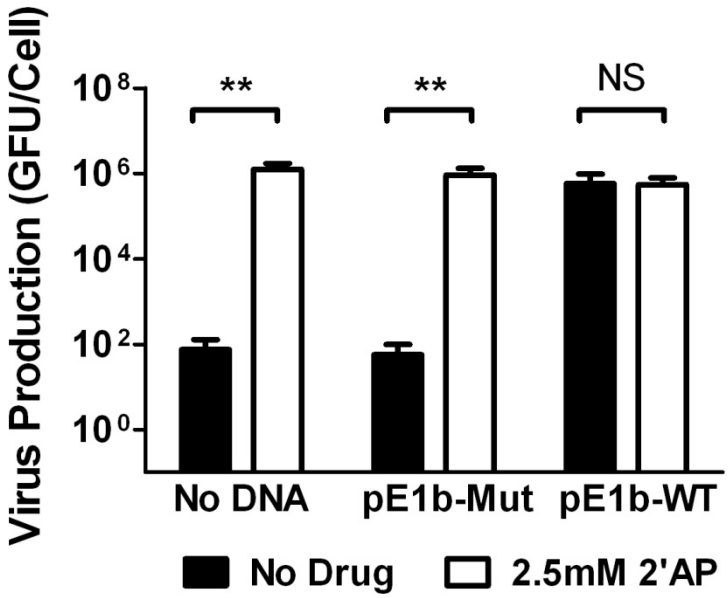

Figure 3.8: 2'AP had no effect on AdΔE1b production in HepG2 cells transiently expressing wildtype E1b-55K.

HepG2 cells were mock transfected or transfected with 1 µg of either a wild-type E1b-55K expression vector or a mutated E1b-55K vector unable to inhibit Mre11 (C454S/C456S). The next day, transfected cells were infected with AdΔE1b at an MOI of 1 GFU per cell and incubated with fresh medium containing no drug or 2.5 mM 2'AP. Four days following infection, cells were harvested and virus yields were determined by titration in Hep3B cells. Error bars correspond to +/- SD of quadruplicates (NS – Not Significant; ** $p<0.01$, t-test).

already high level of AdΔE1b production in HepG2 cells transiently transfected with wild-type E1b-55K. These results suggest that 2'AP treatment might have similar targets as the E1b-55K residues C454 and C456.

To further evaluate the role of these E1b-55K residues in virus replication, we stably transfected HepG2 cells with either wild-type or mutant E1b-55K vectors. The stably transfected HepG2 cells (HepG2-E1b-WT and HepG2-E1b-Mut) as well as the parental HepG2 cells were infected with Ad-dl309, Ad-dl1520 or AdΔE1b then cultured with or without 2'AP treatment for 4 days prior to virus production measurement (Figure 3.9A). While 2'AP had no effect on Ad-dl309 production in any of these cell lines, 2'AP strongly increased production of both Ad-dl1520 and AdΔE1b in parental HepG2 cells as well as HepG2-E1b-Mut cells. As expected, HepG2-E1b-WT at least partially complemented the E1b deletions in Ad-dl1520 and AdΔE1b allowing relatively high virus production, and 2'AP treatment did not further increase yields substantially. Interestingly, AdΔE1b production levels were much lower than Ad-dl1520 even in the complementing HepG2-E1b-WT cells, suggesting that other differences between Ad-dl1520 and AdΔE1b, such as E1b-19K and the E3 death protein encoded by Ad-dl1520, or mCMV-promoter-control of E1a in AdΔE1b, may also contributed to lower AdΔE1b production.

We also generated a time-course of AdΔE1b production with or without 2'AP in the stably transfected cells as well as the parental HepG2 cells (Figure 3.9B). Similar to the results in Figures 3.8 and 3.9A, we found that AdΔE1b production was dramatically increased by 2'AP treatment of the parental HepG2.

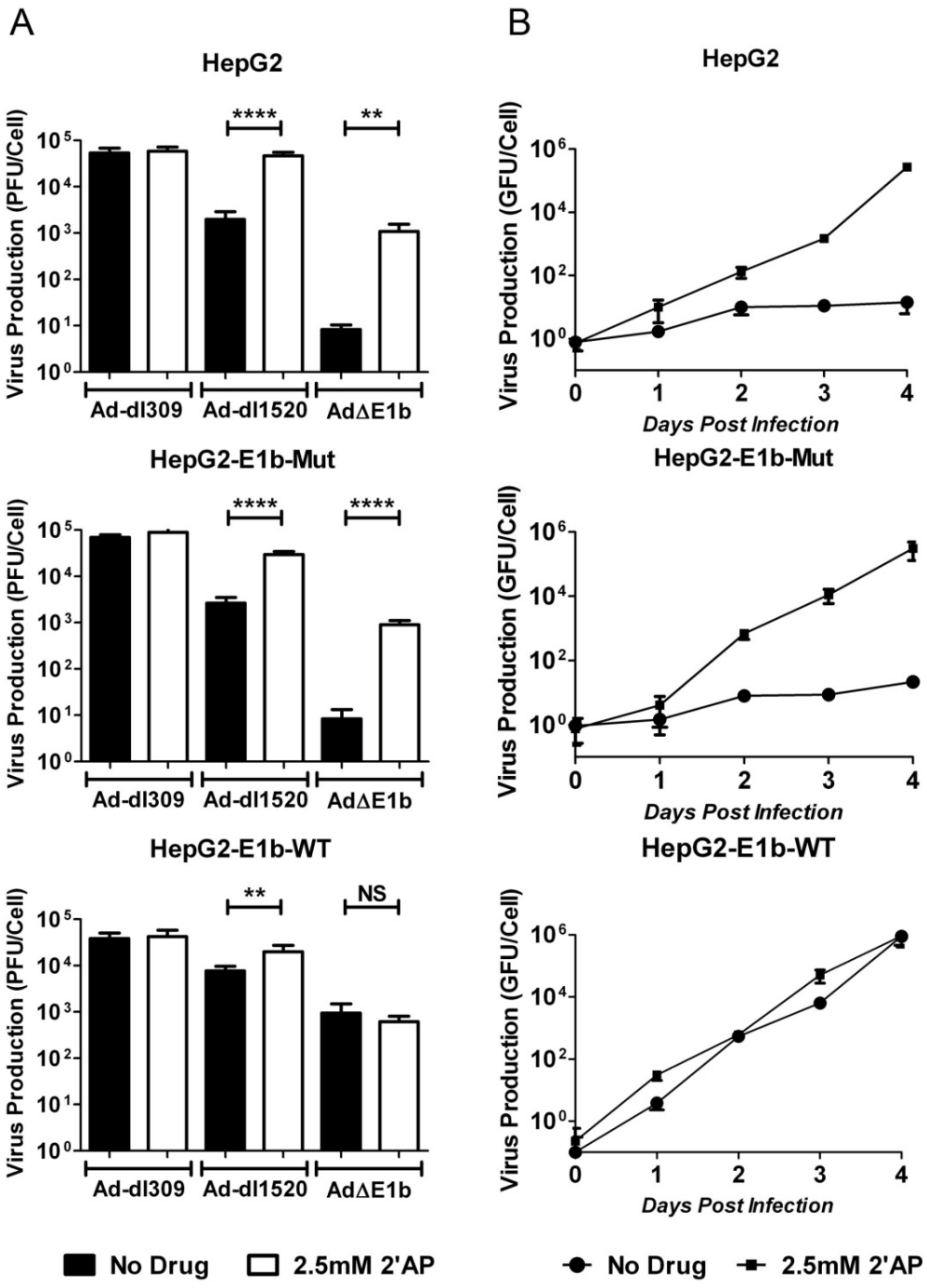
A
B
HepG2
HepG2
Virus Production (PFU/Cell)
Virus Production (GFU/Cell)
****
**
Ad-dl309
Ad-dl1520
AdΔE1b
Days Post Infection
HepG2-E1b-Mut
HepG2-E1b-Mut
****
****
HepG2-E1b-WT
HepG2-E1b-WT
**
NS
No Drug
2.5mM 2'AP

Figure 3.9: 2'AP rescued the C454S/C456S E1b-55K mutation in HepG2 cells.

(A) Parental HepG2, HepG2-E1b-WT and HepG2-E1b-Mut were infected with Ad-dl309, Ad-dl1520 or AdΔE1b at an MOI of 1 PFU/cell for 1 hour prior to treatment with medium containing no drug or 2.5 mM 2'AP. Infected cells and media were harvested 4 days post-infection and virus yields were determine by plaque assays on HEK293 cells. (B) Cells were infected with AdΔE1b at an MOI of 1 GFU/cell for 1 hour prior to treatment with medium containing no drug or 2.5 mM 2'AP. Lysates from infected cells were harvested at 1 hr (day 0) as well as 1, 2, 3 and 4 days post-infection. Virus yields were determined by titration in Hep3B cells as described in the materials and methods. Error bars correspond to +/-SD of quadruplicates (NS – Not Significant; **** $p<0.0001$, ** $p<0.01$, one-way ANOVA).

as well as the HepG2-E1b-Mut cell line. Also, the level of AdΔE1b production in untreated HepG2-E1b-WT was similar to the production levels in 2'AP-treated parental HepG2 and HepG2-E1b-Mut, strongly suggesting that 2'AP can directly compensate for the activities mediated by C454 and C456 residues in E1b-55K.

3.4.7 2'AP enhancement of virus-mediated cell death was independent of E1b-55K expression

Since 2'AP treatment and E1b-55K expression increased E1b-deleted virus production in a complementary manner, we investigated whether complementation would also be observed with virus-mediated cell death. HepG2-E1b-WT, HepG2-E1b-Mut and parental HepG2 cells were infected with the replicating viruses and incubated 6 days with or without 2.5 mM 2'AP prior to survival assessment (Figure 3.10). In the absence of 2'AP, efficient killing by virus at an MOI of 100 VP/cell (about 1 infectious unit/cell) required an intact E1b-55K protein either encoded by the virus (e.g., Ad-dl309 in Figure 3.10A) or provided in *trans* by the cells (e.g., Figure 3.10C). An activity of E1b-55K that is dependent on cysteine residues at 454 and/or 456 appeared to be critical for virus-mediated cell death, as the E1b-55K mutant protein did not complement the E1b-deleted viruses (Figure 3.10B). Furthermore, similar to its effect on virus production (Figure 3.6), 2'AP treatment could substitute for E1b-55K in killing by AdΔE1b and AdΔE1bΔVA (Figure 3.10 A, B). In contrast to AdΔE1b, Ad-dl1520 did not induce greater killing of these cells when combined with 2'AP treatment, which may be due to lower E1a levels in 2'AP-treated cells infected with Ad-dl1520 compared to AdΔE1b. At low virus concentrations (10 VP/cell; ~

0.1 infectious units per cell), a much smaller population of cells would be initially infected, resulting in a more stringent test for virus-mediated killing of HepG2-E1b-WT cells (Figure 3.10D). Surprisingly, we found that 2'AP treatment increased killing by all the viruses except Ad-dl1520, suggesting that this drug induced deregulation of other cellular pathways in addition to those affected by the E1b-55K protein.

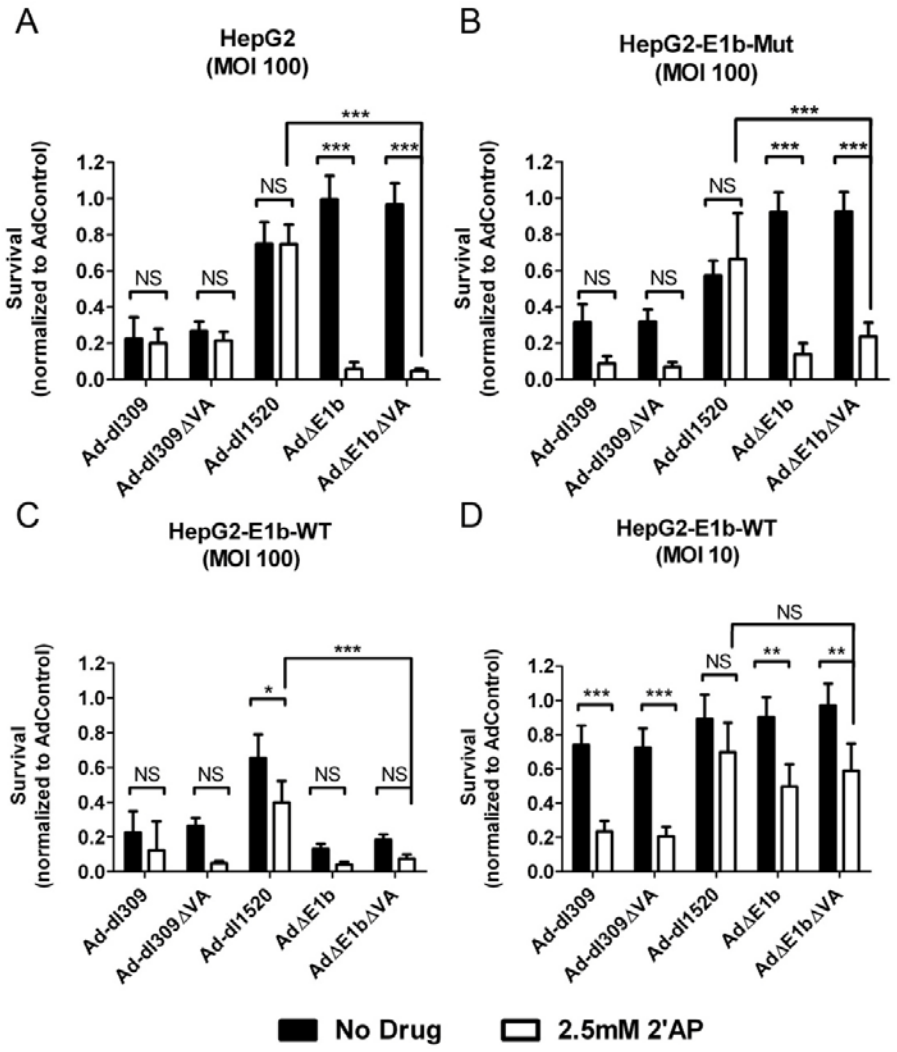

Figure 3.10: 2'AP treatment increased both Ad-dl309 and AdΔE1b mediated HepG2 cell death.

(A) Parental HepG2, (B) HepG2-E1b-Mut and (C, D) HepG2-E1b-WT cells were infected with indicated viruses at an MOI of 10 (D) or 100 (A, B, C) VP/cell and cultured in medium with or without 2.5 mM 2'AP. Survival was determined using an Alamar Blue assay 6 days post-infection. Fluorescence measurements were normalized to AdControl infected wells. Error bars correspond to +/-SD of quadruplicates (NS – Not Significant; *** $p<0.001$, ** $p<0.01$, * $p<0.05$, one-way ANOVA).

3.5 DISCUSSION

Oncolytic adenoviruses with a deletion of either E1b or VA-RNA genes have been extensively studied as potential cancer therapeutics. While these deletions increased the cancer-specificity of the replicating adenoviruses, these mutations often reduce their activity in both virus growth and cell lysis. Therefore, many studies have been performed to determine whether their activity could be increased by co-administration of chemotherapeutic drugs (270; 280; 359; 366; 402; 403; 458; 485). 2'AP has recently been shown to increase the activity of an oncolytic herpes virus with deletions in several genes, including ICP34.5 (486). Similar to E1b-55K and VA-RNAs, ICP34.5 was shown to target both the interferon response as well as the DNA damage response (487-490). Therefore, we sought to determine whether the deletion of both E1b and VA-RNA genes could increase the selectivity of the AdΔE1bΔVA for HCC cells, and whether 2'AP treatment could selectively compensate for the loss of E1b and/or VA-RNAs thus increasing the replicative and lytic activity of this virus.

To determine whether E1b-deleted virus replication was dependent on VA-RNA in HCC cells, we measured the growth rates of AdΔE1b and AdΔE1bΔVA in both normal and HCC cells. We found that the VA-RNA deletion strongly attenuated AdΔE1bΔVA production and lysis of normal cells, but not HCC cells. Our initial tests with 2'AP were designed to elucidate the mechanism of attenuation of the E1b-VA double-deleted virus. However, it was clear that 2'AP could not fully compensate for the combined deletion of VA-RNA and E1b-55K in normal cells although it greatly enhanced replication in HCC cell

lines. The observation of differential enhancement in normal and HCC cells could have important implications for modulating tumor selectivity of the viruses studies here.

Our studies with Ad-dl1520 and AdΔE1b suggest that 2'AP complementation was more closely linked to the E1b deletion than to the VA-RNA deletion. Therefore, we investigated several reported activities of E1b-55K as potential candidates for the activity compensated by 2'AP in our infections. An important role of E1b-55K is blocking host protein synthesis (398; 491). However, 2'AP is reported to inhibit the virus-mediated block in host protein synthesis (241), suggesting that 2'AP counteracts, rather than complements this E1b-55K activity. Furthermore, the 2'AP effect on protein synthesis resulted in reduced adenovirus release (241). Consistent with this, we show here approximately a 10-fold reduction in virus release following 2'AP treatment of HepG2 and MRC5 cells.

Another role of E1b-55K is the promotion of late viral mRNA export (360; 492), which follows viral DNA replication. Consistent with this activity of E1b-55K, in MRC5 cells, increased fiber expression closely correlated to increased virus production. In contrast to MRC5 cells, 2'AP increased fiber expression in HepG2 cells infected with all the replicating viruses, and therefore, increased fiber expression did not correlate with increased virus production in these cells.

E1b-55K was reported to inhibit activation of the host DNA damage response mediated by newly synthesized viral genomes, thereby preventing

genome concatemerization (213). In order to determine whether adenovirus production in HepG2 cells might dependent on the inhibition of the DNA damage response, we measured the growth properties of AdΔE1b in HepG2 cells expressing either wild-type E1b-55K or mutant E1b-55K (C454S/C456S) that is unable to degrade DNA ligase IV and Mre11 (231; 232). In contrast to wild-type E1b-55K, the mutant E1b-55K was unable to significantly increase AdΔE1b or Ad-dl1520 production. Furthermore, our data suggest that 2'AP compensated for the activity mediated by the C454 and C456 residues of E1b-55K. This activity was unlikely to involve p53 inhibition, since the E1b-55K mutant retains the ability to inhibit p53 (231; 232). Consistent with this, we found that 2'AP treatment did not affect p53 levels or activity in AdΔE1b infected HepG2 cells, indicating that 2'AP compensation is not through p53 inhibition.

Although much of our data supports a mechanism for 2'AP enhancement of virus production and cell killing that recapitulates an E1b-55K activity, it is important to note that 2'AP had a stronger effect in infections with the E1b-fully-deleted viruses than with Ad-dl1520, suggesting that 2'AP activity may be more complex than simply compensating for E1b-55K.

In this report, we demonstrate that 2'AP could affect multiple pathways important for completing the adenovirus lifecycle. Furthermore, we show that 2'AP could not increase the replication of AdΔE1bΔVA in normal MRC5 cells to the same level as AdΔE1b. Therefore, although E1b-55K and VA-RNAs may target similar pathways, the VA-RNAs also target pathways that are not complemented by either E1b-55K or 2'AP in normal cells. Further understanding

of how 2'AP preferentially increases AdΔE1bΔVA production as well as virus-mediated cancer cell death, could result in the design of new drugs that can activate attenuated adenoviruses only in cancer cells, thereby reducing toxicities associated with current oncolytic adenoviruses.

3.6 ACKNOWLEDGMENTS

We thank Thomas Dobner and Barbara Hartl (Heinrich-Pette Institute, Germany) for the wild-type and mutant E1b expression vectors, Matthias Dobbelstein (Göttingen University, Germany) and Philip Branton (McGill University, Canada) for selected viruses used in this study, and Frank Graham (McMaster University, Canada) for HEK293 cells and the E1 plasmid. We also thank Kate Agopsowicz for technical assistance.

Chapter 4 - Discussion, Conclusion and Future Directions

Hepatocellular carcinoma is the fifth most common cancer and third leading cause of cancer-related deaths (493). While many risk factors have been identified for this cancer, such as chronic HBV infection (35-37), current therapies have been associated with low response, especially in patients with low liver function (63; 64). Therefore, identification of novel targeted HCC therapies is an active research area. A novel therapy that has been extensively studied for many cancers, including HCC, is adenoviral therapy. The use of adenoviruses as potential cancer therapeutics was originally studied in the 1950's, when clinical studies using replicating Ads of several human serotypes were tested for therapy of various cancer types (302; 303). While these studies have shown that Ad therapy was associated with low side-effects, the anti-tumour response was not complete.

Since these studies were performed, much knowledge has been gained on adenovirus infection and replication. As Ads infect many cell types, both proliferating and quiescent, adenovirus vectors, such as first-generation adenovirus (FGAd) vectors, have been investigated as delivery vehicles to transfer therapeutic genes to cancer cells (308-310). Most FGAd vectors are designed to contain a therapeutic gene in place of the E1 region, rendering them replication-deficient. Furthermore, as the E3 region is non-essential for virus replication *in vitro*, most of this region has also been deleted to increase the cloning capacity of the vector (304; 312).

While these vectors are thought to have a therapeutic potential for many cancer types, the ability of the virus to infect normal cells could potentially result

in high toxicity (146; 307). To reduce Ad vector-mediated toxicity, many targeting modalities have been employed. In some cases targeting is achieved at the transcriptional level by placing the therapeutic gene under the control of promoters from genes such as hTERT, which are highly active in cancer cells but not in normal cells (329-332). In addition to transcriptional targeting, post-transcriptional targeting has also been employed, in which sequences complementary to endogenous miRNAs downregulated in cancer cells, such as miR-122, have been inserted in the 3'UTR of the therapeutic gene (344; 345).

In addition to therapeutic genes, the viral E1a as well as the viral E1a and E1b genes have been re-introduced in the E1/E3-deleted Ad vector backbone in order to allow replication of these viruses (335; 337-339). Similar to transgenes encoded in FGAd vectors, the expression of these E1 genes has been targeted through transcriptional and post-transcriptional mechanisms to induce replication specifically in cancer cells in order to reduce toxicity. In addition, oncolytic Ads have been constructed by deleting viral genes, such as E1b-55K, which are essential for virus replication in normal but not cancer cells (353).

Since the discovery of Ads, several systems have been designed to allow relatively easy construction of modified viruses. The HEK293 cell line, which endogenously expresses the E1 gene products (313), is the most commonly used cell line for the amplification of these Ads. While HEK293 cells support the replication of many replicating and non-replicating adenoviruses, insertion of toxic genes into the Ad genome as well as deletion of essential viral genes outside the E1 region may attenuate virus replication in these cells. Therefore, either

modification of HEK293 cells or identification of other packaging cell lines is often necessary in order to amplify these modified Ads.

4.1 Use of Alternative Packaging Cells for the Amplification of Problematic Ads

4.1.1 Alternative Cell Lines for the Amplification of Ads Lacking Essential Viral Genes

In addition to FGAd-encoded toxic transgenes, Ads with deletions of essential genes outside of the E1 region, such as the VA-RNA genes, are attenuated in HEK293 packaging cells (262). As FGAd vectors can only replicate in E1 complementing cells, such as HEK293 cells, the amplification of VA-RNA-deleted FGAd vectors is extremely challenging. Several systems have recently been developed to amplify these vectors, for example through establishing HEK293 cells stably expressing VA1 (406). Since AdΔE1bΔVA was designed to replicate in cancer cells, we tested Hep3B cells as an alternative packaging cell line for the amplification of E1b/VA-RNA-deleted Ads. Similar to previous reports, we found that Hep3B cells supported the replication of the E1b-55K-deleted Ad, Ad-dl1520 (362; 363). Furthermore, we found that while Ad-dl309ΔVA was attenuated in both Hep3B and HEK293 cells, stronger attenuation was found in HEK293 cells.

Surprisingly, we found that, while the deletion of E1b-55K and a part of the E3 gene (Ad-dl1520) had no effect on virus production in Hep3B cells, full E1b-deletion combined with a larger E3 deletion (AdΔE1b) resulted in high attenuation. A previous study has demonstrated that deletion of the full E1b gene

had no effect on Ad production in Hep3B cells (389). However, in that study, the Ad encoded the full E3 region. Therefore, the deletion of the E3 gene in AdΔE1b may have contributed to the attenuation of AdΔE1b production when compared to Ad-dl309 in Hep3B cells. Nevertheless, the attenuation of AdΔE1b in Hep3B cells was not complete. Furthermore, while AdΔE1bΔVA production was highly attenuated in HEK293 cells, AdΔE1bΔVA production levels were similar to AdΔE1b in Hep3B cells. Therefore, Hep3B cells were used as an alternative for the amplification both ΔE1b viruses.

4.1.2 Infectious Unit Measurements of Attenuated Ads

The plaque assay, which is usually performed in HEK293 cells, is the most common method for the determination of infectious units of Ads (472). However, as replication of certain Ads was found to be attenuated in HEK293 cells, the ability of these Ads to produce plaques in HEK293 cells would likely be reduced. Therefore, the use of plaque forming units as a determination of the infectious units of Ad-dl309ΔVA might have underestimated the total infectious units in our studies.

An additional common method for determination of infectious units is the hexon expression assay, in which the number of hexon positive cells is counted to determine the infectious units applied to the monolayer. This assay relies on late virus gene expression following DNA replication in HEK293 cells, in contrast to the plaque assay, which relies on sequential rounds of virus-mediated cell lysis. However, the hexon expression assay was not used as an alternative for

measurement of VA-RNA-deleted Ads due to the lack of hexon expression in HEK293 cells infected with these viruses.

A previous study showed that the VA-RNA deletion in Ad-dl309ΔVA had no effect on Ad production in HepG2 cells when compared to Ad-dl309 (270). The authors measured infectious units through expression assay of the E2 DNA binding protein in HEK293 cells (270). Unlike hexon expression, the expression of the DNA binding protein is not dependent on viral DNA replication. Therefore, the DNA binding protein expression assay would be more reliable then hexon expression assay for the measurement of infectious units of attenuated Ads.

AdΔE1b and AdΔE1bΔVA encode GFP under the control of the hCMV immediate early promoter, and therefore, similar to the E2 gene products, GFP expression in infected cells is not dependent on virus replication. Therefore, in the experiments presented here, the infectious units of AdΔE1b and AdΔE1bΔVA were measured by GFP expression assay, in which serially diluted viruses were used to infect Hep3B cells and GFP-positive cells were counted three days later to determine the number of infectious units.

4.2 AdΔE1b-Mediated Cell Death Does Not Correlate With Replication

Previous studies have found that E1a gene transfer has therapeutic potential for many cancers due to apoptosis induction as well as inhibition of expression of members of the EGFR family (392; 393; 495-498). Furthermore, E1a was also found to sensitize cancer cells to a number of anti-cancer therapeutic drugs, such as TRAIL, which activates the extrinsic apoptosis pathway (336; 499; 500).

Due to the high anti-cancer potential of E1a, AdΔE1b was constructed with E1a under the control of the strong mCMV promoter. Furthermore, to reduce the anti-apoptotic properties of E1b and E3 gene products during virus replication, both regions were deleted. AdΔE1b infection was found to induce high cell death in the majority of the tested cell lines. However, AdΔE1b-mediated cell death was not correlated with replication in the tested cell lines, suggesting that the deletions in AdΔE1b as well as the high E1a expression resulted in premature cell death prior to completion of virus replication.

4.3 Hep3B Cells Are More Sensitive To AdΔE1b-Mediated Cell Death Than HepG2 Cells

Similar to previous Ad-dl1520 studies (362; 364), we found that both HepG2 and Hep3B cells supported the replication of AdΔE1b although killing of Hep3B cells was more extensive than that of HepG2 cells. Two important differences between the two cell lines are that Hep3B cells are p53-negative and HBV-positive, whereas HepG2 cells are p53-positive and HBV-negative (362-364; 378; 501). Our studies with HepG2 cells engineered to express HBV sequences suggest that the HBV genome was not responsible for the different observations in virus-mediated cell death. An alternative explanation for decreased killing of infected HepG2 cells relative to Hep3B cells is that p53 could have mediated activation of genes in HepG2 cells that can block cell death, such as p21 (364).

4.4 p53-Independent Roles of E1b-55K Increase AdΔE1b Production in HCC Cells

Interestingly, inhibition of p53 was unable to increase AdΔE1b production in HepG2 cells. Furthermore, in contrast to wild-type E1b-55K, the C454S/C456S E1b-55K mutant, which was able to block p53, was unable to increase AdΔE1b production. Therefore, E1b-55K activities modulated by the cysteine residues 454 and/or 456 were important for Ad replication in these cells. Previous studies have shown that these cysteine residues are important for the degradation of key enzymes in the DNA damage response, Mre11 and DNA ligase IV (231; 232), suggesting that loss of the E1b-55K ability to inhibit the host DNA damage response, rather than p53 inhibition, was a major cause of AdΔE1b attenuation in HepG2 cells.

In addition to HepG2 cells, MRC5 normal lung fibroblast cells were also found to support AdΔE1b replication. Furthermore, AdΔE1b induced higher MRC5 cell death than Ad-dl309. As the production levels of AdΔE1b were lower than Ad-dl309, AdΔE1b-mediated MRC5 cell death might have been due to the high levels of E1a in expression. Interestingly, similar to results found with HepG2 cells, transient expression of wild-type but not C454S/C456S mutant E1b-55K resulted in increased AdΔE1b production in MRC5 cells. In summary, compensation of E1b-55K functions, other than p53 inhibition, were required to increase AdΔE1b replication in both HepG2 and the normal MRC5 cells.

4.5 Increased Cancer Specificity of E1b-Deleted Ads

4.5.1 Increased Specificity Through Deletion of The VA-RNA Genes

Our studies showed that AdΔE1b production levels in the normal MRC5 cells and HepG2 cells were similar, suggesting that the combined deletions within AdΔE1b were not sufficient confer cancer selectivity. Since a major goal of this study was the development of a cancer-specific oncolytic agent, we tested whether further modifications of the viral genome would reduce the replication efficiency of the virus in normal cell without dramatically affecting the replication efficiency in cancer cells. A previous study has shown that the VA-RNA-deleted Ad-dl309ΔVA is attenuated in several normal cells, including MRC5 cells, but not in HepG2 cells (270). Also, while co-treatment with IFN further attenuated Ad-dl309ΔVA replication in normal cells, IFN had little effect on virus replication in HepG2 cells (270). Similarly, we found that the VA-RNA deletion in Ad-dl309ΔVA resulted in higher attenuation of Ad production in normal cells than in HCC cells. Recent studies have shown that several functions of the VA-RNAs and E1b-55K overlap. For example, E1b-55K and VA1 are important in the inhibition of the interferon response during virus replication partly through inhibition of PKR activation (251-253; 255; 262-268). Without inhibition of PKR during Ad replication, PKR could prevent virus and host protein synthesis initiation by phosphorylating eIF2α (256).

In addition to the inhibition of the interferon response, E1b-55K and VA1 are also important in the accumulation of late viral mRNA in the cytoplasm as well as the promotion of late viral protein synthesis (233-240). The expression of

E1b-55K and VA1 during virus replication was found to be important for the dephosphorylation of eIF4E, which results in inhibition of cap-dependent translation (398; 502; 503). While most host protein synthesis occurs through a cap-dependent mechanism, viral late protein synthesis occurs through ribosome shunting, similar to heatshock protein synthesis (504-507). Therefore, eIF4E dephosphorylation would allow for preferential synthesis of viral proteins late during infection.

Due to the similar functions of VA-RNAs and E1b-55K, we assessed whether the combined deletion of VA-RNA and E1b genes would further reduce the activity of Ad in the normal MRC5 cells. Reduced activity was determined by virus production and late virus protein expression as well as E1a expression and cell death.

Our studies have shown that MRC5 cells supported AdΔE1b production to a limited extent but not AdΔE1bΔVA. Furthermore, hexon and fiber expression were both low in MRC5 cells infected with AdΔE1bΔVA, consistent with attenuation of virus production. In MRC5 cells infected with AdΔE1b, however, hexon levels were equivalent to that in Ad-dl309 infection, whereas fiber expression was undetectable even by 4 days-post infection. Therefore, while AdΔE1b production was already more attenuated in MRC5 in comparison to that of Ad-dl1520 and Ad-dl309ΔVA, the deletion of the VA-RNA genes in AdΔE1bΔVA resulted in a further restriction of virus production in the normal cells.

In addition to the reduced attenuation of virus production and late protein expression, E1a expression was also found to be reduced in AdΔE1bΔVA-infected MRC5 cells compared to AdΔE1b-infected cells. This result might be due to reduction in viral DNA replication, and therefore, reduction in template DNA. Additionally, the loss of VA-RNAs in AdΔE1bΔVA might have resulted in reduced translational efficiency. Interestingly, the reduction of E1a expression in AdΔE1bΔVA-infected MRC5 cells in comparison to AdΔE1b corresponded with reduction in cell death. Therefore, the attenuation of virus replication as well as reduced cell death in our studies showed that the activity of AdΔE1bΔVA in normal cells was reduced in comparison to AdΔE1b. In contrast to MRC5 cells, the deletion of VA-RNA genes in AdΔE1bΔVA had little effect on virus replication and late protein expression as well as induction of cell death in HepG2 cells. Therefore, the deletion of VA-RNA genes in AdΔE1bΔVA resulted in a more specific oncolytic virus in comparison AdΔE1b.

4.5.2 Increased Specificity Through Insertion of MiR-122T Sites in the 3'UTR of E1a Gene

Several studies have shown that insertion of miRT sites could result in modified tropism of oncolytic Ads (345; 508). The liver-specific miR-122 was shown to be downregulated in many liver cancer cells and insertion of miR-122T sites in the 3'UTR of the E1a gene has been shown by many groups to reduce liver toxicity without affecting replication efficiency in HCC cells (123; 124; 344; 348; 349). Interestingly, we found that the insertion of either two or six miR-122T sites in the 3'UTR of a firefly luciferase gene resulted in similar reduction of

luciferase expression in the miR-122-positive HuH7.5 cells (approximately 10-fold reduction). Therefore, to further reduce AdΔE1bΔVA replication in normal hepatocytes, two miR-122T sites were inserted in the 3'UTR of the E1a gene. This insertion was found to highly inhibit E1a expression in HuH7.5 cells. As previous studies have shown that hepatocytes express much higher miR-122 levels than HuH7.5 cells (345), the tandem miR-122T insertion is predicted to reduce E1a expression in normal hepatocytes even more than in HuH7.5 cells.

Although VA-RNAs were previously reported to block the RNAi pathway (272-275), we found that miR-122 activity was retained during amplification of FGAd-infected 293-miR-122 cells, when VA-RNA would be highly expressed. Furthermore, miR-122T-modified E1a expression was downregulated to the same levels in miR-122-positive Hep3B cells infected with either AdΔE1b or AdΔE1bΔVA. Therefore, any effect of VA-RNA on the RNAi pathway was not sufficient to block miR-122 activity in Hep3B cells. While we have yet to determine whether VA-RNA expression has an effect on miR-122 activity in normal hepatocytes, this would not be a concern for the use of AdΔE1bΔVA as an oncolytic agent.

4.6 Loss of E3 Region In Addition To E1b-55K May Have Resulted In AdΔE1b Attenuation in HCC Cells

AdΔE1b and AdΔE1bΔVA carry additional genomic modifications relative to Ad-dl309 and Ad-dl1520 that may be responsible for the reduced virus production and cell death observed in the HCC cell lines. These modifications include deletion of E1b-19K and a larger deletion in the E3 region. Furthermore,

the E1a promoter in AdΔE1b and AdΔE1bΔVA was replaced with the mCMV immediate early promoter. Interestingly, a previous study reported that a fully E1b-deleted Ad encoding the full E3 region replicated as efficiently as wild-type Ad in both HepG2 and Hep3B cells (389). This is in contrast to our study showing reduced activity of the E3-deleted AdΔE1b compared to Ad-dl309 in these cells. Therefore, E3 expression might be important for the activity of E1b-deleted Ads. Interestingly, we found that transient and stable expression of E1b-55K dramatically increased AdΔE1b production in HepG2 cells, suggesting that E1b-19K-deleted Ad replication is not dependent on the E3 gene products to same extent as the replication of AdΔE1b in these cells.

4.7 2'AP Increased AdΔE1bΔVA Activity Without Affecting Specificity

4.7.1 2'AP Treatment Specifically Increased AdΔE1bΔVA Production in HCC Cells

Similar to VA1 and E1b-55K, 2'AP has been shown to inhibit PKR activation (281). Furthermore, 2'AP treatment was previously found to inhibit the expression of IFNβ, as well as several interferon-stimulated genes (467; 468). Similar to E1b-55K, 2'AP treatment was found to inhibit the DNA damage response through inhibition of ATM and ATR activation, as well as inhibition of p53 (454-456). Since both AdΔE1b and AdΔE1bΔVA were attenuated in the HCC cells in comparison to Ad-dl309, we assessed whether 2'AP could increase the replication of the ΔE1b Ads.

2'AP was found to increase AdΔE1b and AdΔE1bΔVA production in HepG2. Interestingly, while 2'AP treatment increased the Ad-dl1520 production

level in HepG2 cells, the treatment had no effect on Ad-dl309ΔVA production. Therefore, the 2'AP-mediated increase in AdΔE1bΔVA production was through compensating the loss of E1b-55K rather than the VA-RNAs. Furthermore, since Ad replication in MRC5 cells is dependent on VA-RNA expression, 2'AP treatment increased AdΔE1bΔVA production without affecting HCC specificity.

4.7.2 2'AP Specifically Increased Ad-mediated HCC Cell Death

In addition to increased production, 2'AP treatment resulted in increased E1a expression in HepG2 cells infected with AdΔE1b and AdΔE1bΔVA. Furthermore, this increase correlated with increased virus-mediated HepG2 cell death. In contrast, 2'AP treatment was not found to strongly affect E1a expression or cell death of MRC5 cells infected with AdΔE1b or AdΔE1bΔVA.

Interestingly, 2'AP treatment was also found to increase Ad-dl309-mediated HepG2 cell death. Unlike 2'AP's effect on AdΔE1b-infected HepG2 cells, 2'AP treatment did not result in increase E1a expression in Ad-dl309-infected cells. Furthermore, since Ad-dl309 production was unaffected by 2'AP treatment, this drug likely affects AdΔE1b- and Ad-dl309-mediated cell death through different mechanisms. Although Ad-dl309 is missing a small part of the E3 region (509), it is unlikely that 2'AP was compensating for the loss of this region, since previous studies have shown that transient expression of wild-type E3 region reduced Ad-dl309-mediated cell death (301). Surprisingly, 2'AP treatment had no effect on Ad-dl1520-mediated HepG2 cell death. Moreover, Ad-dl1520-mediated cell death was not substantially increased in E1b-55K-positive HepG2 cells regardless of whether 2'AP was added. Therefore, in addition to

E1b-55K expression, other mechanisms might be affected in HepG2 cells infected with Ad-dl1520 compared to Ad-dl309. Previous studies have shown that additional E1b transcripts, which code for smaller E1b-55K variants, are expressed through alternative splicing at different times following infection (203; 510-513). The increase in virus-mediated HepG2 cell death following 2'AP treatment may require the expression of the smaller E1b gene products, which would be absent in Ad-dl1520-infected E1b-55K-positive HepG2 cells.

As our studies have shown AdΔE1bΔVA activity was much lower in normal cells than Ad-dl1520 and Ad-dl309ΔVA. Since 2'AP treatment of HCC cells specifically increased the replication and lytic activity of AdΔE1bΔVA, but not of Ad-dl1520 or Ad-dl309ΔVA, AdΔE1bΔVA in combination with 2'AP might have a stronger therapeutic index than Ad-dl1520 or Ad-dl309ΔVA oncolytic Ads.

4.8 Summary and Future Directions

Previous studies have shown that E1b-55K-deleted oncolytic Ads are highly selective for HCC cells relative to normal cells, both *in vitro* and *in vivo* (362; 363). However, while results from these preclinical studies have shown great promise, results from HCC clinical studies have found little improvement in progression free survival using E1b-55K-deleted oncolytic Ad (379). Therefore, increasing the lytic activity of E1b-55K-deleted Ads may improve their efficacy. In order to increase cell lysis, we constructed an oncolytic Ad containing a deletion of the E1b-19K and E3 genes, in addition to E1b-55K. Furthermore, in order to eliminate E1a negative feedback on its promoter the E1a coding region

was placed under the control of the strong immediate early mCMV promoter (514). In order to reduce the replication efficiency and lysis of normal cells, the VA-RNA genes were deleted, and miR-122T sites were inserted in the 3'UTR of the E1a gene. We found that this novel targeted oncolytic Ad (AdΔE1bΔVA) specifically replicated in and lysed HCC, but not normal cells. However, in contrast to a previous report (389), we found that the full E1b-deletion resulted in reduced Ad replication efficiency in HCC cells. Furthermore, we found that while expression of wild-type E1b-55K increased AdΔE1bΔVA production, the expression of a C454S/C456S mutant E1b-55K had no effect on production, thus E1b-55K functions associated with these cysteine residues are important in AdΔE1bΔVA replication in HCC cells. Interestingly, similar to E1b-55K expression, we found that 2'AP treatment increased AdΔE1bΔVA production, suggesting that 2'AP and the C454/C456 cysteine residues of E1b-55K target similar cellular pathways.

4.8.1 Determination of Mechanism of 2'AP-mediated Rescue of AdΔE1bΔVA Production

Since our study showed that 2'AP compensated for the loss of E1b-55K functions associated with the C454 and C456 residues, further studies on these functions are required to determine the mechanism of 2'AP-enhancement of AdΔE1bΔVA production (candidate pathways are shown in Figure 4.1).

Previous studies have shown that C454S/C456S mutant E1b-55K was unable to induce the degradation of Mre11 and DNA IV ligase, and therefore, this mutant is unable to block the DNA damage response (231; 232). Interestingly,

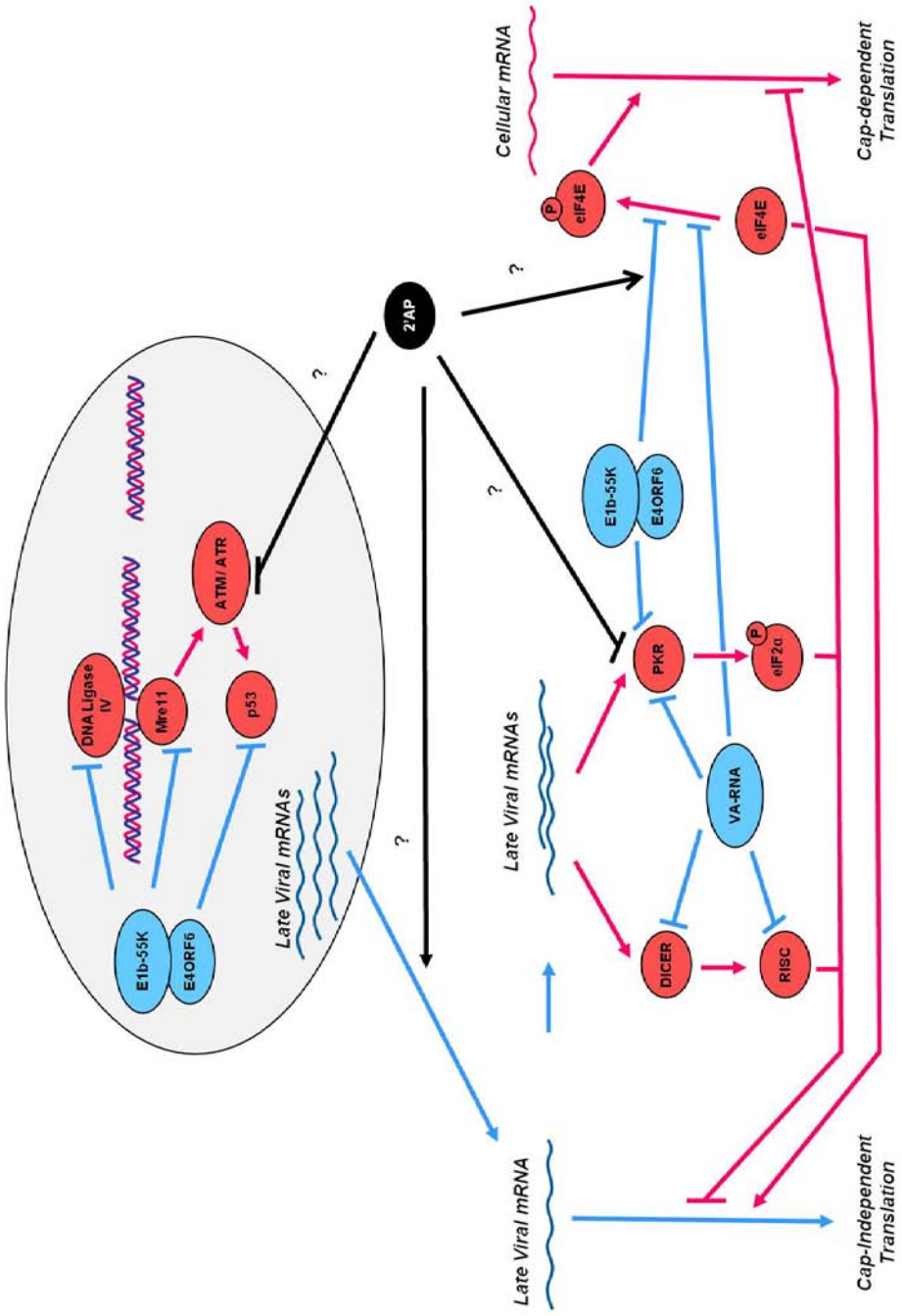

Figure 4.1. Identification of mechanism of 2'AP-mediated compensation of E1b-55K.

Potential mechanisms of 2'AP compensation (black) for the roles of E1b-55K and VA-RNAs (blue) in the inhibition of host proteins (red).

2’AP was previously shown to block the DNA damage response through inhibition of ATM, ATR and p53 following genotoxic stress (456). Since the Mre11 inhibitor mirin (452) was unable to enhance AdΔE1bΔVA production in our studies, inhibition of Mre11 alone may not be sufficient to increase virus production. Therefore, 2’AP treatment might have resulted in increased AdΔE1bΔVA production in HepG2 cells through inhibition of additional proteins within the DNA damage response pathway.

To determine whether 2’AP treatment had an effect on the DNA damage response, ATM and ATR activation and downstream signaling could be assessed in AdΔE1bΔVA-infected HepG2 cells with or without 2’AP treatment. Furthermore, the effect of mutant E1b-55K expression and 2’AP treatment on AdΔE1bΔVA concatemerization in HepG2 cells could be determined in order to assess whether AdΔE1bΔVA production was reduced due to genomic concatemerization.

In addition to the C454S/C456S mutant, other E1b-55K mutants were also found to have lost the ability to block the DNA damage response. Interestingly, studies have shown that H354 insertion mutant blocked viral DNA concatemerization and induced the degradation of Mre11, but not DNA ligase IV (232; 515). In contrast, R443 insertion mutant was unable to block concatemerization or degrade Mre11, but retained the ability to degrade DNA ligase IV (232). Therefore, assessing the effect of these mutants on AdΔE1bΔVA production in HepG2 cells would help determine the role Mre11 and/or DNA ligase IV play in infections with AdΔE1bΔVA.

Previous studies with C454S/C456S mutant E1b-55K focused on activation of the DNA damage response, and therefore, it is currently unknown whether this mutant has also lost other E1b-55K functions, such as inhibition of the interferon response (250-253). Since 2'AP was previously found to inhibit PKR activation and interferon-stimulated gene expression (281; 467; 468), 2'AP enhancement of AdΔE1bΔVA production might have been through inhibition the interferon response. Therefore, determining the effect of C454S/C456S mutant E1b-55K and 2'AP on PKR activation as well as interferon-stimulated gene expression in AdΔE1bΔVA-infected HepG2 cells could also be informative.

Another important function of E1b-55K during replication is promotion of late viral gene expression through promoting late viral mRNA export and protein translation, while inhibiting cellular translation (233-240). Therefore, it would be interesting to examine whether the C454S/C456S mutant E1b-55K protein was able to promote late viral gene expression and if not, to examine whether 2'AP compensated for the loss of this E1b-55K function in AdΔE1bΔVA-infected HepG2 cells.

4.8.2 Effect of E3 Region on AdΔE1bΔVA Production

While a previous study has found that an E1b-deleted Ad encoding the full E3 region replicated in HCC cells as efficiently as wild-type Ad (389), AdΔE1bΔVA production was reduced in our studies, raising the possibility that the E3 gene products have an important role in virus replication *in vitro* especially when the E1b gene products are deleted. Interestingly, we found that AdΔE1b replicated more efficiently and induced greater cell death in HepG2 cells

expressing E1b-55K than in parental HepG2 cells, consistent with the possibility that the dependency on the E3 gene products may be reduced in AdΔE1b when E1b-55K is expressed. Construction of an E3-positive AdΔE1bΔVA and determination of its replication and lytic efficiency in HepG2 cells would confirm that the AdΔE1bΔVA oncolytic activity is dependent on the E3 region. Furthermore, construction of AdΔE1b-55KΔVA and AdΔE1b-19KΔVA viruses with or without the E3 region with would allow the determination of whether the incorporation of either E1b protein reduces the dependency on the E3 region.

4.8.3 Determination of AdΔE1bΔVA Specificity In Vivo

Due to the inability of mouse cells to support human Ad replication, most *in vivo* studies using human Ads rely on immunocompromised mouse xenograft models to determine the oncolytic activity in human tumours (344; 346; 347). However, since high amounts of systemically delivered Ads accumulate in the liver (350; 351), determination of specificity of oncolytic Ads *in vivo* would be more adequately assessed by an animal model that supports human Ad replication in the liver.

Syrian hamster cancer models have therefore been developed for the study of oncolytic Ads (516). Unlike mouse cells, Syrian hamster cells have been shown to support Ad replication *in vitro* and *in vivo* (516; 517). As several Syrian hamster cancer cell lines have been established, this model might be more reliable for the determination AdΔE1bΔVA replication in the liver as well as liver toxicity.

Presently, there is no Syrian hamster HCC cell line that supports Ad

replication. However, previous studies have found that the pancreatic cancer cell line, SHPC-6, and the renal cancer cell line, HaK, were able to support Ad replication (516). While we did not find that SHPC-6 cells supported AdΔE1b and AdΔE1bΔVA replication, the effect of 2'AP on virus replication has yet to be determined in these cells. Furthermore, diethylnitrosamine (DEN)-induced liver cancer has been established as an HCC mouse model (518; 519). Recently, DEN-induced HCC in Syrian hamsters has also been shown to be a representative model for human HCC development (520). Therefore, this HCC Syrian hamster model could potentially be used as an *in vivo* model to determine both the activity as well as the specificity of AdΔE1bΔVA for HCC.

4.8.4 Increasing the Specificity of AdΔE1bΔVA

4.8.4.1 Insertion of Additional miRT Sites

Several groups have shown that the insertion of miR-122T sites in the 3'UTR of E1a gene dramatically reduced the expression of E1a in normal human hepatocytes without affecting virus production in HCC cells (344; 346-349). In humans, miR-122 levels were reduced in the liver of patients with cirrhosis, prior to HCC development, as well as in patients with HCC (123; 521). Furthermore, while interferon treatment was found to have a therapeutic potential for HCC, interferon was also found to reduce miR-122 levels (128; 521; 522). Therefore, the insertion of miR-122T sites in the 3'UTR of the E1a gene may not prevent virus replication in fibrotic liver tissues of AdΔE1bΔVA-treated HCC patients or in normal liver tissues of patients undergoing IFN therapy for HCC. Therefore, insertion of additional miRT sites that are targeted by other miRNAs might

enhance the HCC specificity of E1a expression in certain patients. MiR-199a/b-3p was recently found to be highly expressed in fibrotic livers, and inhibition of this miRNA is thought to induce the progression from fibrosis to HCC (523). Therefore, insertion of miR-199a/b-3pT sites, in addition to miR-122T sites, may reduce E1a expression in both normal and fibrotic noncancerous liver cells without affecting its expression in HCC cells.

4.8.4.2 Reduction of Ad Entry Into Kupffer Cells

While genomic modifications of Ads, such as those in AdΔE1bΔVA, can decrease replication in normal cells, viral entry would not be inhibited. Therefore, extensive studies have been done to modify the capsid proteins in order to specifically target Ads to cancer cells. Interestingly, while the CAR receptor is thought to be the main receptor for Ad2 and Ad5 serotypes, studies have shown that these viruses can enter hepatocytes through a CAR-independent mechanism (143-146; 524-526). This entry is thought to occur by the binding of the hexon to the vitamin K-dependent coagulation factor X (FX). This binding was found to be through the hypervariable region of hexon, and therefore, might be serotype-specific (524; 526; 527). FX binding to hexon allows the binding to heparin sulfate proteoglycans (HSPG) on the surface of the cell (528).

As previous studies have shown that Ad5 vectors that are administered systemically mostly localize to the liver (350; 351), this hepatotropism may have great benefit for HCC-targeted gene therapy. However, in addition to hepatocytes, Ad vectors were also found to infect other cells within the liver, such as Kupffer cells (529). Although Kupffer cells can be infected by Ad5 through a CAR-

independent mechanism using the scavenger receptor A (SR-A), infection can also be facilitated by platelet binding to the viral capsids (530-532). Interestingly, a recent study has shown that reduction of Kupffer cell entry without a loss of hepatocyte entry could be engineered through the replacement of the hypervariable region of Ad5 hexon with Ad6 hexon (533). Therefore, modifications of hexon hypervariable region AdΔE1bΔVA should further increase the specificity of the virus for HCC cells.

4.9 Conclusions

Increasing Ad specificity for cancer cells is a major research area. While post-transcriptional inhibition of E1a expression through insertion of miR-122T sites has the potential to increase HCC specificity without affecting the lytic activity of Ad, we found that low E1a expression occurred in miR-122-positive cells, which could result in liver toxicity. We show in this study that in addition to the insertion of miR-122T sites that inhibited E1a expression, deletions in the E1b and VA-RNA genes increased the specificity of AdΔE1bΔVA to HCC cells by reducing Ad replication and lysis and of normal cells. We also showed that co-treatment with 2'AP increased the activity of AdΔE1bΔVA in HCC cells, but not normal fibroblasts, through complementation of E1b-55K activities. Further studies to determine the mechanism of 2'AP complementation and the role of the E3 gene products in E1b-deleted Ad replication could allow the construction of enhanced oncolytic Ads for future treatment of HCC.

Chapter 5 - References

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Appendix - Post-Transcriptional Inhibition of a Pro-apoptotic Gene Encoded within a First Generation Adenovirus Vector Using the Liver-Specific miR-122

A.1 INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third leading cause of cancer-related deaths (493). While tumour resection and transplantation are the preferred treatment option, most patients are presented with an inoperable tumour. Radiation and chemotherapy treatment have been shown to promote only a limited increase in progression free survival (534). Therefore, much research has been done to identify novel HCC therapies (535). Gene therapy has been extensively studied as a potential therapeutic agent against many cancers, including HCC. In this approach genes are specifically delivered to the cancer cells in order to induce a therapeutic effect. Various viruses and non-virus particles have been used to deliver therapeutic gene and one of the most studied is the first-generation adenoviral (FGAd) vector (304; 312; 439; 536; 537). This vector has a deletion in the E1 region, and therefore, is non-replicating. Furthermore, a large deletion is often introduced in the viral E3 region to allow for cloning therapeutic genes of approximately 8 kilobases (kb) in size.

FGAd vectors have been used to transfer many different genes to tumours to induce a therapeutic effect, such as suicide and pro-apoptotic genes (535). For example, co-treatment of FGAd vectors encoding the herpes simplex virus (HSV) thymidine kinase (TK) gene with the prodrug ganciclovir were shown to induce a therapeutic effect in HCC cells both *in vitro,* in animal models and in clinical trials (538-540). Furthermore, FGAd vectors encoding the pro-apoptotic Bax gene have also been shown to induce HCC cell death directly through apoptosis induction (324; 325).

One of the main challenges in the use of FGAd vector-mediated expression of toxic genes, such as suicide and pro-apoptotic genes, is the ability of these vectors to infect and subsequently express the toxic genes in normal hepatocytes. To reduce liver toxicity, different targeting modalities have been utilized to repress transgene expression in hepatocytes. Since the discovery of the RNA interference (RNAi) pathway, several studies have assessed post-transcriptional gene targeting in order to block gene expression in normal hepatocytes (345; 347; 348).

The liver-specific microRNA (miRNA), miR-122 (116), was found to have an important role in hepatocyte function as well as to have tumour suppressive activity (116; 541; 542). Furthermore, miR-122 expression was found to be downregulated in many HCC cells (118; 120; 123-126; 543). Therefore, addition of completely complementary miR-122 target (miR-122T) sites to the 3' untranslated region (3'UTR) of therapeutic genes may block gene expression post-transcriptionally in normal hepatocytes while not affecting expression in HCC cells.

Several studies have shown reduced reporter gene expression in mouse hepatocytes infected with an FGAd vector encoding a luciferase reporter gene containing miR-122T sites in the 3'UTR compared to a luciferase reporter gene not containing miR-122T sites, both *in vitro* and *in vivo* (344; 346). Furthermore, these studies have shown that while miR-122T sites do not affect luciferase gene expression in infected miR-122-negative HCC cells, the miR-122T-containing

luciferase expression was downregulated in the miR-122-positive HCC cell line, HuH7 (346).

In addition to reporter gene expression, miR-122T sites were also inserted in the 3'UTR of an HSV-TK gene encoded within an FGAd vector (345). This study has shown that co-administration of this vector with ganciclovir resulted in reduced liver toxicity when compared to an FGAd vector encoding HSV-TK without miR-122T sites (345).

Unlike FGAd vectors encoding the HSV-TK gene, construction and amplification of FGAd vectors encoding pro-apoptotic genes are highly challenging due to the induction of apoptosis of the packaging cells (407). Several systems have been designed to reduce expression of these toxic transgenes in the packaging cells, such as transcriptional inhibition using tetracycline-regulated and lac repressor systems (325; 411). Furthermore, other systems have been designed to block apoptosis through stable expression of anti-apoptotic genes, such as crmA and Bcl-2 (544; 545).

A system was also designed to increase the concentrations of an FGAd vector encoding a nontoxic gene in HEK293 packaging cell line through post-transcriptional gene silencing (415). This study found that higher titers of an FGAd vector were obtained when amplified in HEK293 cells stably transfected with a small hairpin RNA (shRNA) targeting the transgene. Therefore, we examined whether the insertion of miR-122T sites used to post-transcriptionally inhibit FGAd-encoded toxic gene expression in hepatocytes could also be exploited to produce high titers of these FGAds.

A.2 MATERIAL AND METHODS

A.2.1 Cell Culture

All cells were maintained in medium supplemented with 10 % fetal bovine serum (FBS; Gibco, Cat # 12483), 1X antibiotic-antimycotic solution (100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B; Cat # 15240) and 2 mM L-glutamine (Gibco, Cat # 25030) except where noted.

A.2.1.1 Maintenance of Packaging Cell Lines

Human embryonic kidney 293 (HEK293) cells (a gift from Dr. Frank Graham, McMaster University, ON, Canada), were previously established through transformation of HEK cells with the E1 region of the human adenovirus serotype 5 (hAd5) (313), and maintained in minimum essential medium (MEM; Gibco, Cat # 61100-087). 293-miR-122 cells express the miR-122-containing woodchuck hcr gene under the control of a Tet-on promoter (546). Similarly, 293-miR-122-Mut cells express a mutant miR-122-containing woodchuck hcr gene under the control of a Tet-on promoter, with the seed sequence of miR-122 mutated at positions 3, 4 and 6 (546). Both cell lines (gifts from Dr. Timothy Block, Drexel University College of Medicine, PA, USA) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Cat # 12800-058).

A.2.1.2 Maintenance of Other Cell Lines

The human HCC cell lines, HuH7.5 (547) (a gift from Dr. Lorne Tyrrell, University of Alberta, AB, Canada), Hep3B (378; 548) (a gift from Dr. Roseline

Godbout; University of Alberta, AB, Canada) and HepG2 (549) (ATCC, HB-8065), were maintained in high glucose DMEM. The human colorectal cancer cell lines, HCT116 Bax +/- and HCT116 Bax -/- (550) (gifts from Dr. Raymond Lai; University of Alberta, AB, Canada), were maintained in McCoy's 5α medium (Thermo Scientific HyClone, Cat # SH3020001).

A.2.1.3 Cell Culture Growth Conditions

All cells were maintained at 37˚C in a humid atmosphere containing 5 % CO_2 and passaged every 4 to 5 days using 0.25 % trypsin (Gibco, Cat # 15090) in versene [8 g/L NaCl (Fisher, Cat # BP358-212), 0.2 g/L KCl (Fisher, Cat # P330-3), 1.15 g/L Na_2HPO_4 (Sigma, Cat # S-3264), 0.2 g/L KH_2PO_4 (Fisher, Cat # P-286), 0.2 g/L ethylenediaminetetraacetic acid (EDTA; Fisher, Cat # S311-500, 0.0075 g/L phenol red (MCB Chemicals, Cat # PX535)]. Live cells were counted on a hemocytometer using 0.4 % trypan blue stain (Gibco, Cat # 15250).

A.2.2 Plasmid Construction

pEGFP-C3 (GenBank accession # U57607) was a kind gift from Dr. David Evans (University of Alberta, AB, Canada) and pmCherry-C1 was a gift from Dr. Robert Campbell (University of Alberta, AB, Canada). phTERT-Bax-miR-122Tx0 (pML13), pAD5 and pSJ7 adenoviral shuttle plasmids were previously constructed in our lab. The pmiR-122Tx2 plasmid (synthesized by Integrated DNA Technologies) was designed to encode two repeats of a target sequence completely complementary to miR-122 (pmiR-122Tx2) flanked by the restriction enzyme sites, PstI, at the 5' end, and NsiI, at the 3' end (Figure A.1).

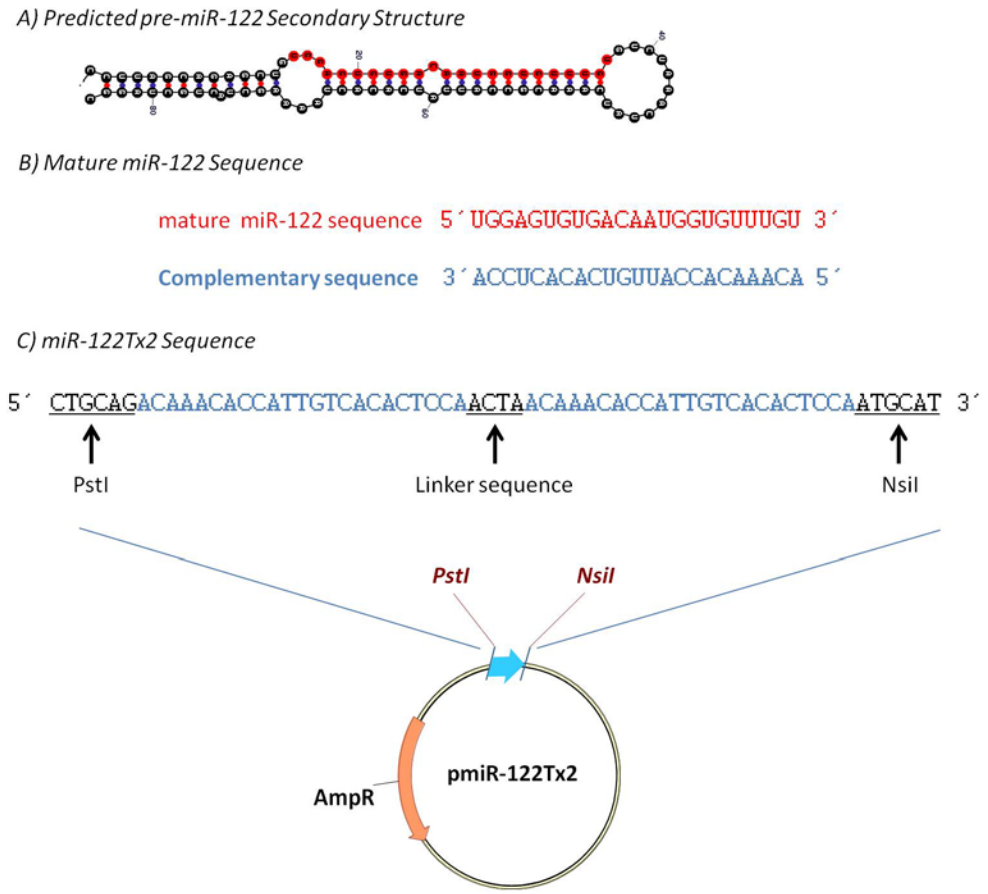

Figure A.1: Design of miR-122 targeted sequences.

Predicted human miR-122 secondary structure was obtained from miRNAMap version 2.0 (551).

The two miR-122T sites were separated by four nucleotides (5’ ACTA 3’) to allow enhanced binding by RISC-bound miR-122.

Plasmids were constructed by restriction enzyme cleavage and ligation as shown in Figures A.2 and A.3. Restriction enzymes were obtained from Fermentas, except for NsiI, which was obtained from New England Biolabs.

A.2.3 Adenovirus Construction, Amplification and Purification

A.2.3.1 Construction of FGAd Vectors

FGAd vectors used in this study contain deletions in the E1 region (between 480 – 3533 bp) and the E3 region (between 28130-30820 bp). These vectors were constructed by Cre-mediated recombination using the AdMax system (552) by co-transfection of 293-miR-122 packaging cells (546) with the adenovirus genomic plasmid, pBHGloxΔE1,3Cre (Microbix, Cat #PD-01-64) and the E1 shuttle plasmids phTERT-Bax-miR-122Tx6, pmCMV-Bax-miR-122Tx6, pmCMV-Luc-miR-122Tx0 and pmCMV-Luc-miR-122Tx6 (construction shown in Figure A.2 and A.3). One day following transfection, the medium was replaced with fresh medium containing 10 µg/mL tetracycline (Sigma, Cat # T7660) in order to induce miR-122 expression. Furthermore, an additional 1 mL medium containing tetracycline was added once a week until infection was detected.

A.2.3.2 Isolation of First Generation Adenoviral Vectors

Wells with cells showing cytopathic effect (CPE) containing virus infected cells were harvested and freeze-thawed three times and the lysate was then used to infect 6 well plates containing fresh 293-miR-122 cells in medium containing 10 µg/mL tetracycline. Once complete CPE was observed, the wells were harvested

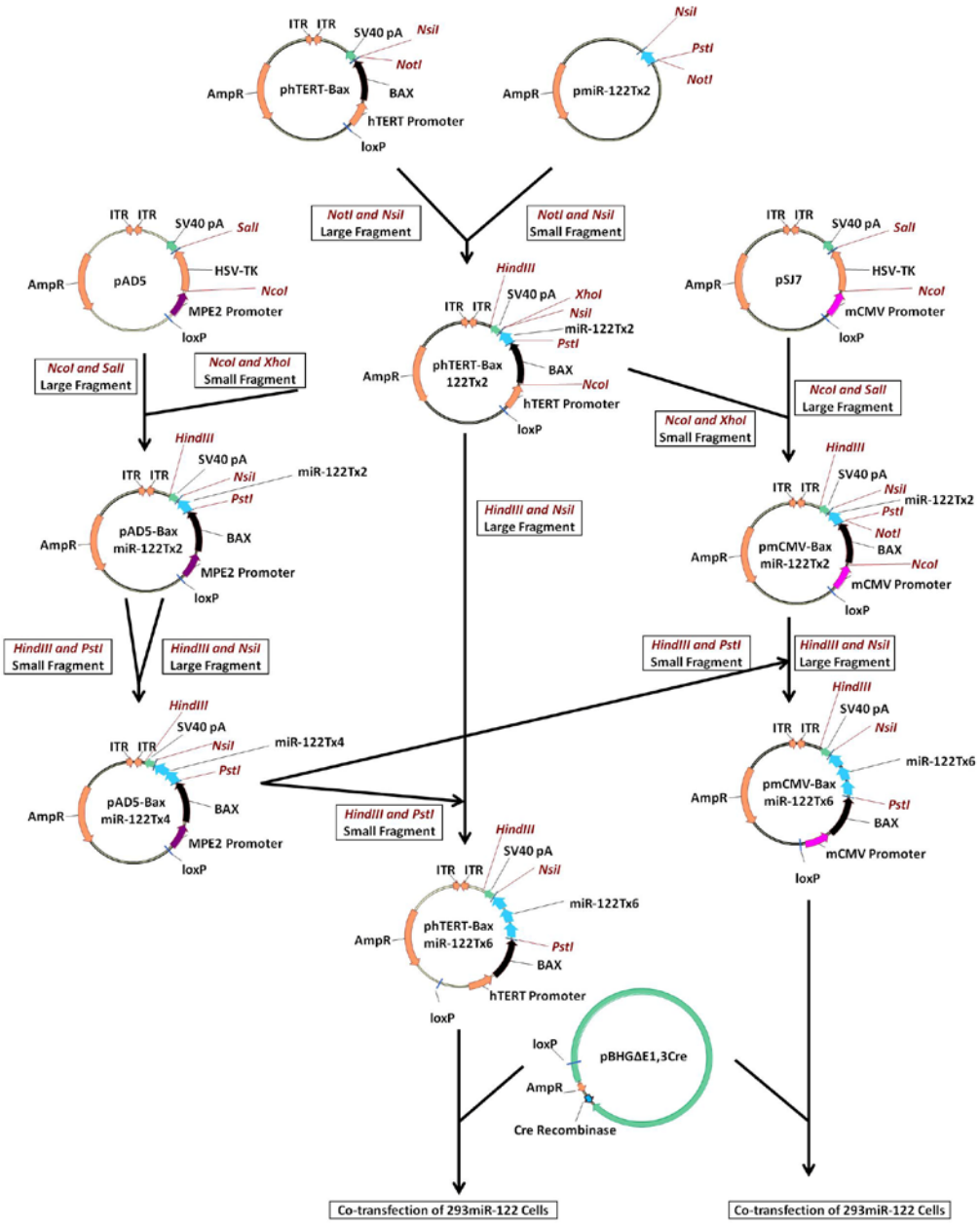
ITR ITR
SV40 pA
NsiI
NotI
AmpR
phTERT-Bax
BAX
hTERT Promoter
loxP
pmiR-122Tx2
PstI
NotI and NsiI
Large Fragment
NotI and NsiI
Small Fragment
pAD5
SalI
HSV-TK
NcoI
MPE2 Promoter
pSJ7
mCMV Promoter
HindIII
XhoI
miR-122Tx2
phTERT-Bax
122Tx2
NcoI and SalI
Large Fragment
NcoI and XhoI
Small Fragment
pAD5-Bax
miR-122Tx2
pmCMV-Bax
HindIII and PstI
Small Fragment
HindIII and NsiI
Large Fragment
miR-122Tx4
pAD5-Bax
miR-122Tx4
miR-122Tx6
pmCMV-Bax
miR-122Tx6
phTERT-Bax
miR-122Tx6
pBHGΔE1,3Cre
Cre Recombinase
Co-transfection of 293miR-122 Cells
Co-transfection of 293miR-122 Cells

Figure A.2: Cloning strategy for the construction of Bax-encoding FGAd vectors.

The E1 shuttle plasmids pmCMV-Bax-miR-122Tx6 and pmCMV-Bax-miR-122Tx6 were constructed by standard cloning techniques (554). Briefly, plasmids were cleaved with the appropriate restriction enzymes (brown) and the resulting DNA fragments were resolved in 1 % agarose gel. The appropriate DNA fragments were gel purified and ligated overnight using T4 DNA Ligase. The next day, electrocompetent bacterial cells were transformed with the ligated DNA prior to plating on Luria-Bertani (LB)-agar plates containing 100 µg/mL ampicillin. The plates were incubated overnight 3[illegible] and the next day, bacterial colonies were isolated and used to inoculate 3 mL LB containing 100 µg/mL ampicillin. The bacterial solutions were incubated in a 37˚C shaking incubator at 200 rpm for 16 hours prior to purification of plasmid DNA using TENS plasmid minipreparations protocol (555). Once plasmid DNA was verified by restriction enzyme digestion, bacteria containing correct plasmid were grown in 100 mL LB containing 100 µg/mL for 16 hours in a 37˚C shaking incubator. The next day, plasmid DNA was purified by the HiSpeed Plasmid Midi Kit (QIAGEN, Cat # 12643) using the manufacturer's protocol. FGAd vectors were constructed by Cre-mediated recombination using the AdMax system (552) by co-transfection of 293-miR-122 packaging cells (546) with the appropriate E1 shuttle plasmid and the adenovirus genomic plasmid pBHGloxΔE1,3Cre. miR-122T – miR-122 target sites; mCMV promoter – murine cytomegalovirus immediate early promoter; hTERT promoter – human telomerase promoter; SV40 pA – Simian virus 40

polyadenylation signal; ITR – adenoviral inverted terminal repeat; AmpR – ampicillin resistance gene.

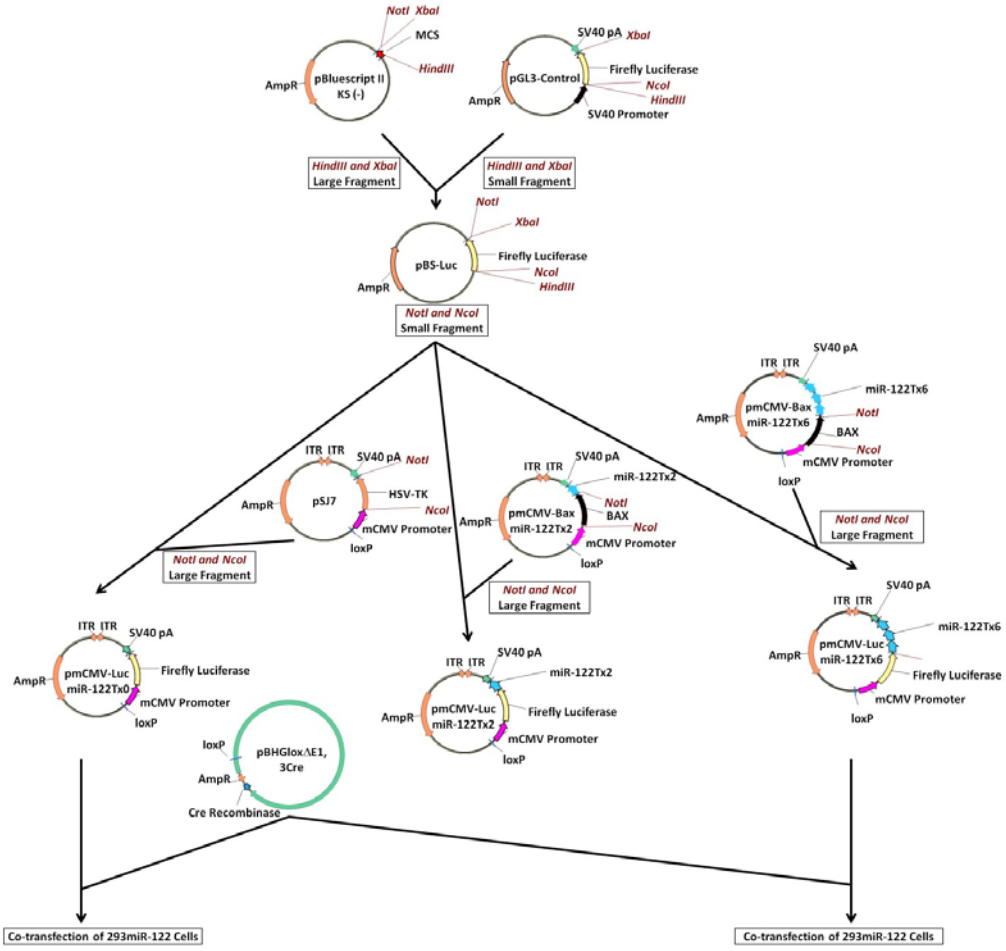

Figure A.3: Cloning strategy for the construction of luciferase-encoding FGAd vectors.

The E1 shuttle plasmids pmCMV-Luc-miR-122Tx6 and pmCMV-Luc-miR-122Tx6 were constructed as described in Figure A.2. miR-122T – miR-122 target sites; mCMV promoter – murine cytomegalovirus immediate early promoter; SV40 pA – Simian virus 40 polyadenylation signal; ITR – adenoviral inverted terminal repeat; AmpR – ampicillin resistance gene.

and freeze-thawed three times. In order to isolate single adenovirus recombinants, the lysate was serially diluted on a 96 well plate containing 293-miR-122 cells in medium containing 10 µg/mL tetracycline. Once complete CPE was observed, single wells were harvested and added to 500 µL of phosphate-buffered saline (PBS). The virus lysate was freeze-thawed three times prior to infection of 6 well plates containing fresh 293-miR-122 cells in tetracycline-containing medium until complete CPE was observed. These infected wells were harvested and 1 mL of the lysate was used to isolate DNA for determination of correct recombinants by restriction enzyme analysis with HindIII (552). The rest of the lysate was freeze-thawed three times.

A.2.3.3 Amplification and Purification of First Generation Adenoviral Vector

Once a correct first generation adenoviral vector recombinant was identified, 500 µL of the correct viral lysate was used to infect one 150 mm plate of 293-miR-122 cells in tetracycline-containing medium until complete CPE was observed. The infected plate was harvested and freeze-thawed three times and entire viral lysate was used to infect forty 150 mm plates containing 293-miR-122 cells in tetracycline-containing medium. Once complete CPE was observed, the infected cells were harvested and purified by cesium chloride gradient as previously described (472). The final sample was dialyzed against 10 mM Tris pH 8.0 (Fisher, Cat # BP154-1), glycerol (Fisher, BP229-4) was added to approximately 10% and virus was stored at -80˚C.

A.2.3.4 Determination of Adenovirus Titers

A.2.3.4.1 Determination of Concentration in Virus Particles Units

To determine concentration of virus in particle numbers, 20 µL of purified virus was diluted in 980 µL TE/SDS solution [10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 0.1 % sodium dodecyl sulfate (SDS; Biorad, Cat # 161-0302)] and incubated at 65˚C for 20 min. For a blank, 20 µL of 10 % glycerol in 10 mM Tris pH 8.0 was also diluted in 980 µL of TE/SDS solution. The OD260 was determined using a spectrophotometer. VP/mL was calculated by multiplying the OD260 by 50 (the dilution factor) and 1.1 x 10e12 (472; 553).

A.2.3.4.2 Determination of Concentration in Plaque Forming Units

HEK293 cells were seeded in 12 well plates and infected the next day in duplicate with serially diluted purified virus in 200 µL of PBS. After 1 hour incubation, 2 mL of MEM and agarose (EMD, Cat # 2125) solution (1 mL of 2xMEM supplemented with 10 % FBS, 2 mM L-glutamine and antibiotics mixed with 1 mL 1 % melted agarose) was added. The plates were incubated at room temperature for 15 min to allow the medium to solidify and then incubated at 37˚C for 10 days. Following the incubation, plaques were counted by eye. The concentration in PFU/mL was calculated by multiplying the number of plaques by the virus dilution factor in the counted well and dividing by 0.2 mL (the volume added to the well).

A.2.4 SDS-PAGE and Western Blot Analysis

A.2.4.1 Protein Isolation

Cells were harvested from a 24 or a 12 well plate by removing the medium

and washing the cells twice with PBS prior to the addition of 100 µL of RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate (Fisher, Cat # 302-95-4), 0.1 % SDS, 50 mM Tris, pH 8.0) containing 1mM phenylmethylsulfonyl fluoride (PMSF; Sigma, Cat # P7626) and 1x Protease Inhibitor Cocktail Set III EDTA-Free (Calbiochem, Cat # 539134).

In some cases, cells and medium were harvested and spun down at 1000 xg for 5 min. The supernatant was removed and the pellet was washed with PBS and spun down at 1000 xg for 5 min twice. The pellet was then resuspended in 100 µL RIPA supplemented with PMSF and protease inhibitor cocktail.

Cell lysates were incubated at 4˚C for 20 min prior to freezing at -70˚C. The next day, lysates were thawed and spun down at 4˚C for 20 min at 14000 rpm and the cleared lysates were transferred to new tubes. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Cat # 23225) and the FluoStar Optima (BMG) or FluoStar Omega (BMG) plate readers.

A.2.4.2 SDS-Polyacrylamide Gel Electrophoresis and Protein Transfer

All gels were prepared, run and transferred using the Bio-Rad miniPROTEAN apparatus. The appropriate protein amounts were from samples were mixed with 5x loading dye [15 % glycerol, 75 mM Tris pH 6.8, 2 % SDS, 0.15 mg/mL Bromophenol Blue (ANALCO, Cat # 201 3179), 200 mM 2-mercaptoethanol (Fisher, Cat # BP176-500)]. The solutions were mixed and denatured at 95˚C for 10 min prior to loading on a 12 % polyacrylamide resolving gel with a 5 % polyacrylamide stacking gel. PageRuler Prestained Protein Ladder (Fermentas, Cat # SM0671) was used on all gels for band size determination.

The protein samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) at 100 V in Tris-glycine running buffer [25 mM Tris, 192 mM glycine (Fisher, Cat # PB381-1), 0.1 % SDS] for 2 hours. Resolved proteins were then transferred on to a nitrocellulose membrane (Bio-Rad, Cat # 162-0115) for 2 hours at 350 milliamps or overnight at 30V at 4˚C in transfer buffer [Tris-glycine, 20 % methanol (Fisher, A412P-4)].

A.2.4.3 Western Blot Analysis

Membranes were blocked with 5 % skim milk in PBS containing 0.1 % Tween 20 (PBST; MP Biomedicals, cat # 194724) for 1 hour at room temperature on a vertical orbital rotator followed by overnight incubation with the appropriate primary antibody at 4˚C. The primary antibodies used were mouse antibody against Bax (Enzo Life Sciences, Cat # ADI-AAM-140) at a dilution of 1/2000 in 5 % milk in PBST and rabbit antibody against caspase-3 (Stressgen, Cat # AAP-113) at a dilution of 1/1000 in 5 % milk in PBST.

The next day, the membranes were washed with PBST and then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Cat # 115-035-003) or goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Cat # 315-005-045) at a dilution of 1/2000 in 5 % milk in PBST for 1 hour at room temperature. The membranes were washed with PBST, horseradish peroxidase chemiluminescent substrate (GE Healthcare, Cat # RPN2106) was added and signal was detected on X-ray film (Fujifilm, Cat # 47410 19238) using the Kodak X-OMAT 2000A or the Konica SRX-101A film processors.

A.2.5 Plasmid DNA Transfection

Transfections were performed in 24 well plates using Lipofectamine 2000 reagent (Invitrogen, Cat # 11668-019) according to the manufacturer's protocol with a lipofectamine:DNA ratio of 1 µL:1 µg in 100 µL OptiMEM. In some cases, transfection was normalized by co-transfecting 50 ng of an expression vector encoding the *Renilla* luciferase reporter gene under the control of the mCMV promoter (pSK5).

A.2.6 Transfection of miRNA Mimic

Miridian microRNA mimic negative control # 1 (Cat # CN-001000-01-05) and Miridian Human hsa-miR-122 mimic (Cat # C-300591-05) were obtained from Dharmacon (Fisher Scientific). Transfections were performed in 24 well plates using HiPerFect Transfection Reagent (QIAGEN, Cat # 301705) according to the manufacturer's protocol at a ratio of 3 µL reagent: 3 µL of 5 µM microRNA mimic in 100 µL OptiMEM.

A.2.7 Virus Infection

One day prior to infection, cells were seeded in 24 well plates with the appropriate medium. The next day the cells were counted, the medium was removed and cells were infected with the indicated viruses in 200 µL PBS per well at the indicated multiplicity of infection (MOI). One hour following infection, fresh medium was added and the cells were incubated at 37˚C.

A.2.8 Luciferase Reporter Assay

Transfected or infected cells in 24-well plates were washed with PBS prior to addition of 100 µL of 1x Passive Lysis Buffer (Promega, Cat # E194A) or 1x

Reporter Lysis Buffer (Promega, Cat # E3971). The plates were incubated at 4C for 20 minutes prior to freezing at -70˚C. Next, the plates were thawed and the lysates were transferred to a centrifuge tube. The lysates were spun at 14000 rpm for 5 min and the supernatant was transferred to a new tube. 20 µL of cleared lysate was transferred to a flat-bottomed white polystyrene 96 well plate (Costar, 3912). Luciferase expression was then measured using the Dual-Luciferase Reporter Assay System (Promega, Cat # E1960) or the Luciferase Assay System (Promega, Cat # E1501) according to the manufacturer's protocol with detection by FluoStar Optima (BMG) or FluoStar Omega (BMG) plate readers.

A.3 RESULTS

A.3.1 Validation of Mir-122-Targeted Gene Expression in HCC Cells

In order to determine whether endogenous miR-122 could regulate expression of exogenous genes containing miR-122T sites, miR-122-positive HuH7.5 cells were transfected with an expression vector carrying 6 miR-122T sites in the 3'UTR of the Bax gene (pmCMV-Bax-miR-122Tx6). Mock transfected cells or cells transfected with an mCherry expression vector were used as negative controls (No DNA and pmCherry, respectively). HepG2 cells (miR-122-negative control HCC cells), which has similar transfection efficiency as HuH7.5 cells, were also transfected with the same expression vectors. As additional controls, HCT116 cells with deletion of one or both Bax alleles (HCT116 Bax +/- and HCT116 Bax -/-) were also transfected with the expression vectors. Bax expression was determined 2 days post-transfection by western blot analysis (Figure A.4). High level of Bax expression was detected in the miR-122-negative cell lines HepG2, HCT116 Bax -/+ and HCT116 Bax -/- following transfection with pmCMV-Bax-miR-122Tx6 when compared to transfections with pmCherry and to untransfected cells. In contrast, in HuH7.5 cells, Bax expression following transfection with pmCMV-Bax-miR-122Tx6 was similar to that following transfection with pmCherry. Since the tested cells have similar transfection efficiencies and the experiments were done in duplicate, these results suggest that miR-122 expression in HuH7.5 cells specifically inhibited Bax expression, however, additional studies including loading controls are required to validate these results.

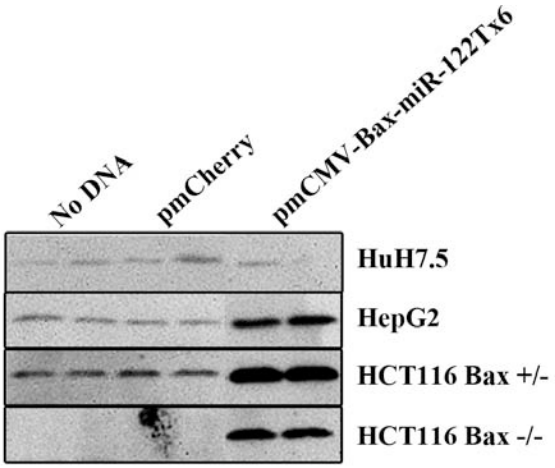

Figure A.4: MiR-122T insertion reduced Bax expression in miR-122-positive HuH7.5 cells.

Cells were transfected in duplicate with Bax expression vector containing 6 miR-122T sites (pmCMV-Bax-miR-122Tx6). Mock Transfected cells (No DNA) or cells transfected with pmCherry expression vector were used as negative controls. Two days post-transfection, cells were washed and lysed with RIPA. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed with a Bax specific antibody.

A.3.2 Insertion of MiR-122T Sites Reduced Transgene Expression in miR-122-Positive Cells

In order to determine whether miR-122 would quantitatively reduce the expression of miR-122T-modified genes encoded within an FGAd vector, we first analyzed the effect of miR-122 on modified reporter genes. MiR-122-positive HuH7.5 cells were infected with FGAd vectors encoding a luciferase reporter gene either containing or not containing the six miR-122T sites (Ad-mCMV-Luc-miR-122Tx6 and Ad-mCMV-Luc-miR-122Tx0, respectively) at an MOI of 1000 VP/cell. MiR-122-negative HepG2 and Hep3B cells were also infected with these FGAd vectors as controls. Luciferase expression was determined two days post-infection (Figure A.5). As expected, luciferase expression levels were similar in both miR-122-negative cell lines, HepG2 and Hep3B, after infection with either virus. In contrast, luciferase expression was reduced by 1 log in HuH7.5 infected with Ad-mCMV-Luc-miR-122Tx6 when compared to infection with Ad-mCMV-Luc-miR-122Tx0.

In order to verify that the inhibition of luciferase gene expression was mediated by miR-122, HepG2 cells were transfected with a miR-122 mimic a day prior to infection with Ad-mCMV-Luc-miR-122Tx6 or Ad-mCMV-Luc-miR-122Tx0 at an MOI of 1000 VP/cell. As a control, cells were transfected with a synthetic miRNA mimic that does not target any known mammalian genes (miR-Neg). Luciferase expression was determined two days post-infection (Figure A.6). Similar to observations with HuH7.5 cells (Figure A.5), luciferase expression was inhibited in the miR-122-transfected HepG2 cells infected with Ad-mCMV-Luc-

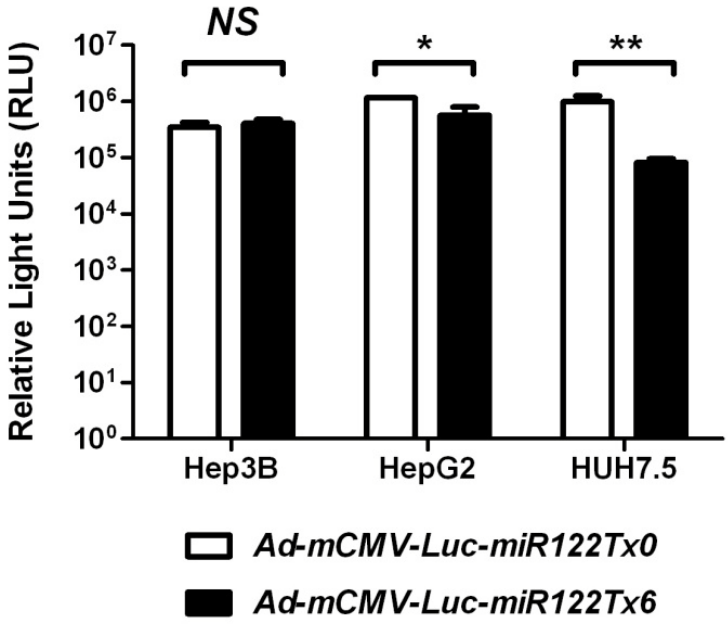

Figure A.5: Inhibition of luciferase expression in miR-122-positive HuH7.5 cells infected with FGAd vector containing six miR-122T sites.

Cells were infected with FGAd vectors encoding firefly luciferase gene either containing or not containing 6 miR-122T sites (Ad-mCMV-Luc-miR-122Tx6 or Ad-mCMV-Luc-miR-122Tx0, respectively). Two days post-infection, cells were harvested and luciferase expression was determined. Error bars correspond to +/- SD of triplicate wells (NS – not significant, * - $p < 0.05$, ** - $p < 0.01$; t-test).

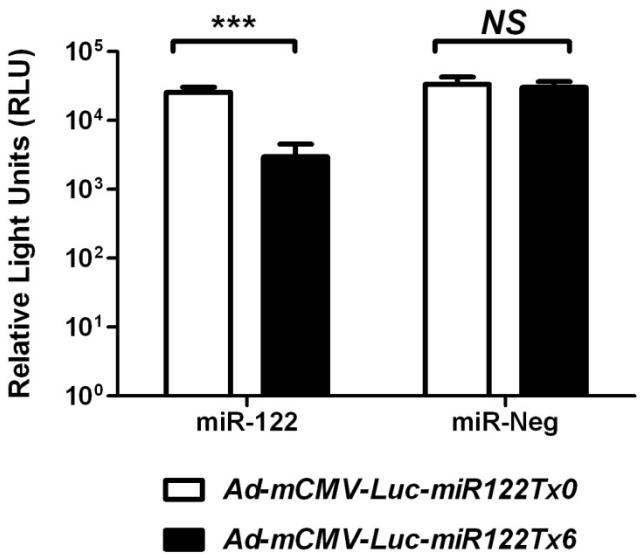

Figure A.6: Inhibition of luciferase expression in miR-122-transiently expressing cells infected with FGAd vector containing six miR-122T sites.

Cells were transfected with a miR-122 mimic or a nontargeted miRNA mimic (miR-Neg). The next day, cells were infected with FGAd vectors encoding firefly luciferase gene either containing or not containing 6 miR-122T sites (Ad-mCMV-Luc-miR-122Tx6 or Ad-mCMV-Luc-miR-122Tx0, respectively). Two days post-infection, cells were harvested and luciferase expression was determined. Error bars correspond to +/- SD of triplicate wells (NS – not significant, *** - $p < 0.001$; t-test).

miR-122Tx6 compared to Ad-mCMV-Luc-miR-122Tx0. These results suggest that miR-122 specifically induced a strong inhibitory effect on the expression of genes containing the six miR-122T sites in the 3'UTR.

Next we examined whether our results with the reporter could be extrapolated to modulation of expression of the pro-apoptotic Bax gene in an FGAd vector. To determine whether insertion of miR-122T sites would reduce FGAd vector-encoded Bax gene expression, HuH7.5 cells were infected with FGAd vectors encoding the Bax gene with the six miR-122T sites under the control of the mCMV promoter or the hTERT promoter (Ad-mCMV-Bax-miR-122Tx6 and Ad-hTERT-Bax-miR-122Tx6, respectively) at an MOI of 1000 VP/cell. Mock infected cells and cells infected with an FGAd vector encoding the EGFP gene (AdControl) were used as negative controls. As an additional control, miR-122-negative HepG2 cells were infected with the viruses. Bax expression was determined using western blot analysis two days post-infection (Figure A.7). Bax expression was low in HuH7.5 cells under all conditions, consistent with downregulation of Bax by miR-122. In contrast, Bax expression was found to be higher in HepG2 cells infected with the Bax-encoding viruses when compared to uninfected or AdControl-infected cells. These results suggest that endogenous miR-122 in HuH7.5 cells inhibited Bax expression, however further studies including loading controls are required to validate these results.

A.3.3 Transient Transfection of MiR-122 Reduced MiR-122T-Containing Transgene Expression in HEK293 Cells Infected With FGAds

In order to determine whether miR-122 would reduce exogenous gene

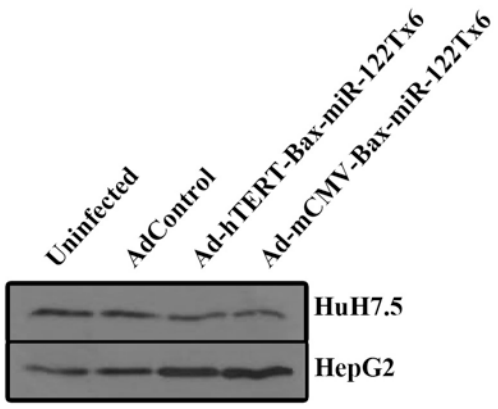

Figure A.7: Insertion miR-122T reduced miR-122T-containing Bax expression in miR-122-positive HuH7.5 cells

Cells were mock infected or infected with Bax FGAd vectors containing 6 miR-122T sites under the control of the mCMV or hTERT promoters (Ad-mCMV-Bax-miR-122Tx6 and Ad-mCMV-Bax-miR-122Tx6, respectively) at an MOI of 1000 VP/cell. As a negative control, cells were infected with AdControl vector. Two days post-infection, cells were washed and lysed with RIPA. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed with a Bax specific antibody.

expression in cells supporting FGAd vector replication, HEK293 cells were transfected with the miR-122 mimic a day before infection with Ad-mCMV-Luc-miR-122Tx6 or Ad-mCMV-Luc-miR-122Tx0 at an MOI of 100 VP/cell. Cells were also transfected with miR-Neg as a control. One day post-infection, luciferase expression was determined (Figure A.8). Luciferase expression was inhibited by approximately 2-fold in miR-122-transfected HEK293 cells infected with Ad-mCMV-Luc-miR-122Tx6 compared to Ad-mCMV-Luc-miR-122Tx0. Interestingly, approximately 20 % inhibition was found in untransfected as well as miR-Neg transfected HEK293 cells infected with Ad-mCMV-Luc-miR-122Tx6 compared to Ad-mCMV-Luc-miR-122Tx0, suggesting that the insertion of the six miR-122T sites may have reduced luciferase expression in a miR-122-independent mechanism.

A.3.4 MiR-122T Insertion Increased FGAd Vector Amplification in HEK293 Cells Transfected with MiR-122

Due to the ability of miR-122 mimic to partially reduce the expression of transgenes containing miR-122T sites in HEK293 cells, we tested whether transfection with miR-122 mimic would increase production of FGAd vectors encoding the Bax gene, since poor yields have been a major stumbling block in pro-apoptotic gene therapy with viral vectors. HEK293 cells were transfected with the miR-122 mimic or the miR-Neg a day before infection with Ad-mCMV-Bax-miR-122Tx6. Cells were also infected with Ad-mCMV-Luc-miR-122Tx6 and Ad-mCMV-Luc-miR-122Tx0 as Bax-negative control vectors. Virus production was determined four days post-infection by plaque assays in HEK293

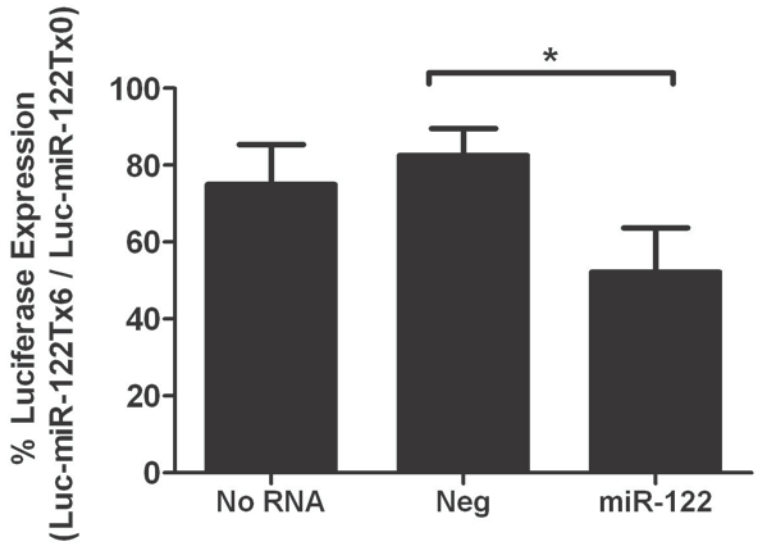

Figure A.8: Inhibition of luciferase expression in transiently-transfected HEK293 packaging cells infected with FGAd vector containing six miR-122T sites.

HEK293 cells were mock transfected (No RNA) or transfected with either a miR-122 mimic or a nontargeted miRNA mimic (Neg). The next day, cells were infected with FGAd vectors encoding luciferase gene either containing or not containing 6 miR-122T sites. One day post-infection, cells were harvested and luciferase expression was determined. % luciferase expression was determined by normalizing luciferase values obtained from cells infected with Ad-mCMV-Luc-miR-122Tx6 to Ad-mCMV-Luc-miR-122Tx0. Error bars correspond to +/- SD of triplicate wells (* - $p < 0.05$; t-test).

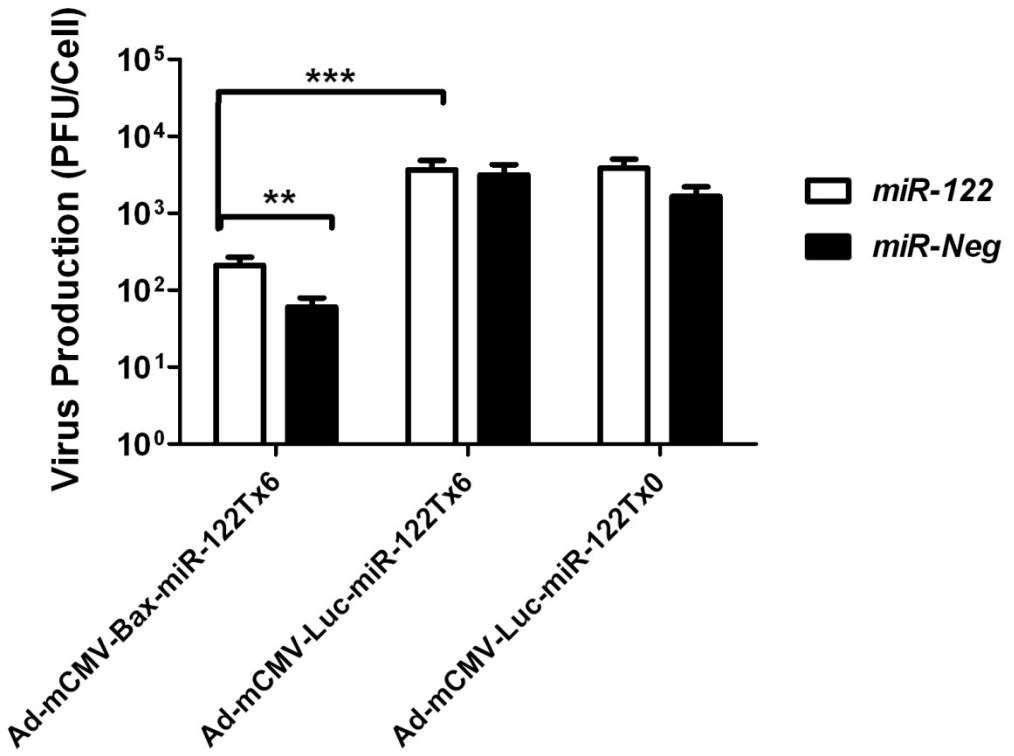

Figure A.9: Enhanced Bax-miR-122T-encoding FGAd vector production in HEK293 packaging cells transiently transfected with miR-122 mimic.

Cells were transfected with a miR-122 mimic or miR-Neg a day prior to infection with Ad-mCMV-Bax-miR-122Tx6, Ad-mCMV-Luc-miR-122Tx6 or Ad-mCMV-Luc-miR-122Tx0. Four days post-infection, cells were harvested and virus production was determined by plaque assays in HEK293 cells. Error bars correspond to +/- SD of duplicate infections (** - $p < 0.01$, *** - $p < 0.001$; t-test).

cells (Figure A.9). While Ad-mCMV-Bax-miR-122Tx6 production was slightly increased in HEK293 cells transfected with miR-122 mimic when compared to HEK293 cells transfected with miR-Neg, the level of production was not as high as with Ad-mCMV-Luc-miR-122Tx6. Therefore, transfection of miR-122 mimic was not sufficient to highly increase production of the Bax-encoding FGAd vector in HEK293 cells.

Interestingly, transient transfection of miR-122 mimic had no effect on Ad-mCMV-Luc-miR-122Tx6 production, suggesting that the previously reported role of miR-122 in the regulation of the interferon response (127) had no effect on FGAd vector production in HEK293 cells.

2.3.5 MiR-122T Insertion Reduced Plasmid-Encoded Gene Expression in HEK293 Cells Stably Expressing miR-122

We next determined whether stable expression of miR-122-containing hcr gene in HEK293 cells (293-miR-122 cells) would increase transgene inhibition relative to transient transfection of miR-122 mimic. 293-miR-122 cells were transfected with pmCMV-Bax-miR-122Tx6 with or without supplementation of the medium with 10 µg/mL tetracycline to induce miR-122 expression (546). Mock transfected cells or cells transfected with an EGFP expression vector (pEGFP) were used as negative controls. In addition, 293-miR-122-Mut, which contains mutations in the seed sequence of the mature miR-122, was transfected as an isogenic miR-122-negative control cell line. Bax expression was determined using western blot analysis two days post-transfection (Figure A.10). Bax expression was undetectable in negative control samples. Importantly, tetracycline

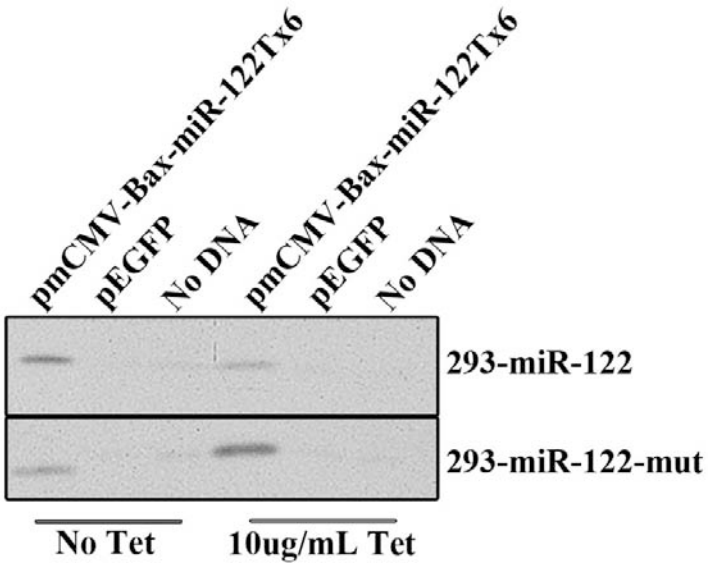

Figure A.10: Inhibition of exogenous Bax expression in 293-miR-122 cells following induction of miR-122 by tetracycline treatment.

Cells were seeded in a 24 well plate with or without the addition of 10 µg/mL tetracycline to induce miR-122 expression. The next day, cells were mock transfected or transfected with a Bax expression vector containing 6 miR-122T sites (pmCMV-Bax-miR-122Tx6) or an EGFP expression vector (pEGFP), as a Bax negative control. Two days post-transfection, cells were washed and lysed with RIPA. 10 µg protein was separated by SDS-PAGE, transferred to a western blot and probed with a Bax specific antibody.

treatment of 293-miR-122-mut cells did not reduce exogenous Bax expression, whereas tetracycline induction of miR-122 in 293-miR-122 cells resulted in significant inhibition of Bax expression following transfection with the modified Bax gene. However, further studies that include loading controls are required to validate these results.

In order to quantitate miR-122-inhibitory activity, tetracycline-treated 293-miR-122 and 293-miR-122-Mut cells were transfected with pmCMV-Luc-miR-122Tx6 and pmCMV-Luc-miR-122Tx0 and assayed for luciferase expression two days later (Figure A.11). The level of luciferase expression in 293-miR-122 cells transfected with pmCMV-Luc-miR-122Tx6 was found to be approximately 15 % of the luciferase expression in cells transfected with pmCMV-Luc-miR-122Tx0. Furthermore, luciferase expression in 293-miR-122-Mut cells transfected with pmCMV-Luc-miR-122Tx6 was lower than with pmCMV-Luc-miR-122Tx0. These results suggest that the insertion of the six miR-122T sites reduced luciferase gene expression in 293-miR-122 cells through miR-122-dependent and independent mechanisms.

A.3.6 MiR-122T Insertion Reduced FGAd Vector-Encoded Gene Expression in 293-miR-122

MiR-122-mediated inhibition of Bax expression was also determined in 293-miR-122 cells infected with FGAd vectors encoding the Bax gene with or without inserted miR-122T sites. Tetracycline-treated 293-miR-122 cells were infected with Ad-mCMV-Bax-miR-122Tx6, Ad-hTERT-Bax-miR-122Tx6 and Ad-hTERT-Bax-miR-122Tx0 at an MOI of 100 VP/cell. Cells were also infected

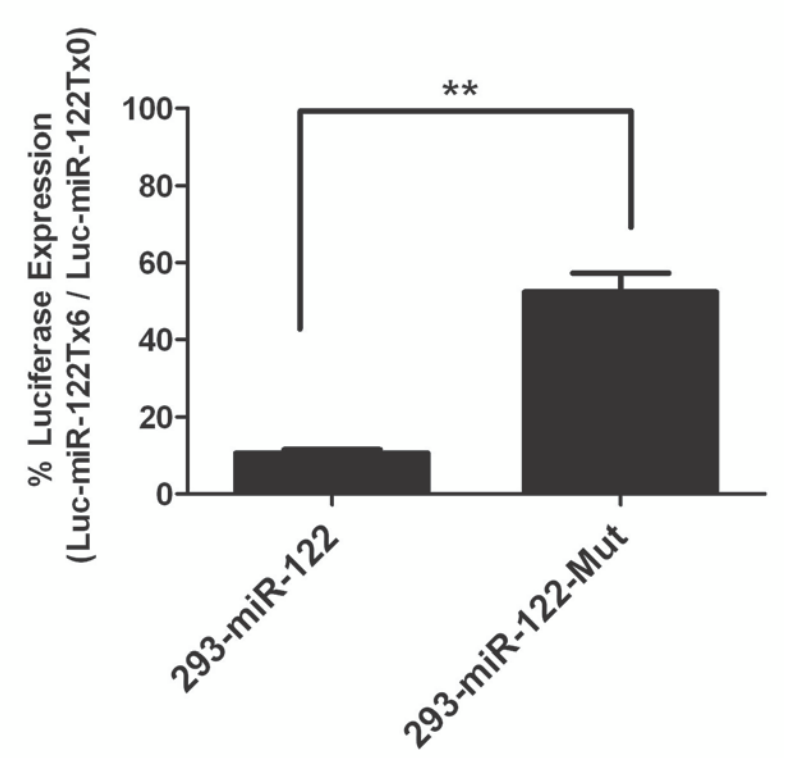

Figure A.11: Inhibition of luciferase expression in 293-miR-122 cells transfected with an expression plasmid containing six miR-122T sites.

10 µg/mL tetracycline-treated cells were transfected with firefly luciferase expression vectors with or without 6 miR-122T sites (pmCMV-Luc-miR-122Tx6 and pmCMV-Luc-miR-122Tx0, respectively) as well as a *Renilla* luciferase reporter plasmid as a transfection control. Two days post-transfection, cells were harvested and luciferase expression was determined. Firefly luciferase values were normalized to *Renilla* luciferase values in each well. Furthermore, % luciferase expression was determined by normalizing luciferase values obtained from cells transfected with pmCMV-Luc-miR-122Tx6 to pmCMV-Luc-miR-122Tx0. Error bars correspond to +/-SD of triplicate wells (** - $p < 0.01$; t-test).

with AdControl as a negative control. In addition, these viruses were used to infect tetracycline-treated 293-miR-122-Mut cells as a miR-122-negative control cell line. Two days post-infection, Bax and caspase-3 expression levels were determined by western blot analysis (Figure A.12). Bax and active caspase-3 levels were higher in 293-miR-122 cells infected with Ad-hTERT-Bax-miR-122Tx0 compared to infections with the Bax vectors containing miR-122T sites or AdControl. In contrast, Bax expression and caspase-3 cleavage levels were higher in 293-miR-122-Mut cells infected with the three Bax encoding vectors compared to AdControl. Since duplicate samples demonstrated reproducible results, and the cells had similar adenovirus transduction efficiencies, these results suggest that miR-122 specifically inhibited FGAd-encoded gene expression, however further studies including loading controls are required to validate these results.

In order to measure the miR-122-inhibitory level of expression of an FGAd vector-mediated gene in 293-miR-122 cells, tetracycline-treated 293-miR-122 and 293-miR-122-Mut cells were infected with Ad-mCMV-Luc-miR-122Tx6 and Ad-mCMV-Luc-miR-122Tx0. Two days post-infection, luciferase expression was determined (Figure A.13). Similar to the results found in transfections of Bax expression vectors, luciferase expression levels in both 293-miR-122 and 293-miR-122-Mut cells infected with Ad-mCMV-Luc-miR-122Tx6 were lower than Ad-mCMV-Luc-miR-122Tx0. However, a stronger inhibitory effect was found in 293-miR-122 cells compared to 293-miR-122-Mut cells.

Due to the ability of miR-122 to inhibit FGAd vector-mediated gene expression in 293-miR-122 cells, the effect of stable miR-122 expression on Bax-encoding FGAd vector production was also determined. Tetracycline-treated 293-miR-122 cells were infected with Ad-mCMV-Bax-miR-122Tx6 at an MOI of 1 PFU/cell for 4 days prior to determination of virus production by plaque assay in HEK293 cells. Cells were also infected with Ad-mCMV-Luc-miR-122Tx6 as a Bax-negative control vector. In addition, virus production was determined in HEK293 cells and tetracycline-treated 293-miR-122-Mut cells as miR-122-negative controls (Figure A.14). Similar to virus production in HEK293 cells transiently transfected with miR-122 mimic, Ad-mCMV-Bax-miR-122Tx6 production was increased in 293-miR-122 cells when compared to 293-miR-122-Mut and HEK293 cells. However, Ad-mCMV-Bax-miR-122Tx6 production did not reach as high as Ad-mCMV-Luc-miR-122Tx6, suggesting that stable miR-122 expression was not sufficient to allow completely unhindered production of the FGAd vector encoding Bax.

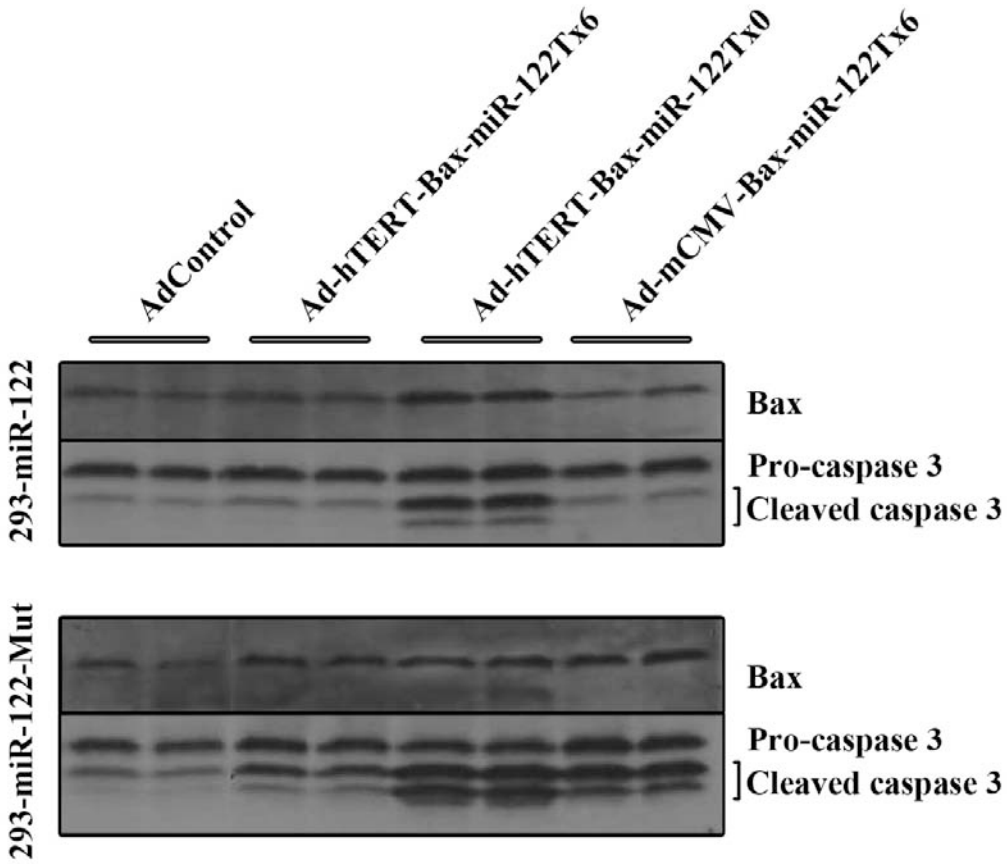

Figure A.12: Inhibition of Bax expression and caspase 3 activation in 293-miR-122 cells infected with FGAd vectors containing six miR-122T sites.

10 µg/mL tetracycline-treated cells were infected in duplicate with Bax-encoding FGAd vectors with or without 6 miR-122T sites (Ad-mCMV-Bax-miR-122Tx6, Ad-hTERT-Bax-miR-122Tx6 or Ad-hTERT-Bax-miR-122Tx0). As a Bax negative control, cells were infected with AdControl. Two days post-infection, cells were washed and lysed with RIPA. 10 µg of protein was separated by SDS-PAGE, transferred to a western blot and probed with a Bax or a caspase 3 specific antibody.

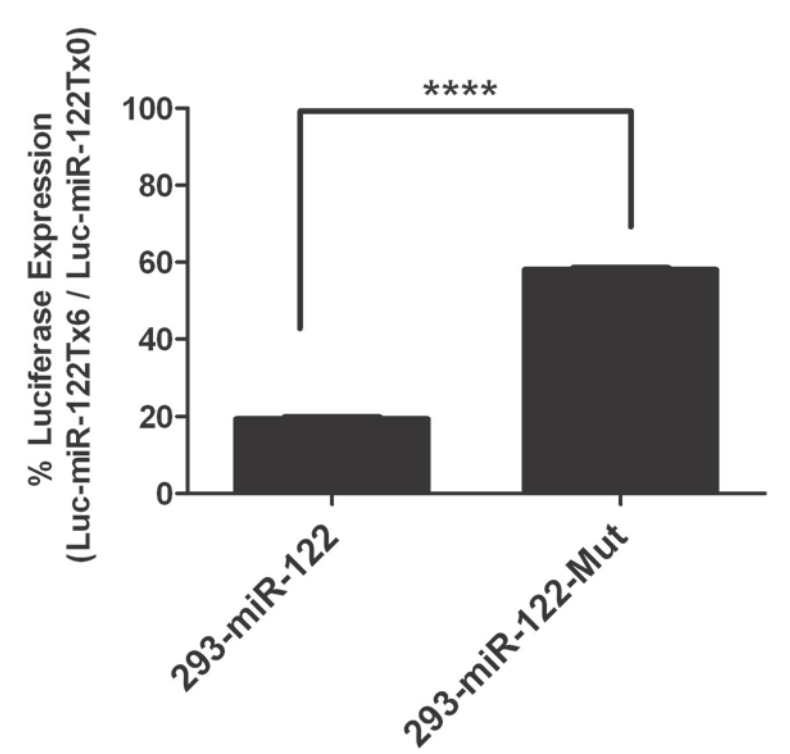

Figure A.13: Inhibition of luciferase expression in 293-miR-122 cells infected with FGAd vector containing six miR-122T sites.

10 µg/mL tetracycline-treated cells were infected with firefly luciferase-encoding FGAd vectors with or without 6 miR-122T sites (Ad-mCMV-Luc-miR-122Tx6 and Ad-mCMV-Luc-miR-122Tx0, respectively). Two days post-infection, cells were harvested and luciferase expression was determined. % luciferase expression was determined by normalizing luciferase values obtained from cells infected with Ad-mCMV-Luc-miR-122Tx6 to Ad-mCMV-Luc-miR-122Tx0. Error bars correspond to +/-SD of triplicate wells (** - $p < 0.01$; t-test).

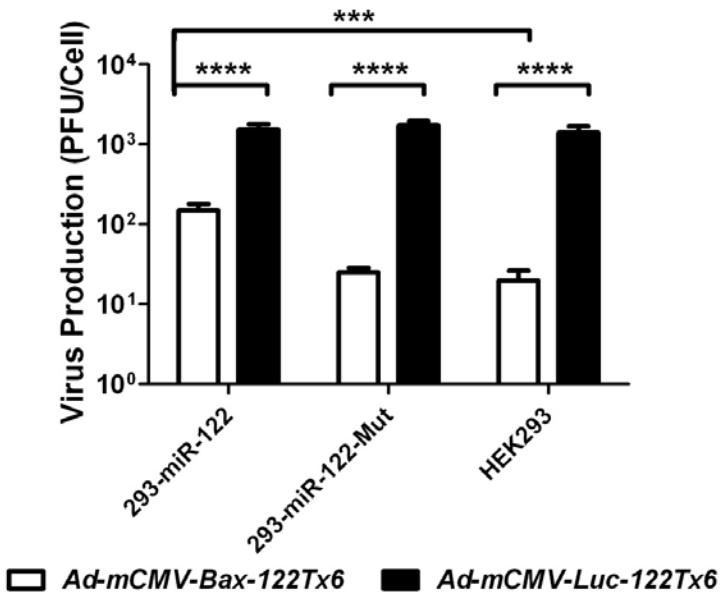

Figure A.14: MiR-122T insertion increased Bax-encoding FGAd vector production in 293-miR-122 cells.

Cells were infected with Ad-mCMV-Bax-miR-122Tx6 or Ad-mCMV-Luc-miR-122Tx6 vectors. Four days post-infection, cells were harvested and virus production was determined by plaque assays in HEK293 cells. Error bars correspond to +/- SD of duplicate infections (*** - $p < 0.001$, **** - $p < 0.0001$; ANOVA).

A.4 DISCUSSION

FGAd-encoded toxic genes have high potential for cancer gene therapy, however, the amplification of these vectors has been highly challenging due to the induction of packaging cell death. Therefore, many strategies have been pursued to inhibit apoptosis induction of infected packaging cells. One study has shown that stable expression of the poxvirus anti-apoptotic protein crmA in HEK293 cells allowed increased titers of an FGAd encoding the pro-apoptotic Fas gene (544). However, as induction of cell death was found to be important in virus release (545; 556), stable expression of anti-apoptotic genes may reduce FGAd titers. Another method to reduce packaging cell death is through the inhibition of FGAd-mediated transgene expression.

Previous studies have found that the insertion of endogenous miRNA targeted sequences in the 3'UTR of transgenes post-transcriptionally inhibited virus-mediated transgene expression (345; 557). Furthermore, the addition of miR-122T sites to a firefly luciferase reporter gene was found to reduced gene expression in miR-122-positive HCC cells and mouse hepatocytes (344-346), therefore, we wanted to determine whether insertion of miR-122T sites in the 3'UTR of an FGAd-encoded Bax gene would reduce Bax expression, and thus, increase virus titers in miR-122-positive packaging cells.

In order to validate miR-122-mediated inhibition, a sequence containing six miR-122T sites was designed and inserted into the 3'UTR of an FGAd-encoded firefly luciferase gene. MiR-122-mediated inhibition of luciferase expression was then measured in miR-122-positive and -negative infected HCC

cells. Similar to other reports (345), we found that the insertion of miR-122T sites reduced luciferase expression by 1 log in the miR-122-positive HuH7.5 but by very little in the miR-122-negative HCC cells, HepG2 and Hep3B. As further validation of miR-122-mediated luciferase inhibition, HepG2 cells transiently transfected with a miR-122 mimic also showed a 1 log reduction in luciferase expression following infection with the FGAd carrying the miR-122T sites.

In comparison to the results with HepG2 cells, however, transient transfection of miR-122 in HEK293 packaging cells resulted in reduced miR-122-mediated inhibition of gene expression. Replicating Ads were previously shown to inhibit the RNAi pathway through saturation of key enzymes, which bind and process the adenoviral VA-RNAs into miRNAs (272-275). While studies have shown that nonreplicating FGAds had no effect on miRNA processing (558), the high level of VA-RNA expression during FGAd replication in HEK293 cells might have resulted in reduced miR-122-mediated inhibition. In addition to VA-RNA saturation of the RNAi pathway, the high levels of miR-122T-containing transcripts might have saturated the miR-122 molecules, which might have also resulted in reduced miR-122-mediated inhibition.

Because miRNA mimics enter the RNAi pathway at the RISC-binding level, we wished to determine whether miR-122-mediated inhibition could be enhanced in HEK293 cells expressing pri-miR-122 (293-miR-122) (546), in which miR-122 processing would require the full RNAi pathway. MiR-122-mediated inhibition was higher in these packaging cells than in HEK293 cells transiently transfected with miR-122 (80% vs. 50%). Furthermore, the level of

miR-122-mediated inhibition found in 293-miR-122 was similar to that in the miR-122-positive HuH7.5 cells (80% and 90%, respectively).

Interestingly, although the mutation in the seed sequence of miR-122-Mut was expected to lack miR-122 activity, inhibition of miR-122T-containing luciferase expression was also found in these cells. Similarly, inhibition was observed following infection of these cells with the miR-122T-containing Bax vectors. These results might be due to incomplete loss of miR-122 activity even with mutations in the seed sequence. However, inhibition of gene expression might have been due to a miR-122-independent mechanism, since inhibition of miR-122T-containing luciferase expression was also observed in miR-122-negative HepG2 and HEK293 cells. This miR-122-independent mechanism might have been a result of secondary structures in the 3'UTR resulting from the 6 tandem repeats of miR-122T sequences.

While the insertion of miR-122T sites reduced FGAd-mediated Bax expression in 293-miR-122 infected cells through miR-122-mediated post-transcriptional inhibition as well as through miR-122-independent mechanism, this reduction in gene expression was not sufficient to increase the levels of virus production to that of the luciferase-encoding virus. Therefore, in addition to RNAi-mediated post-transcriptional gene silencing, additional gene inhibitory mechanisms, such as transcriptional regulation, would be required for adequate inhibition to allow for toxic gene-encoded FGAd amplification in miR-122-positive packaging cells.