Improving adenovirus efficacy with p14 fusion associated small transmembrane protein expression for cancer treatment

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ABSTRACT

Adenovirus (Ad) has been one of the most commonly used vectors in gene therapy for many years. One of the limitations of using Ad for cancer therapy is that Ad does not spread within a tumor well. As such, fusogenic proteins have been incorporated into these vectors in an attempt to improve spread through cell-cell fusion. Fusogenic protein mediated cell fusion is also associated with syncytiosome release and cell death, which promote an immune response and suggests that fusogenic proteins may be effective as a sole therapeutic for cancer treatment. In this study, I examined whether the reptilian reovirus p14 fusion-associated small transmembrane (FAST) protein can improve Ad efficacy in cancer models *in vitro* and *in vivo.* Ad-mediated FAST protein expression induced cell-cell fusion in human embryonic kidney 293 cells and in human lung adenocarcinoma A549 cells. Infected cells had decreased membrane integrity, cellular metabolic activity and increased cleaved caspase-3 expression. In an A549 xenograft nude mouse model, AdFAST did not induce tumor cell fusion or promote mouse survival compared to control mice. In murine 4T1 mammary carcinoma cells, FAST protein expression had modest effects on cell fusion and metabolic activity *in vitro*. When AdFAST was administered intratumorally in a 4T1 syngeneic mouse model, there were no obvious signs of cell fusion or improved mouse survival. As FAST protein expression promotes membrane permeability, whether AdFAST could enhance the bystander effect of cancer therapies or promote uptake of anti-cancer drugs was investigated *in vitro*. FAST protein expression in combination with thymidine kinase/ganciclovir or etoposide administration did not have an enhanced effect. Addition of Smac-mimetic compound (SMC) was not effective in the SMC resistant A549 cell line, but the combinational therapy was able to decrease metabolic activity in SMC susceptible SNB75 cells. AdFAST and administration of the membrane impermeable chemotherapeutic

drug bleomycin decreased metabolic activity and promoted cleaved caspase-3 expression in A549 cells. Overall, my results suggest that FAST protein expression could improve adenovirus efficacy and could be used with other anti-cancer treatments.

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LIST OF ABBREVIATIONS

Δ24: E1A deleted of 24 amino acids
aa: amino acid
α-tubulin: alpha-tubulin
Ad: adenovirus
Ad5: adenovirus serotype 5
ANOVA: analysis of variance
ATP: adenosine triphosphate
β-Gal: beta-galactosidase
BGHpA: bovine growth hormone polyadenylation sequence
calcein AM: calcein acetoxymethyl ester
CAR: Coxsackie-adenovirus receptor
cDNA: complementary DNA
cFLIP: cellular FLICE-inhibitory protein
CMV: cytomegalovirus
DC: dendritic cell
DMEM: Dulbecco's Modified Eagle Medium
DMSO: dimethyl sulfoxide
E1: early region 1
E3: early region 3
ER: endoplasmic reticulum
FAST: fusion-associated small transmembrane protein
FADD: Fas-Associated protein with Death Domain
FBS: fetal bovine serum
FOLFOX: 5-fluorouracil, leucovorin and oxaliplatin
GALV: Gibbon-ape leukemia virus
GFP: green fluorescent protein
GMCSF: granulocyte macrophage colony-stimulating factor
HA: hemagglutinin
H/E: hematoxylin and eosin
HeLa/CD4+: HeLa cells expressing the HIV-1 receptor CD4
HIV-1: human immunodeficiency virus type 1
hpi: hours post infection
HRP: horseradish peroxidase
HSV-1: Herpes Simplex Virus-1
HUVEC: human umbilical vein endothelial cell
IgG: immunoglobulin G
IAP: inhibitor of apoptosis
IFN: interferon
IFNγ: interferon-gamma
IL: interleukin
IRF3: interferon regulatory factor-3
ISG: interferon stimulated genes
ITR: inverted terminal repeat
LDH: lactate dehydrogenase
MEM: Minimum Essential Media

MEF: mouse embryonic fibroblast
MHC: major histocompatibility complex
mL: millilitre
MLP: major late promoter
MOI: multiplicity of infection
MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MV: Measles virus
NDV: Newcastle disease virus
NK: natural killer
orf: open reading frame
PBS: phosphate buffered saline
PBM: polybasic motif
pfu: plaque forming units
PVDF: polyvinylidene fluoride
Rb: retinoblastoma protein
RFP: red fluorescent protein
RSV: respiratory syncytial virus
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
Smac: second mitochondria-derived activator of caspase
SMC: SMAC-mimetic compound
TK: thymidine kinase
TERT: telomerase reverse transcriptase
TNFα: tumor necrosis factor-alpha
TRAIL: tumor necrosis factor-related apoptosis-inducing ligand
VV: Vaccinia virus
VSV: Vesicular stomatitis virus

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CHAPTER 1: Introduction

1.1 The problem of cancer

Cancer cells have distinct capabilities, also known as the hallmarks of cancer, first reviewed by Hanahan and Weinberg in 2000 (1, 2). Such hallmarks promote tumorigenesis through persistent proliferative signaling and unresponsiveness to growth suppressors, leading to cell death resistance and indefinite cell division. Cancer cells also promote cell invasion/metastasis and angiogenesis. Research over the years has identified new characteristics and potential hallmarks (2). The two new characteristics include mutations or genome stability and inflammation that promote tumorigenesis, which lead to acquisition of the hallmarks of cancer. Cells in the tumor microenvironment such as cancer-associated fibroblasts, immune cells and pericytes, can also play a role in cancer progression. The new potential hallmarks involve energy metabolism reorganization and being able to avoid eradication by the immune system. Given the complexity of cancer, finding effective therapeutics to combat the disease is an ongoing effort.

Currently, the most common treatments for cancer are surgery, chemotherapy and radiation therapy (3). Depending on the type and stage of cancer, these treatments may be used in combination to maximize the therapeutic benefit and promote patient survival (3). While established treatments are quite effective, there is currently only an overall 63% relative survival rate of at least 5 years after diagnosis (4). Naturally, there is variation in relative survival rate depending on the type of cancer. For example, thyroid cancer patients have a 98% 5 year survival rate while those with pancreatic cancer have a survival rate of 8% (4). As such, more effective therapeutics or combinational therapies need to be developed.

1.2 Viruses in cancer therapy

Viruses used for cancer therapy can be separated into two classes. Viruses in the first class are rendered replication defective through deletion of genes essential for virus replication, but can be engineered to express exogenous anti-cancer genes, such as cell suicide genes or immunostimulatory molecules (5). Alternatively, viruses can be modified to allow for selective replication in cancer cells while leaving normal cells intact, and these vectors are termed oncolytic or conditionally-replicating vectors (6).

Viruses for cancer therapy currently in late stages of clinical trial are JX-594 (Jennerex Inc.), a Wyeth strain vaccinia virus (VV) deficient of thymidine kinase (TK) and expressing the immunostimulatory protein granulocyte-macrophage colony-stimulating factor (GMCSF) and β-galactosidase (β-Gal) (7), Oncovex(GMCSF) (Amgen/Biovex), which is derived from the Herpes Simplex virus-1 (HSV-1) JS1 strain and also expresses GMCSF (8, 9) and the reovirus serotype 3 Dearing strain Reolysin® (Oncolytics Biotech Inc.) (10).

At this time, there are three approved viruses for cancer therapy. One of them is an ECHO 7 virus, known as Rigvir and was made available in Latvia as of 2005, for treatment of patients with melanoma (11, 12). Rigvir is an oncolytic virus that promotes expression of tumor-associated differentiation antigens while downregulating expression of tumor promoting, melanoma-associated antigens (12). The other two approved viruses are adenovirus-based vectors and will be discussed in the following section.

1.3 Adenoviruses for cancer treatment

1.3.1. Adenovirus genome and life cycle

Ads are non-enveloped viruses with a double stranded DNA genome and were first discovered in adenoid tissue and as a cause of respiratory infection (13-15). Ads are

typically 70-100 nm in size and have genomes ranging from 30 to 40 kb (15). The Ad capsid is made of facets and vertices constructed from trimers of protein II (hexon) and five copies of protein III (penton), respectively (16). The fibre protein, which is composed of three protein IV copies, extend from the penton (16). Minor capsid proteins such as IIIa, IVa2, VI, VIII and IX help maintain the capsid structure (16, 17).

One of the most studied human Ads, which is also used in gene therapy is Ad serotype 5 (Ad5) (18). Its genome is ~36 kDa (Figure 1.1, adapted from (19)). The inverted terminal repeats (ITRs) are the origins of replication and are found at both ends of the genome. The packaging element, responsible for DNA packaging into viral particles, is located directly after the left ITR. Ad proteins are classified into two groups: early and late proteins. The early proteins are first transcribed and expressed and are essential in mediating viral replication and immune evasion. Early region 1 (E1) A is a main transactivator of other Ad promoters and one of its functions is to inhibit the tumor suppressor retinoblastoma protein (Rb) to allow for transformation and cell cycle progression (20-23). E1B proteins (19kDa and 55kDa) can inactivate the tumor suppressor p53 to promote unregulated cell division and inhibit apoptosis (24, 25). The E2A and E2B proteins are involved in Ad DNA replication (26). The E3 region encodes various proteins such as the Ad death protein and 19K that function to evade the immune response and improve virus release (27-29). Finally, E4 region proteins promote viral replication and inhibit cellular function (30-32). After DNA replication, there is a switch from early to late protein expression (33). Major late proteins include structural proteins (i.e. proteins for fibre, penton, hexon) and are expressed under the control of the major later promoter (MLP) and via alternative splicing of the corresponding transcript (L1-L5 in Figure 1.1) (34-37). Apart from the major late proteins,

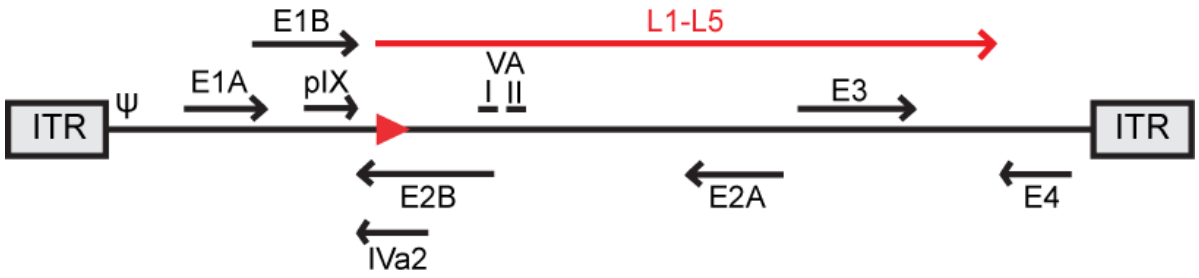

E1B
L1-L5
VA
I II
E1A
pIX
ITR
Ψ
E3
ITR
E2B
E2A
E4
IVa2

Figure 1.1: Simplified schematic of the Ad5 genome. Transcripts encoding early gene proteins (E1-E4), protein IX, IVa2 and RNA VA are depicted by the black arrows. The major late promoter (red arrow) produces L1-L5 transcripts through alternative splicing. The inverted terminal repeats act as initiators of replication and are shown as "ITR" at both ends of the genome. The packaging element (Ψ) is located at the left terminal end of the genome. Of note, the diagram is not to scale. The schematic is based on the diagram published by Giberson *et al.* (19).

there are also intermediate/late expressed gene products such as protein IX, IVa2, and the VA RNA products (38-40).

Ad5 infection is initiated through the interaction between the Ad fibre protein and the Coxsackie-adenovirus receptor (CAR) (41, 42). Initial binding can also be mediated through direct and indirect interactions with heparan sulfate proteoglycans (43-46). Secondary Ad interactions with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins promote virus internalization via endocytosis (47, 48). The Ad capsid is partially disassembled in the endosome and escapes the early endosome using protein VI (49-51). Ad then uses the microtubule network to travel to the nucleus and the capsid is completely disassembled during transport, to allow for the Ad DNA and protein VII complex translocation into the nucleus via the nuclear pore (49, 50, 52-54). Within the nucleus, viral replication and progeny assembly occurs. Viral progeny are released through breakdown of the nuclear envelope and cell death (28, 29).

Ad can be made replication defective through deletion of E1 genes (5). First generation Ads are typically deleted of the E1 and E3 regions while second generation Ads include further deletions in the E2 or E4 region (5). Ad vectors that do not contain any viral genes are termed helper dependent Ads (5). Replication defective Ads are mostly used to express transgenes for gene therapy (5). Conditionally replication competent Ads are modified to specifically target and replicate in cancer cells (5). This can be accomplished through using cancer specific promoters or viral gene deletions (5).

1.3.2. Approved adenovirus-based cancer treatments

Two Ad-based vectors are commercially available for cancer therapy in China (55-57). The first virus, also known as Gendicine®, is the very first gene therapy vector sold on the market for cancer treatment (55-57). It is a replication defective Ad expressing human p53 under the control of a Rous sarcoma virus promoter and a bovine growth hormone

polyadenylation sequence (BGHpA) (55, 56). Gendicine® was initially used for treatment of head and neck cancer and typically combined with radiotherapy, but has since been tested for other cancers (55, 56).

The second approved Ad, Oncorine® (H101) is an oncolytic Ad5 vector with deletions in the adenovirus E1B-55kDa protein (55, 57). It works essentially the same as ONYX-015 (discussed in section 1.3.3), where cancer cells are able to circumvent the loss of E1B-55kDa-mediated viral mRNA export (55, 58). Its development was also for the treatment of head and neck cancer and has been tested in various carcinomas (55).

1.3.3. Adenovirus vectors in clinical trials

One of the most well known Ads tested in clinical trial is ONYX-015. ONYX-015 is a chimeric Ad2 and 5 vector with an ~800 bp deletion in the adenovirus E1B-55kDa gene (55, 59, 60). As E1B normally inactivates the tumor suppressor p53 to allow for virus replication, ONYX-015 would selectively replicate in cancer cells with defective or inactivated p53 (59, 60). Later studies showed that the virus replication was not dependent on p53 activity and that the E1B deletion allowed for specific replication in cancer cells that were able to mediate late viral mRNA export (58, 61). ONYX-015 completed phase I and II clinical trials in North America and later, Shanghai Sunway Biotech, who initially developed Oncorine®, bought the rights to ONYX-015 (55, 60, 62-66).

While ONYX-015 is one of the most known Ads tested in clinical trial, numerous trials have been conducted using replication defective Ads. In this section, we will highlight the replication defective Ads that have completed clinical trials since 2000. Even though Gendicine® has been approved for head and neck cancer, it is currently being tested extensively for its use for other cancers. Gendicine® administration with fractionated stereotactic radiotherapy for hepatocellular carcinoma increased 1 yr disease free survival,

overall survival rate and median survival time (67). In a phase II study, Gendicine® was tested using a trans-tracheal injection method and in combination with the chemotherapeutic drug docetaxel (68). While the treatment was well tolerated, it unfortunately did not improve overall survival or efficacy compared to the control group given docetaxel. In another study, Gendicine® and 5-fluorouracil did not improve overall response rates in hepatocellular carcinoma patients who underwent transcatheter arterial chemoembolization compared to the control group, but increased p53 mRNA and p53-targeted gene mRNA expression in a majority of treated patients (69). With Gendicine® administration combined with radiotherapy after oral cancer radical surgery, significantly decreased overall recurrent rates and increased the 3 yr disease-free survival rates were observed in the treatment group compared to the control patients (70). A phase III trial investigating Gendicine® with chemotherapy in the treatment of oral cancer showed a better response rate and increase in p53 related activity ~80% of patients (71). Overall, the numerous clinical studies involving Gendicine® highlight the efficacy of this virus for various cancer types, making it one of the most promising replication defective Ad vectors for cancer treatment to date.

Another replication defective Ad expressing p53 has been tested in various clinical trials in the United States. INGN 201, also known as Advexin®, expresses p53 under the control of the cytomegalovirus (CMV) promoter (72-75). In a phase I study, intraveneous administration of INGN 201 in patients with solid tumors showed mostly grade I and II adverse effects and vector-mediated p53 expression was detected in tumor biopsies from a majority of treated patients 4 days post transfusion. However, no objective response rates were observed (74). In another phase I study, INGN 201 was administered intraperitoneally into patients with platinum and paclitaxel resistant ovarian cancer (75). Only one partial response was observed in a patient that received 30 rounds of treatment (5 daily injections

every 3 weeks). As the study focused on safety and the number of treatments varied between patients (ranging from 1-30 cycles), the study could not conclude the ideal dosage, treatment regime or determine efficacy (75). A phase I/II study used INGN 201 in combination with radiation therapy in a 6-week regime for non-small cell lung cancer patients that could not undergo surgery or chemoradiation (73). Three months after treatment was completed, 63% of patients showed a complete or partial response while 16% had stable disease. Most patients had increased viral p53 DNA and mRNA expression one day post injection and were significantly higher than in pretreatment biopsies. In a phase II study, subjects with stage III breast cancer given INGN 201 and chemotherapeutic drugs led to an increase in p53 mRNA expression and all patients showed a partial response, even though the trial was terminated early (72). Taken together, Gendicine® and INGN 201 indicate that non-replicative Ad vectors can effectively express p53 and in combination with other cancer treatments, hold promise for future therapies.

Another replication defective Ad that has clinical potential is E10A, an Ad that expresses the anti-angiogenic endostatin gene under the control of a CMV promoter and SV40 polyadenylation signal (76, 77). In a phase I trial, patients with solid tumors were intratumorally injected with E10A weekly for two weeks. The treatment was well tolerated and increased endostatin in the circulation was observed (76, 77). Progression to a phase II trial with head and neck cancer patients in combination with chemotherapeutic drugs resulted in a longer progression-free duration, higher overall disease control rate, compared to patients receiving chemotherapy only (78). Three to four treatment cycles of E10A and chemotherapy every three weeks was deemed most effective between patient groups. Endostatin expression was detected in the circulation and biopsy samples, indicating

effective and persistent Ad-mediated transgene expression. Given that the treatments were well tolerated, a phase 3 clinical trial is currently in progress.

Numerous replication deficient Ads modified to express immunostimulatory molecules have also advanced to clinical studies. A phase I trial involving patients with cutaneous T and B cell lymphomas intralesionally injected with Ad expressing the IFN-gamma (IFNγ) cytokine under the control of the CMV promoter (TG1042) showed a decent safety profile with 5 out of 9 patients having a complete or partial response (79). IFNγ mRNA production was observed in 7 patients. Infiltration of immune cells was observed in patient biopsies and increased Ad neutralizing antibodies were present following TG1042 treatment. Neutralizing Abs did not appear to affect transgene expression. A subsequent phase II resulted in a complete or partial response in 53% of evaluable patients and had effects on uninjected tumors in 4 out of 15 patients (80). Some patients had an increase in cytokine expression and Ad-neutralizing antibodies, but there did not correlate with clinical outcomes. A phase II trial involving recurring cutaneous B cell lymphoma patients resulted in 54% complete responses and 31% partial responses in injected tumors (81), highlighting the efficacy of TG1042 in cutaneous lymphoma patients.

While several non-replicative Ads show promise in the clinic, others lack efficacy in clinical studies or have just completed phase I trials, making determination of efficacy difficult. An Ad expressing the CD40 ligand for bladder carcinoma treatment showed efficient transgene expression and well tolerated treatments, but the study did not directly assess efficacy and had a small number of patients (82). In another study intratumoral injections of an Ad vector expressing interleukin (IL)-12 in pancreatic, colorectal or liver cancer patients resulted in stable or progressive disease with increased immune cell infiltration in some patients (83). No significant increase in serum IL-12 levels was observed,

suggesting poor transduction and/or limited transgene expression. A phase I/II study in which patients with solid tumors or melanoma were intratumorally given cycles of replication deficient Ad expressing IL-2 (TG1024) treatment resulted in well tolerated administration and an increase in IL-2 production following injection (84). Only 6 patients (17%) achieved an objective response. In phase I trials using a replication defective Ad expressing IFNβ for patients with malignant mesothelioma and pleural effusion, a single intrapleural dose allowed for efficient gene expression and led to immune system activation in 70% of patients (85). Two treatments of the virus were tolerable, but limited gene expression was observed and patients also had increased Ad neutralizing antibodies titres (86). Ad expressing tumor necrosis factor-alpha (TNFα) controlled by the chemoradiation EGR-1 promoter, in combination with chemotherapy and radiation caused a response rate of 83.3% in head and neck cancer patients (87). The same Ad used in a phase I/II trial involving pancreatic cancer patients led to a 8% response rate (i.e. complete and partial response) (88). A phase III trial was initiated, but has been halted due to lack of efficacy (88).

Replication defective Ad combined with prodrug based treatments have also been examined in clinical trials. For example, a replication deficient Ad expressing the *Escherichia coli* nitroreductase (CTL102), which converts the compound CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) into a DNA cross linking molecule, has been tested (89-91). In a phase I clinical trial, intratumoral injections into patients with liver or hepatic metastatic colorectal cancer and administrated CB1954 demonstrated no major adverse effects and dose-dependent transgene expression in resected tumor cells (90). In a phase I/II trial with prostate cancer subjects, Ad-mediated transgene expression was observed in injected prostates (91). Virus and prodrug treatment reduced prostate-specific antigen levels

(>10% decrease) in 7 out of 19 patients. Pre-existing Ad immunity, Ad neutralizing antibodies and T cell numbers were not indicative of treatment outcome (89, 91). Only 3 out of 11 subjects showed an increase in tumor-specific T cells (89). Improvements of virus delivery or treatment regime could be made and a phase III trial would provide a clear analysis of whether clinical responses can be obtained (91).

Numerous studies have also been conducted with non-replicative Ads expressing HSV-1 TK and will be discussed in more detail in chapter 5. It is important to note that most phase I or II studies have a small number of patients, which may not be reflective of clinical applications and focus on safety, rather than virus efficacy. Overall, all these studies using replication deficient Ad show that virotherapy resulted in Ad-mediated transgene expression and well-tolerated treatments, holding promise for new emerging therapies.

1.3.4. Limited intratumoral Ad spread in cancer treatment

While virotherapy for treating cancer has great potential, efficacy is limited due to poor virus distribution in solid tumors and this issue is more prominent with intratumoral injections (92). Limited viral distribution can be attributed to intratumoral pressure gradients, cancer cell heterogeneity, physical barriers such as the tumor vasculature and extracellular matrix, and effectiveness of viral replication and progeny release for oncolytic vectors (92-94). In one study, human A549 cells were infected with an Ad that does not express the E3 14.7K, 14.5K and 10.4K proteins (Ad309) and mixed with non-infected human A549 cells at a ratio of 1:100 before inoculation into nude mice (95, 96). No tumor formation was observed in the nude mice, but when Ad309 was injected into established tumors, it could not completely facilitate tumor regression (95). Virus, however, was detected 100 days post injection, suggesting intratumoral virus distribution was limited. Similarly, an oncolytic Ad with a deletion in the E1B-19kDa protein was able to prevent tumorigenesis when mixed

with uninfected tumor cells at a ratio of 1:1000 at initial injection, but failed to promote complete tumor eradication in a treatment model (95). In another study, a population of 5% ONYX-015-infected cells at the time of initial injection was enough to inhibit tumorigenesis (97). Again, intratumoral injections in established tumors reduced tumor size, but complete regression was not observed in treated mice (97). Furthermore, ONYX-015 was not able to significantly inhibit tumor growth in distal uninjected tumors (98), suggesting that a major limiting factor in Ad efficacy is vector distribution.

Ad distribution has also been studied in various cancer tumor models. In an A549 xenograft nude mouse model, 10^9 virus particles of Ad309 were intratumorally injected and studies were conducted over an 8 week period (94). Tumors excised at various timepoints in the first 24 h contained a maximum of 10^6 plaque forming units (pfu) of virus and intratumoral Ad titre increased and stabilized after 24 h. The decrease in intratumoral Ad was likely due to circulation dissemination and clearance by innate immunity. Ad was initially detected in the circulation in the first 24h and at 4 and 8 weeks post injection. Virus also localized to the liver and lungs at 8 weeks post injection. Ad was distributed unevenly in excised tumors 60 days post injection and also found near intratumoral blood vessels, suggesting that Ad re-infected the tumor after circulation. It is important to note that Ad does not replicate in murine cells, which would mean that murine-derived connective tissue could act as physical barriers for virus spread throughout the tumor. This was observed in excised tumor sections as Ad mostly localized between necrotic areas and connective tissue (94). In another study, an oncolytic Ad with an E2F-1 promoter controlling expression of E1A protein deleted of the 24 amino acids (Δ24) responsible for Rb binding was intravenously injected into nude mice with A549 tumors (21, 99). While treatment slowed tumor growth, it did not cause complete tumor regression. Staining of excised tumors

showed that patches of Ad-positive staining surrounded by murine connective tissue, indicating that murine cells can act as a physical barrier for intratumoral Ad spread, and thus, limiting vector efficacy (99). In syngeneic cancer models, intratumoral injections of a replication defective Ad expressing green fluorescent protein (GFP) under the control of the CMV promoter showed most GFP fluorescence localized to the liver, spleen and lung with minimal GFP expression in areas near the administration site 24 h post injection (100).

When a non-replicative Ad expressing p53 was tested in a phase I clinical trial with recurrent glioma patients, p53 staining of specimens after treatment showed the transgene expression was limited to an average of 5 mm around the injection tract (93). Apoptotic cells were also centered near the injection site, suggesting that while the vector was effective, it did not disseminate through the tumor well. A phase I/II study with CTL102 showed that transgene expression was limited to a small area around the injected tract of intratumorally injected prostate cancer tumors (91). Virally-transduced areas did, however, increase with injection volumes, suggesting a better treatment regime to improve virus distribution and subsequent, transgene expression.

In another phase I study, patients with various types of advanced cancer were injected intratumorally with INGN 241, a replication defective Ad expressing the IL-24 (101, 102). Within the tumor, viral DNA and RNA levels were highest at the injection site and decreased with further distance from the injection site (101). Similarly, staining of tumor biopsies showed IL-24 transgene expression at the injection site with decreased expression at distal regions ($\geq$ 12mm) (102). IL-24 expression was detected as far as 3 cm, where viral vector was not detected, from the injection sites in some samples (101). Areas expressing IL-24 also correlated with TUNEL staining, indicating the vector and transgene expression promoted apoptosis (102). Overall, high intratumoral viral copies were found in the first 48

h and decreased drastically by day 4 post injection. Only 0.0001% of initial injected vector was detected at day 30 (102). This correlated with high (740-900) copies of viral vectors per cell in the first 48 h, which decreased to about one viral genome per cell by day 4 post injection (101). Hence, improvement of Ad retention or distribution within a tumor could provide a better anti-cancer effect. Taken together, these studies highlight the challenges of limited intratumoral Ad distribution in cancer therapy.

1.4 Expression of fusogenic proteins for cancer treatment

1.4.1. Fusogenic protein expression from viral vectors

To overcome the problem of limited vector efficacy, various groups have utilized vector-mediated expression of membrane fusion proteins. The idea stems from the fact that virus fusogenic proteins induce cell-cell fusion, which could allow for lateral transfer of viral genes and anti-cancer transgenes. In combination with syncytia cell death and viral lysis, an enhanced therapeutic effect could be achieved (103).

Previous studies have highlighted the efficacy of viral vectors expressing membrane fusogenic proteins and have focused on fusogenic proteins from enveloped viruses. Administration of lentiviral vectors pseudotyped with VSV-G expressing Gibbon ape leukemia virus (GALV) fusogenic protein into mice with fibrosarcoma xenografts allowed for complete tumor regression in all treated mice with small tumors (i.e. 1-4 mm diameter) (104). Isolated tumor cells after 48 h post injection and grown *in vitro* showed syncytia formation while cells extracted from mice treated with the control virus and phosphate buffered saline (PBS) did not, suggesting that GALV fusogenic protein can be used as a sole therapeutic.

GALV fusogenic protein was also able to show anti-tumor effects when expressed from an oncolytic HSV vector. HSV expressing the GALV fusogenic protein not only

enhanced killing of various cancer cell lines *in vitro*, but also was able to reduce tumor size or completely regress tumors in liver and lung xenograft models of cancer compared to the virus control (105, 106). Syncytia formation was observed in liver tumors treated with GALV-expressing viruses while no syncytia were seen in tumor sections from control virus and mock-treated mice (105). In human bladder cancer models, GALV fusogenic protein expression enhanced the cancer cell killing abilities of an oncolytic HSV expressing the cytosine deaminse and uracil phopho-ribosyltransferase suicide gene (107). In the absence of the prodrug, increased HSV progeny production was observed with cells treated with HSV expressing the fusogenic protein and suicide genes compared to the HSV expressing GFP. *In vivo* studies showed that virus expressing GALV fusogenic protein alone was able to reduce tumor size compared to the controls, but this was not statistically significant. With addition of the prodrug, a significant decrease in tumor volume was observed compared to virus alone and the PBS/prodrug control. As such, the GALV fusogenic protein could enhance cell death and virus production in a cancer therapy.

Other fusogenic proteins that have been examined to improve virus spread are the human immunodeficiency virus type-1 (HIV-1) envelope proteins (108). A replication competent Ad expressing HIV-1 envelope proteins (AdHIV) promoted syncytia formation in HeLa cells expressing the HIV-1 receptor CD4 (HeLa/CD4+) while normal HeLa cells remained unfused. When HeLa/CD4+ cells were co-infected with AdHIV and Ad expressing GFP, single and fused HeLa/CD4+ cells expressed GFP, suggesting that fusogenic proteins can improve transgene spread. Furthermore, syncytia formation did not affect transgene expression as HeLa and HeLa/CD4+ cells co-infected AdHIV and Ad expressing luciferase had similar luciferase activity levels. Fusogenic protein expression also promoted Ad particle nuclear distribution and increased viral progeny production and

release in the first 72h. Thus, cell-cell fusion can improve virus and transgene spread to improve virus efficacy.

The Newcastle disease virus (NDV) fusion protein with a L289A substitution allows for the protein to induce syncytia formation in the absence of its accessory protein hemagglutinin-neuraminidase (109). Its expression from a vesicular stomatitis virus (VSV) vector induced cell-cell fusion and cell killing in human hepatocellular carcinoma cells *in vitro*. When the virus was infused into a syngeneic hepatocellular carcinoma model *in vivo*, syncytia formation was observed in excised tumors and treated rats showed significant prolonged survival compared to the control virus and mock-treated rats (109). Furthermore, studies using an NDV containing the L289A substitution in a further modified fusion protein also showed similar enhancing anti-cancer properties in hepatocellular carcinoma models (110). In a study using a sygeneic metastatic colorectal rat model, VSV expressing NDV fusion protein with L289A promoted tumor cell death and improved rat survival compared to control rats (111). Therefore, the NDV fusion protein with cell-cell fusion abilities can be used as a sole therapeutic against certain cancer models.

Ad-mediated expression of GALV, respiratory syncytial virus (RSV), measles virus (MV) or VSV fusion proteins have shown efficacy in xenograft and immunocompetent murine models of cancer (112-115). An oncolytic Ad expressing the GALV fusogenic protein under the control of the MLP (ICOVIR16) was able to limit tumor growth in nude mice with subcutaneous human melanoma SkMel28 and human pacreatic NP18 tumors compared to the virus control (ICOVIR15), and PBS-treated mice (112). Furthermore, even though mice were subjected to only one IV injection, some of the ICOVIR16-treated mice showed complete tumor regression. Tumors of ICOVIR16-treated mice through the IT route showed signs of syncytia formation and had larger necrotic areas compared to the controls.

However, the extent of immune cell infiltration into the tumors did not differ between mice treated with ICOVIR16 and ICOVIR15. The two viruses exhibited a similar toxicity profile in Syrian hamsters that are permissive for Ad replication, which holds promise for future studies.

Intratumoral injections of a non-replicative Ad expressing fusion proteins from RSV, MV (H and F protein) or VSV significantly reduced the tumor size in injected tumors in immunocompetent mice compared to mock infected mice (113-115). Furthermore, distal uninjected tumors also showed reduction in size, supporting the use of Ad-mediated fusogenic protein expression as a cancer therapeutic. In terms of effects of viral replication and spread, in E1-complementing HER911 cells, Ad-mediated expression of the MV fusion proteins caused synyctia formation and enhanced cytotoxicity (116). However, there was no difference in viral progeny production, Ad protein and DNA levels compared to the control virus or with the administration of a fusion inhibitory peptide. Interestingly, a common observation was that while many nuclei were positive for Ad proteins, only one nuclei of the syncytium was positive for viral DNA, suggesting that Ad replicated in the nuclei of the initially infected cell. Nevertheless, these studies highlight the efficacy of fusogenic protein expression from non-replicative Ad vectors to provide a more robust anti-cancer effect. Taken together, these studies suggest that fusogenic protein expression can, for certain viruses and models, be used as a sole therapeutic to increase virus and transgene spread, virus production and cancer cell killling.

1.4.2. Fusogenic protein expression in combinational therapy

Fusogenic protein expression has also been tested in preclinical studies in combination with other anti-cancer treatments. In one study, while mice with syngeneic murine colon and renal carcinoma tumors injected with plasmids expressing the VSV

fusogenic glycoprotein caused intratumoral syncytia formation and inhibited tumor growth, co-administration of a plasmid expressing the immunostimulatory cytokine, IL-12 was more effective in reducing tumor burden and regression compared to individual treatments (117).

In a recent study by Hu *et al.* (118), chemotherapy resistant leukemia cells retrovirally transduced to express VSV fusogenic glycoprotein cocultured with leukemia cells expressing HSV-1 TK and given ganciclovir had decreased metabolic activity compared to HSV-1 TK transduced cells alone. An enhanced combinational effect was also observed with cells administered the common anti-leukemia molecule, all-trans retinoic acid, suggesting that along with fusogenic protein-mediated cell death, cell fusion with non-transduced cells could promote suicide gene or anti-cancer molecule effects. In another HSV-1 based vector (OncdSyn), syncytial promoting mutations were introduced into HSV glycoproteins B and K to allow for cell-cell fusion (119). In a syngeneic mammary cancer mouse model, mice given the immunomodulatory molecule thalidomide combined with OncdSyn intratumoral injections was more effective at controlling the tumor volume at early timepoints, compared to OncdSyn or thalidomide treatment alone. Increased cytokine production (i.e. IL-2, IL-5) was also observed in mice given virus and thalidomide compared to individual treatments. Thus, virotherapy and immunostimulatory drugs can produce a more prounounced therapeutic benefit.

In a study using modified oncolytic Sendai virus that causes cell-cell fusion and expresses IFN-β (Bioknife-IFNβ), the authors observed an increase in viral genomes in cell-fusion permissive gliosarcoma murine cells *in vitro* compared to their non-permissive counterparts, suggesting that cell-cell fusion can improve virus spread (120). Interestingly, higher genome copies were present in cells infected with the Bioknife-IFNβ versus a Sendai virus expressing GFP, suggesting the increase in IFN-β expression promotes viral replication.

Enhanced cytotoxicity was observed with Bioknife-IFNβ infected cells compared to the Sendai virus expressing GFP and given IFN-β protein. This indicates that gene transfer is more effective compared to protein administration. Single intratumoral injections into an orthotopic rat brain tumor model showed Bioknife-IFNβ increased transgene expression compared to the non-fusogenic Sendai virus expressing IFN-β. Furthermore, Bioknife-IFNβ significantly improved rat survival compared to the control viruses (i.e. Sendai virus expressing GFP, non-fusogenic virus expressing IFN-β), suggesting while cell fusion and IFN-β expression separately has anti-cancer properties, the combination of the two provides a more complete therapeutic effect as no rats died during the duration of the experiment and complete tumor regression was observed.

Several studies have also highlighted the efficacy of Ad-mediated fusogenic protein expression in combination with other anti-cancer moities. Intratumoral injections of Ad expressing RSV, MV or VSV fusogenic proteins reduced tumor burden in immunocompetent mice in both injected and uninjected contralateral tumors. Enhanced effects could be seen through Ad-mediated co-expression of immunostimulatory genes such as interleukin (IL)-2, IL-21 and GMCSF, suggesting that fusogenic proteins can be used in a multi-faceted regime to improve therapeutic outcome (113-115).

In another study, Ad-mediated expression of MV H and F glycoprotein in combination with the chemotherapeutic drug gemcitabine worked synergistically *in vitro* to promote apoptosis (121). *In vivo*, the multi-modal therapy reduced tumor burden and promoted survival compared to indvidual treatments in a xenograft mouse model of pancreatic cancer. A more extensive study examining Ad-mediated expression of MV fusogenic proteins and administration of chemotherapeutic drugs including 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX) showed that the combinational treatment was able to

reduce the dose required to cause a 50% cytotoxic effect in established colorectal cancer cell lines and primary cultures compared to individual treatments (122). Combination treatments also increased the apoptotic marker, Annexin V and caspase-3 and -7 activity compared to the controls. In a colorectal xenograft model, intratumoral injections of Ad expressing the fusogenic glycoproteins and FOLFOX significantly improved mouse survival in comparison to individal therapeutics, highlighting enhanced efficacy of combinational treatments of viruses and anti-cancer molecules.

1.4.3. Fusogenic membrane protein-mediated cell death and immune system stimulation

Unfortunately, how fusogenic membrane proteins promote cell death or stimulate the immune system has not been fully elucidated. Most of the work studying the mechanism of cell fusion-mediated cell death involve fusogenic proteins from enveloped viruses. Fusion of cells occurs primarily during the G_2 or M phase and the resulting synyctia are initially viable, (103, 123, 124). Nuclear fusion has been observed in some syncytia (103, 125). Generally, after several days, syncytia show a decline in cell viability, which ultimately, results in a non-apoptotic cell death (103, 123, 125).

The mechanism of cell death by fusogenic proteins remains unclear. Overexpression of fusogenic membrane proteins in cancer cells results in heat shock protein 70, gp96 expression and retention of the NFκB inhibitor, IκBα, in the cytoplasm, leading to NFκB pathway inhibition and cell death via a necrotic-like mechanism (124, 125). One study showed mitochondria depolarization, resulting in cytochrome-c release and depletion of adenosine triphosphate (ATP) stores are the cause of cell death while another study did not observe any cytochrome-c release (103, 123). Instead, at late timepoints, the authors observed signs of autophagy such as increased vacuolation and lysosome acculumation (103). Blebbing was also observed from viable syncytia, resulting in the release of exosome-like

particles termed syncytiosomes (103). These syncytiosomes contained higher amounts of tumor antigen, caveolin and lower levels of major histocompatibility complex (MHC) class I compared to tumor-derived exosomes. Fused cells and syncytiosomes can promote macrophage and dendritic cell (DC) activation and cross presentation, resulting in activation and production of tumor antigen specific T cells and long lasting immunity (103, 126-128). Cell fusion associated syncytiosomes are released at higher quantities compared to other cell death methods (i.e. irradiation, freeze-thaw, osmotic-shock, HSV-1 TK/ganciclovir treatment) and are also more effective in stimulating an immune response in comparison (103).

Previous studies have shown that Ad-mediated fusogenic protein expression reduces tumor burden in bilateral immunocompetent mouse models through a tumor-specific T cell response (113-115). Both injected and distal tumors showed infiltration of innate immune cells such as macrophages and natural killer (NK) cells, indicating that fusogenic proteins are capable of inducing a strong immune response against the tumor. Interestingly, a study by Hoffmann *et al.* showed while Ad-mediated expression of the RSV glycoprotein and fusion protein enhanced syncytia formation compared to RSV fusion protein alone *in vitro*, the two vectors had comparable efficacy in immunocompetent mouse models of cancer, suggesting that the immune system plays an integral part in tumor regression (129). The importance of cell fusion is further supported by the fact that treatment with Ad expressing a non-fusogenic form of the fusion protein was not effective in promoting mouse survival or tumor regression. While the use of fusogenic proteins as a cancer therapy is promising, it is important to note that most fusogenic proteins are quite large in size. Their size makes them difficult to incorporate Ad vectors with other regulatory elements (i.e. for creation of oncolytic Ads), often closely reaching or exceeding the cloning capacity of ~38kb (112, 122, 130).

1.4.4. p14 fusion-associated small transmembrane protein

1.4.4.1. Protein structure and function

The reptilian reovirus p14 FAST protein is a 125 amino acid (aa) type III transmembrane protein (N_{exo}/C_{cyt}) with limited sequence homology to other orthoreovirus and FAST proteins (131, 132). Structually, p14 FAST protein has a myristoylated N terminus containing a hydrophobic region, transmembrane domain, a membrane proximal polybasic motif, and a C terminus containing a string of prolines (133, 134). The membrane proximal polybasic motif (PBM) [Q**KRR**E**RRR**Q] contains tribasic Golgi export signalling domains, which are dependent on the number of basic residues, rather than the net charge, to exert its function. Insertion of the motif near the transmembrane domain of a chimeric protein was sufficient to facilitate its export out of the Golgi complex and to the plasma membrane (133). Interestingly, the location of the PBM causes different trafficking signals (135). PBM localization to the C-terminus results in the protein endoplasmic reticulum (ER) retention while an internal PBM signal (i.e. within the C terminus) allows for ER, but not Golgi export. Furthermore, introduction of an extra PBM can also alter FAST protein trafficking. The combination of a transmembrane-proximal and C-terminal PBM results in ER export and retrieval with a majority of protein retained in the ER. Finally, presence of a membrane-proximal and internal PBM allows for regular transport to the plasma membrane.

The p14 FAST protein is considered as a non-structural protein as it is not essential for virus entry or release (131, 136). Expression of the p14 FAST protein alone is sufficient to induce membrane fusion and its expression in cells results in extensive cell fusion, and induces apoptosis, leading to membrane permeability (i.e. efflux and influx of small molecules across the cell membrane) (131, 134). During the process of FAST protein-mediated fusion, however, the membrane is intact (134). The exact mechanism by which

FAST protein mediates cell fusion has yet to be elucidated. FAST protein traffics through the ER/Golgi complex, an event that appears necessary for homomultimerization of the protein (137). Homomultimerization is predicted to be mediated through weak ectodomain interactions. Localization studies have shown p14 FAST protein in the perinuclear region, in foci within the cytoplasm and in the plasma membrane (131). After reaching the plasma membrane, FAST protein localizes to detergent-resistant heterogeneous microdomain populations and near adheren junctions (138, 139). As the FAST protein does not have receptor specificity and its small ectodomain makes it unable to form a hairpin structure that many enveloped virus fusogenic proteins use to promote membrane apposition, it is unlikely the FAST protein itself is involved in mediating the initial membrane-membrane contact (139). Only one of the membranes needs to express the FAST protein in order to initiate the fusion process (140).

It is proposed that initial contact between two membranes is through weak nectin, cadherin or other surrogate adhesion interactions (139). Through actin remodelling, adherin junction formation increases the contact area between cellular membranes, allowing the clusters of FAST protein to initiate the cell fusion process. Cadherins can enhance FAST protein-mediated cell-cell fusion, but are not essential to the process (139). Random fluctuations or oscillations in the cellular membranes may also allow for membrane apposition for FAST protein-mediated cell-cell fusion, but this is not as efficient compared to active adhesion acquired membrane apposition. It is possible that apposition is strengthened through interactions of the FAST protein ectodomain, C terminal aromatic residues and its hydrophobic residues with the donor membrane, leading to lipid layer mixing (hemifusion) (139, 141). Through an uncharacterized mechanism, hemifusion leads to initial pore formation and expansion of these structures into stable micropores, and finally, syncytia

formation. Pore expansion leading to syncytia formation (syncytiogenesis) is sensitive to the positive membrane curvature-inducing lipid lysophosphatidylcholine, and requires cellular annexin A1 (140, 142).

Myristylation of the N terminus and the endodomain of the p14 FAST protein are essential for mediating cell-cell fusion (131, 143). The endodomain is involved in stable pore formation and expansion and is dependent on amino acid composition, rather than direct sequence (143). A naturally proteolytically-cleaved 68 aa endodomain, in combination with the full length protein, has been found to enhance formation of syncytia after pore formation (131, 143, 144). The soluble endodomain fragment can be found in the nucleus and cytoplasm as opposed to the full length protein which resides in the cytoplasm and plasma membrane (144). The exact mechanism of how the soluble endodomain promotes syncytiogenesis has not been elucidated. It does not directly interact with the full length protein or plasma membrane and has no effect on the actin cytoskeleton. Furthermore, only a low level of the soluble endodomain is required to enhance syncytiogenesis. It is suggested that the endodomain activates other intracellular signalling pathways to promote syncytiogenesis (144).

p14 FAST protein mediated cell-cell fusion can also induce an antiviral state in certain cell types. In human embryonic lung fibroblasts, FAST protein expression induces IFN-stimulated genes (ISGs) such as ISG56K, MxB, and IFN-inducible protein 10 (145). However, in African green monkey kidney Vero cells and human osteosarcoma U2OS cells, FAST protein expression did not induce ISG expression. In another study, mouse embryonic fibroblasts (MEFs) were treated with Lipofectamine and full length p14 FAST protein or a non fusogenic truncated version of the p14 FAST protein (p14Δ30) (146). When these cells were infected with VSV expressing GFP, full length p14 FAST protein treated cells had

lower levels of GFP expression compared to p14Δ30-treated cells. This suggests that cell fusion induced an antiviral state. Cells treated with p14 FAST protein showed increased transcription in various ISG and IFN receptor regulatory factor-related genes. Furthermore, no detectable levels of extracellular IFN were observed in the experiments, suggesting that the antiviral state is not dependent on external IFN signalling. However, the antiviral state requires interferon regulatory factor-3 (IRF3), a major activator of IFN production during virus infection, as experiments using IRF3-deficient MEFs showed comparable levels of GFP expression between wildtype and truncated p14 FAST protein- treated cells.

1.4.4.2. Effects of virus-mediated p14 FAST protein expression

The p14 FAST protein has been shown to increase the pathogenicity of VSV, a virus that does not normally induce syncytia formation (147). In immunocompetent mice, intranasal administration of VSV expressing the FAST protein infected a larger brain area and increased viral replication compared to VSV expressing GFP. There was also increased neuropathology in treated mice, suggesting that the FAST protein can act as a virulence factor to promote virus spread.

In another study, co-infection of oncolytic VSVΔ51 (148) expressing the p14 FAST protein and a doubly-deleted VV (deficient in the viral TK and vaccinia growth factor (149)) increased vector efficacy (150). In 786-O kidney cancer cells, co-infection of these two viruses promoted viral spread and increased the yield of VV titre by ~100 fold relative to the combination of VV and native VSVΔ51. Increased viral spread and enhanced cell killing was also observed in co-infected primary human colon tumor cells. Taken together, these studies suggest that virus-mediated FAST protein expression can improve virus spread and cancer cell killing.

1.5 Rationale, Hypothesis, Objectives

Since the FAST protein has previously shown to promote virus spread and efficacy (147, 150) and its small size can allow for incorporation of other anti-cancer genes into the same vector, the FAST protein is an ideal candidate to incorporate into an Ad vector to help improve virus distribution and anti-cancer effects. The small size of the FAST protein also allows for insertion of large regulatory regions in oncolytic vectors, which are normally restricted due to the large size of other fusogenic proteins (112, 122, 130). Furthermore, the immunostimulatory effects of the fusion process and subsequent cell death could also improve therapeutic outcome. My hypothesis is that Ad-mediated FAST protein expression will improve virus distribution and efficacy as a cancer therapeutic. The objectives include:

i) Examine the effects of a replication defective Ad expressing FAST protein *in vitro* and in a nude mouse xenograft model

ii) Investigate Ad-mediated FAST protein expression in murine cell lines *in vitro* and in an immunocompetent syngeneic mouse tumor model

iii) Examine combinational therapies with Ad-mediated FAST protein expression for cancer treatment

CHAPTER 2: Materials and Methods

2.1 Cell culture

Human embryonic kidney 293 (151), 293N3S (152) cells and A549 human lung adenocarcinoma cells were grown in Minimum Essential Media (MEM) (Sigma Aldrich, Invitrogen) containing 10% (v/v) fetal bovine serum (FBS) (Sigma Aldrich), 2mM GlutaMAX (Invitrogen) and 1x antibiotic-antimycotic (Invitrogen). A549 cells that constitutively express GFP from an integrated lentivirus vector (A549::WPI) were also maintained in MEM media with all supplements mentioned above. Mouse colon carcinoma CT26, melanoma B16F10, mammary carcinoma 4T1 cells, human cervical cancer HeLa and glioblastoma SNB75 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Invitrogen) with the same supplements as mentioned above. Ad infected cells were maintained in their corresponding media supplemented with 5% FBS, 2mM GlutaMAX and 1x antibiotic-antimycotic. All cells were kept at 37°C and in a 5% CO_2 atmosphere.

2.2 Ad constructs and adenovirus purification

Serotype 5 Ad constructs used for these experiments are depicted in Figure 2.1. All Ad are E1 and E3 deleted, and were generated using a combination of conventional and RecA-mediated bacterial cloning (153). To summarize, the vector lacking a transgene and deleted of E1 and E3 regions is denoted as AdEmpty. The AdRFP construct encodes a monomeric Red Fluorescent Protein (RFP), a kind gift of R. Tsien (University of California San Diego (154)), under control by the CMV enhancer/promoter and BGHpA, replacing the E1 deletion (155). The FAST cDNA was removed from plasmid pVSV-FAST (150), provided by Dr. Bell (Ottawa Hospital Research Institute), by digestion with NcoI/NheI, and cloned into NcoI/SpeI digested pPOLY (156), generating pRP2868. pRP2868 was digested

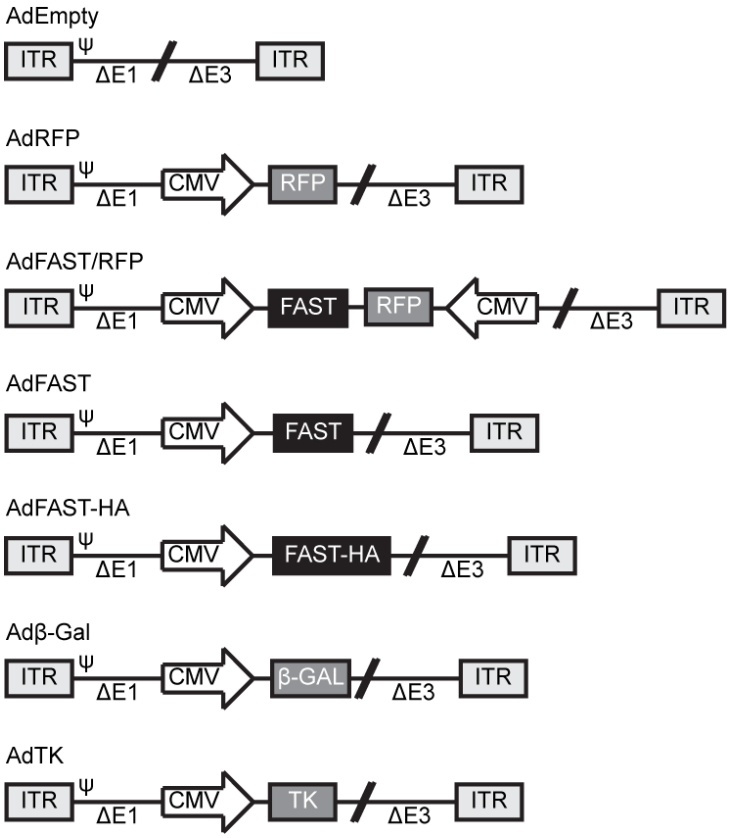

AdEmpty
ITR
Ψ
ΔE1
ΔE3
ITR
AdRFP
ITR
Ψ
ΔE1
CMV
RFP
ΔE3
ITR
AdFAST/RFP
ITR
Ψ
ΔE1
CMV
FAST
RFP
CMV
ΔE3
ITR
AdFAST
ITR
Ψ
ΔE1
CMV
FAST
ΔE3
ITR
AdFAST-HA
ITR
Ψ
ΔE1
CMV
FAST-HA
ΔE3
ITR
Adβ-Gal
ITR
Ψ
ΔE1
CMV
β-GAL
ΔE3
ITR
AdTK
ITR
Ψ
ΔE1
CMV
TK
ΔE3
ITR

Figure 2.1: Schematics of adenoviruses used in the studies. All viruses are early region 1 (E1) and early region 3 (E3) deleted. RFP is the red fluorescent protein and CMV represents the cytomegalovirus enhancer/promoter. AdFAST-HA has a single HA tag linked to the C terminus through a glycine linker. β-Gal represents the gene encoding β-galactosidase and Herpes Simplex Virus-1 thymidine kinase is represented as TK. ITR denotes inverted terminal repeats and Ψ is the packaging signal.

with EagI, and the fragment was cloned into NotI digested pRP2645, an Ad left end shuttle plasmid containing the CMV enhancer/promoter, a multiple cloning site and BGHpA, generating pRP2870. pRP2870 was recombined into an Ad genomic plasmid, pRP2014 (157), generating pRP2872 and the resulting virus AdFAST. A XhoI/SalI fragment from pRP2483 (155) containing a CMV-mRFP-BGHpA expression cassette was cloned into SalI digested pRP2870, with transcription directed opposite to the FAST expression cassette, generating pRP2873. pRP2873 was recombined into the pRP2014 Ad genomic plasmid, generating pRP2874 and the resulting virus AdFAST/RFP. AdFAST-HA is similar to AdFAST, but contains a C-terminal HA epitope tag. The HA tag was added with a glycine linker and amplified from pRP2868 using the sense primer 5'-CCGCCATGGGGAGTGGACC-3' and antisense primer 5'-CTAACTAGTCTAAGCGTAGTCTGGGACGTCGTATGGGTACGAGCCACCGCCACC AATGGCTGAGACATTATCGATGTTGACG-3'. The resulting PCR fragment was digested with NcoI/SpeI and cloned into pRP2868, replacing the native FAST cDNA, generating pCW100. pCW100 was sequenced to verify its integrity. An EagI fragment from pCW100 was cloned into NotI digested pRP2645, generating pCW101, and subsequently recombined into the pRP2014 Ad genomic plasmid, generating pCW103 and the resulting virus AdFAST-HA. Adβ-Gal has the *E. coli* β-galactosidase gene under control by the CMV enhancer/promoter (158).

The HSV-1 TK cDNA was amplified from pBEneoTK vector, kindly provided from Dr. M. Rudnicki (Ottawa Hospital Research Institute) using the sense primer 5'-ACAGGTACCATGGCTTCGTACCCCTGCCATC-3' and antisense primer 5'-TCTGGCGCGCCTCAGTTAGCCTCCCCCATCTCCC-3'. The PCR product was digested with KpnI and AscI and cloned into KpnI/MluI-digested pRP2483 (155) to generate

pCW108, a vector that expresses TK under the control of CMV enhancer/promoter and BGHpA. pCW108 was recombined with the Ad genomic plasmid pRP2014 using RecA mediated cloning to generate pCW110 and the corresponding virus AdTK. Vectors were propagated and purified using standard techniques (159).

2.3 Immunoblotting

All immunoblots were developed using the Pierce Enhanced Chemiluminescent Western Blotting Substrate (Thermo Scientific). Densitometry was performed using AlphaEaseFC (Alpha Innotech).

2.3.1. Analysis of FAST protein expression and effects on Ad gene expression

To confirm FAST protein expression, 293 and A549 cells were seeded at $1x10^6$ and $0.8x10^6$ cells per 35mm dish, respectively. Next day, the cells were infected for 1 h at a multiplicity of infection (MOI) of 10 with AdEmpty or AdFAST-HA. Following a 48 h incubation, whole cell lysates were collected using the cell lysis buffer as described by Lieber *et al.* (160). Samples were separated on a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). The resulting membrane was probed with a mouse anti-HA tag monoclonal antibody (1:1000, Cell Signaling #2367) and goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (1:10 000, Bio-Rad #170-6516). To confirm Ad replication, the membrane was reprobed for Ad5 fibre (1:20 000 mouse anti-fibre monoclonal antibody, clone 4D2, Neomarkers). Ad fibre protein is a late gene and is only expressed after DNA replication (33). Thus, we used fibre expression as a marker of active virus replication. The membrane was stipped and also probed with antibody to alpha (α)-tubulin to confirm equal loading (1:5000 rabbit anti-α-tubulin antibody, AbCam #ab15246, 1:5000 goat anti-rabbit IgG conjugated to HRP, Bio-Rad #170-6515).

To examine whether FAST protein expression affects Ad gene expression, 293 cells were plated at a density of $1x10^6$ cells per 35mm dish. Cells were infected at an MOI of 1 with AdEmpty or AdFAST the next day for 1 h. Cells were washed two times with PBS before adding 5% FBS/MEM. Whole cell lysates were collected at varying timepoints using cell lysis buffer as described above and probed for Ad5 fibre to compare adenoviral gene expression between the two vectors. Tubulin was used as a loading control.

2.3.2. Analysis of TK expression

293 cells were seeded at $1x10^6$ per 35 mm dish while A549 and HeLa cells were plated at $0.8x10^6$ per dish and grown to confluency. Cells were infected at a MOI of 10 with AdEmpty or AdTK for 1 h. Forty-eight hpi, whole cell lysates were collected with 2x Laemmli loading buffer (62.5mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol). Samples were resolved on a 12% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed for TK expression using 1:500 goat anti-HSV-1 TK antibody (Santa Cruz Biotechnology) and 1:5000 donkey anti-goat immunoglobulin G (IgG) HRP (Santa Cruz Biotechnology). The blot was stripped and reprobed for Ad fibre and α-tubulin was used as a loading control.

2.3.3. Cleaved caspase-3 expression

A549 cells were seeded $0.8x10^6$ cells per 35mm dish and grown to confluency. Cells were infected for 1 h with AdEmpty or AdFAST at an MOI of 10. Whole cell lysates were collected 72 hpi with 2x Laemmli loading buffer. Samples were resolved on a 15% SDS-PAGE gel and transferred onto a PVDF membrane. Membranes were probed with rabbit anti-cleaved caspase-3 monoclonal antibody (1:1000, Cell Signaling #9664). A mouse antibody against α-tubulin was used as a loading control.

For experiments examining whether AdFAST in combination with other anti-cancer therapies induces apoptosis, A549 and SNB75 cells were seeded $0.8x10^6$ cells per 35mm dish and grown to confluency. Cells were infected for 1h with AdEmpty or AdFAST at an MOI of 50 for SMC experiments and at an MOI of 10 for bleomycin experiments. Stocks of SMCs LCL161 and OICR720 were diluted in PBS to working stocks of 100 μM and 10 μM, respectively. Twenty four hours post infection, for SMC experiments, cells received a final concentration of 0.05% dimethyl sulfoxide (DMSO), 5 μM LCL161 or 0.25 μM OICR720. For bleomycin treated cells, a final concentration of 100 μM bleomycin was added. Whole cell lysates were collected 24 h after SMC administration or 48 h after bleomycin treatment with 2x Laemmli loading buffer. Samples were resolved on a 15% SDS-PAGE gel and transferred onto a PVDF membrane. Membranes were probed for cleaved caspase-3 and alpha-tubulin as described above.

2.4 Ad progeny titration

To determine whether FAST protein expression affects virus production, the supernatant fractions were collected from the experiments conducted for analysis of adenovirus gene expression (section 2.3.1). Viral progeny titre was determined using plaque assays (159).

2.5 Ad infectivity and promoter activity assays

4T1 and A549 cells were plated at a density of $0.8x10^6$ cells per 35mm dish. When plates were confluent, cells were infected with Adβ-Gal at an MOI of 1. Twenty-four hours post infection, cells were either fixed and stained with 50 μL/mL X-gal overnight (161) or collected to determine β-gal activity using the Galacto-Star β-Galactosidase Reporter Gene Assay System for Mammalian Cells (Applied Biosystems). Chemiluminescent activity was determined using a 20/20n luminometer (Turner Biosystems).

2.6 Giemsa staining

293 and A549 cells were seeded in 35mm dishes at densities of $1x10^6$ and $0.8x10^6$ cells per dish, respectively, and grown to confluency. 4T1 cells were seeded in 4 well plates ($0.15x10^6$/well) and grown to confluence. 293 cells were infected at an MOI of 1 with AdEmpty or AdFAST. Cells were fixed with methanol and stained with Giemsa stain (Merck Millipore) 18 hpi. A549 cells were infected at an MOI of 50 with AdEmpty or AdFAST while 4T1 cells were infected at an MOI of 1000. A549 and 4T1 cells were stained at 48 hpi. Stained cells were visualized using the 20x objective of the Zeiss Axiovert 200M microscope.

2.7 Fluorescence microscopy

Thirty-five mm dishes of 293 and A549 cells were seeded at a density of $0.6x10^6$ cells. Next day, the cells were infected with AdRFP or AdFAST/RFP at varying MOI in 200 µL incoculum. Following a 1 h infection, 2 mL of 5% FBS/MEM was added. The cells were visualized at varying times post infection using a Zeiss Axiovert 200M microscope (20x objective).

For the A549 co-culture assay, A549 cells were infected with AdRFP or AdFAST/RFP at an MOI of 50 in the manner as described above. After 6 hours, the infected A549 cells were mixed at a 1:1 ratio with A549::WPI cells. Cocultures were observed using fluorescence microscopy at 24 h intervals using the 20x objective.

CT26, B16F10 and 4T1 cells were seeded at a density of $0.8x10^6$ cells per 35mm plate. Next day, the cells were infected at an MOI of 50 with AdRFP or AdFAST/RFP for 1 h in 200 µL inoculum. Two mL of media was added and cells were viewed using the 20x objective every 24 h. All images were compiled in Adobe Photoshop CS4.

2.8 Fluorescence based cell viability assay

293 cells were seeded in 24 well plates at densities of $0.2x10^6$ cells per well and grown to confluency. The cells were changed to media containing 5% FBS immediately prior to infection. Cells were infected with AdEmpty or AdFAST by adding the virus directly to the wells without removing the media. To determine whether AdFAST caused overt cytotoxicity compared to the control virus, the LIVE/DEAD® Cell Viability Assay (Invitrogen) was performed after 48 hpi. Media was removed to a final volume of 500 µL for each well, and the cells were stained for 30 min at a final concentration of 2 µM calcein acetoxymethyl ester (calcein AM) and 10 µM ethidium homodimer-1. Stained cells were visualized using the 20x objective of the Zeiss Axiovert 200M microscope and processed using Adobe Photoshop CS4.

2.9 MTS metabolic activity assays

All experimental conditions were conducted in triplicate. Metabolic activity was assessed using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) and a 1 h incubation with MTS substrate (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Absorbance was determined at 490 nm using the SpectraMax 190 plate spectrophotometer (Molecular Devices).

2.9.1. AdFAST-infected human and murine cell lines

In 96 well plates, 293 were seeded at $2.5x10^4$ cells per well while A549 and 4T1 cells were seeded at $2x10^4$ cells per well. Next day, the cells were infected at varying MOI with AdEmpty or AdFAST in 25 µL inoculum for 1 h, followed by addition of media supplemented with 5% FBS to a final volume of 100 µL. Relative metabolic activity was assessed 72 hpi. A second set of 293 and A549 cells were infected at an MOI of 10 with AdEmpty or AdFAST and the relative metabolic activity was monitored using the MTS

assay over 96 h at 24 h intervals. Another timecourse with 4T1 cells infected at an MOI of 1000 was conducted in the same manner.

2.9.2. AdTK-infected A549 and HeLa cells

A549 and HeLa cells were plated in 96 well plates at a density of $1x10^4$ cells per well. The next day, at a confluency of 50-60%, the cells were infected with $3.2x10^5$ pfu of AdEmpty or AdTK in 25 μL inoculum. After 1 h of infection, MEM or DMEM supplemented with 5% FBS was added to a final volume of 100 μL. Following infection, stock solutions of ganciclovir (CalBiochem) dissolved in DMSO were diluted in media containing 5% FBS and added to varying final concentrations in 25 μL following infection and metabolic activity was assessed 24 hpi. In a separate experiment, HeLa cells were infected in triplicate wells with $9.6x10^4$ pfu (~MOI 3), $3.2x10^5$ pfu (~MOI 10) and $8.0x10^5$ pfu (~MOI 25) in 25 μL of AdEmpty or AdTK for 1 h. Seventy-five μL of DMEM supplemented with 5% FBS was added after infection. A final ganciclovir concentration of 10 μM or media was supplemented to the wells up to a final volume of 125 μL. An MTS assay was conducted 72 hpi to determine relative metabolic activity.

2.9.3. AdFAST and AdTK combination experiments

HeLa cells were plated at $1x10^4$ cells per well in 96 well plates. At subconfluency, cells were co-infected with AdEmpty or AdTK at an MOI of 1 and AdEmpty or AdFAST at MOIs of 1, 3 and 10 for 1 h in 30 μL inoculum. Media was added to a final volume of 100 μL. Forty-eight hpi, a final concentration of 10 μM ganciclovir was added to the wells infected with AdTK while other wells were given 5% FBS/DMEM. The relative metabolic activity was assessed as described above 24 h after ganciclovir administration.

2.9.4. AdFAST and SMC, chemotherapeutic drug treatments

A549 or SNB75 cells were seeded into 96 well plates at a density of $1.5x10^4$ cells/well and grown to ~80-90% confluency. Cells were infected with AdEmpty or AdFAST at varying MOI in 25 μL inoculum for 1 h. Media with 5% FBS was added to a final volume of 100 μL. Twenty four hours post infection, for SMC experiments, cells were either given a final concentration of 0.05% DMSO, 5 μM LCL161 or 0.25 μM OICR720. Cells were incubated with the SMAC mimetics for 24 h before assessing relative metabolic activity as described above.

For studies involving etoposide or bleomycin, A549 cells were seeded in the same manner and infected at an MOI of 10 with AdEmpty or AdFAST in 25 μL inoculum. After an hour infection, media was added to the wells up to 100 μL. Following a 24 h incubation, stock solutions of bleomycin (Santa Cruz Biotechnology) in water or etoposide (Sigma Aldrich) were further diluted in 5% FBS/MEM and 25 μL was added to each well at varying final concentrations. Cells were incubated for 48 h before the relative metabolic viability of treated cells were examined. Another set of A549 cells were infected with varying MOI of AdEmpty or AdFAST and following a 24 h incubation, were treated with 100 μM of bleomycin for 48 h before assaying metabolic activity.

2.10 Membrane integrity assays

293 cells was seeded at a density of $2.5x10^4$/well while A549, HeLa, CT26, B16F10, and 4T1 cells were seeded at $2x10^4$ cells/well in 96 well plates and grown to sub-confluency. Media was removed and cells were infected with AdEmpty or AdFAST at varying MOI in 25 μL inoculum for 1 h. The VSVΔ51 virus (148) was used as a positive control. Media was added to a final volume of 125 μL for 72 h. Prior to collecting the supernatants, a series of wells containing uninfected cells were lysed with 9% Triton X-100 for 45 min to provide

a maximum release control. All treatments were completed in triplicate. Fifty μL of supernatant was transferred to a new 96 well plate and the CytoTOX-ONE™ Homogeneous Membrane Integrity Assay kit (Promega) was used to assess membrane integrity. Fluorescence was determined using the 544 nm excitation filter and 590 nm emission filter with a FluoSTAR Galaxy fluorimeter (BMG Labtech).

2.11 *In vivo* studies

All animal experiments were performed according to the guidelines and protocols approved by the Animal Care Committee at the University of Ottawa. Mice were obtained from Charles River Laboratories (Wilmington, MA). Tumor size was monitored three times a week using digital calipers. Theresa Falls (Ottawa Hospital Research Institute) conducted the subcutaneous and intratumoral injections, and removal of tumors and livers from sacrificed mice. For histological analyses, samples were fixed with 10% formalin overnight and submerged in 70% ethanol until processed. Tissues were processed and stained with hematoxylin and eosin (H/E) at the Morphology Unit of the Department of Pathology and Laboratory Medicine at University of Ottawa.

2.11.1. A549 xenograft in CD-1 mice

Six week old CD-1 nude mice were injected in the right hind flank with 10^6 A549 cells resuspended in 100 μL PBS. When tumors reached approximately 5x5mm in size, mice were injected intratumorally with PBS, $5x10^8$ pfu AdEmpty or AdFAST in 50 μL. Endpoint was considered when the tumor reached $1687mm^3$ by volume (length x width^2 /2) or evident ulceration was observed. Another group of mice were injected when A549 tumors reached 7x7 to 10x10 mm in size with the same virus dose as above. The second set of mice was sacrificed 5 days post injection for tumor and liver samples.

2.11.2. Subcutaneous 4T1 tumors in Balb/C mice

Six week old Balb/C mice were injected with $1x10^5$ 4T1 cells resuspended in 100 μL PBS in the right hind flank. When tumors reached approximately 5x5mm in size, mice were injected intratumorally with PBS or $1.9x10^9$ pfu AdFAST in 50 μL PBS. Mice were euthanized when the tumors reached 15x15 mm in size (endpoint). For tissue analyses, a second group of mice were injected when 4T1 tumors reached 7x7 to 10x10mm in size and treated as described above. A third group of mice were injected with $1.4x10^8$ pfu AdEmpty. All mice used for histological analysis were sacrificed day 3 post infection and the tumor and liver were collected for further analysis.

2.12 Statistical analysis

SigmaStat 2.0 or Graphpad Prism 6.0 was used to determine statistical significance. One-way analysis of variance (ANOVA) tests and Tukey's test were applied when normality was observed while Kruskal-Wallis one-way ANOVA on Ranks was used when samples did not follow a normal distribution. Dunn's test was used in place of Tukey's test when required. The student's t-test was used for analysis of cleaved caspase-3 expression. Two-way ANOVA and Tukey's test were applied to the combinational therapy experiments. For animal studies, the log-rank test was used to determine statistical significance of the Kaplan-Meier survival curve. All statistical tests were completed with $p=0.05$.

CHAPTER 3: Effects of a replication defective Ad expressing FAST protein *in vitro* and in a nude mouse xenograft model

3.1 Introduction

In preclinical and clinical studies for cancer treatment, Ad, as well as many other viral vectors, has limited distribution throughout a tumor (92, 93, 95, 97, 99). Since previous studies expressing fusogenic proteins from viral vectors provided benefit as a sole therapeutic (105-109, 112-115), we proposed that FAST protein expression from an Ad vector could promote Ad distribution and improve its efficacy as a cancer therapy vector. To test this hypothesis, we generated an E1-deleted Ad vector encoding p14 FAST protein (Figure 2.1), and examined its effect on cell function under conditions of active replication (i.e. in the E1-complementing 293 cell line) and under non-replicating conditions in human lung adenocarcinoma A549 cancer cells. We also explored the efficacy of AdFAST as a sole therapeutic in a murine xenograft model of cancer.

3.2 Results

3.2.1. Ad-mediated FAST protein expression in mammalian cells causes cell fusion

To confirm FAST protein was expressed from the viral constructs, we infected 293 and A549 cells with AdEmpty and AdFAST-HA at an MOI of 10 and examined the relative FAST protein expression 48 hours later by immunoblot of whole cell protein lysates (Figure 3.1A). As expected, AdFAST-HA produced a protein of appropriate size in both 293 and A549 cells, although the level of FAST protein expression was higher in 293 cells. This result is likely due to the increase in template copy number caused by active virus replication in E1-complementing 293 cells. Untagged versions of the FAST protein were used for all other experiments to ensure FAST protein function would not be affected by the presence of the HA tag.

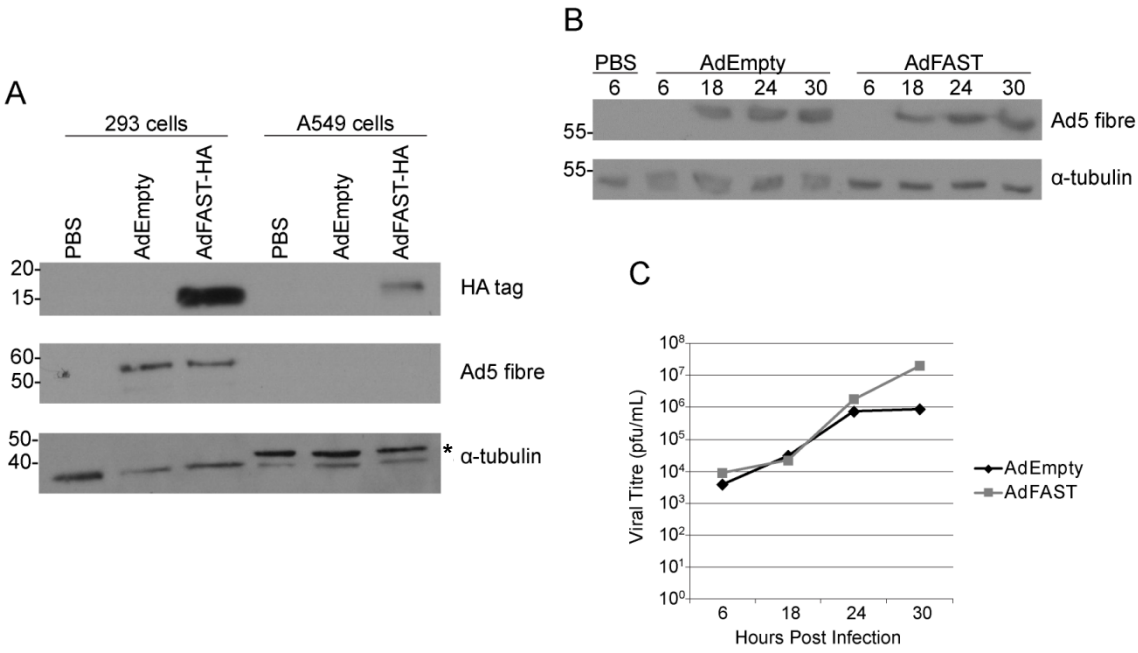

A
293 cells
A549 cells
PBS
AdEmpty
AdFAST-HA
PBS
AdEmpty
AdFAST-HA
20-
15-
HA tag
60-
50-
Ad5 fibre
50-
40-
*
α-tubulin
B
PBS
AdEmpty
AdFAST
6
6
18
24
30
6
18
24
30
55-
Ad5 fibre
55-
α-tubulin
C
Viral Titre (pfu/mL)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
6
18
24
30
Hours Post Infection
AdEmpty
AdFAST

Figure 3.1: Ad-mediated FAST protein expression does not affect virus production. A) 293 and A549 cells were infected with the control virus AdEmpty or AdFAST-HA at an MOI of 10. Whole cell lysates were collected 48 hpi. Samples were probed for the HA tag, Ad5 fibre and alpha-tubulin as a loading control. The bands denoted by * in the A549 samples are non-specific bands. **B)** 293 cells were infected with AdEmpty or AdFAST at an MOI of 1 and whole cell lysates were collected at 6, 18, 24 and 30 hpi. Samples were probed for Ad5 fibre and alpha-tubulin was used as a loading control. **C)** Supernatants collected from 293 cells infected with AdEmpty or AdFAST at an MOI of 1 were used to conduct plaque forming assays to determine the number of viral progeny at various hpi.

To determine whether FAST protein expression affected Ad replication, we performed a timecourse of infection and monitored the expression of fibre, a late gene that is expressed only after active DNA replication, by immunoblot (Figure 3.1B) and virus yield by plaque assay (Figure 3.1C). No difference in fibre expression was observed between AdEmpty and AdFAST infected cells at late timepoints. In addition, there was no difference in the number of Ad progeny up to 24 hpi as determined using a plaque forming assay (Figure 3.1C). Thus, FAST protein expression did not affect virus replication and progeny production.

To investigate whether Ad-mediated FAST protein expression can promote cell fusion, we infected 293 cells with AdEmpty or AdFAST at an MOI of 1 and stained the cells with Giemsa stain 18 hpi (Figure 3.2A). Cells infected with AdEmpty showed no discernible difference relative to PBS-treated control. In contrast, cells treated with AdFAST showed evidence of cell fusion, and the formation of multinucleated syncytia at this early time point. In another experiment, AdFAST/RFP-fused cells from the treatment of an MOI of 10 and 48 h incubation lifted from the plate and adopted a large spherical structure, which was not observed for cells treated with control virus (Figure 3.2B). Mock infected cells did not show any signs of fusion or fluorescence (not shown). Thus, expression of the FAST protein from an Ad vector in 293 cells caused extensive cell fusion.

3.2.2. Ad-mediated FAST protein expression decreases cell membrane integrity and metabolic activity in 293 cells

After confirming that Ad-mediated expression of the FAST protein induced cell-cell fusion, we next examined the effect of the FAST protein on cell function, initially using 293 cells in which both AdFAST and the control vector can readily replicate. FAST protein expression in cells is reported to ultimately reduce membrane integrity (134). To examine

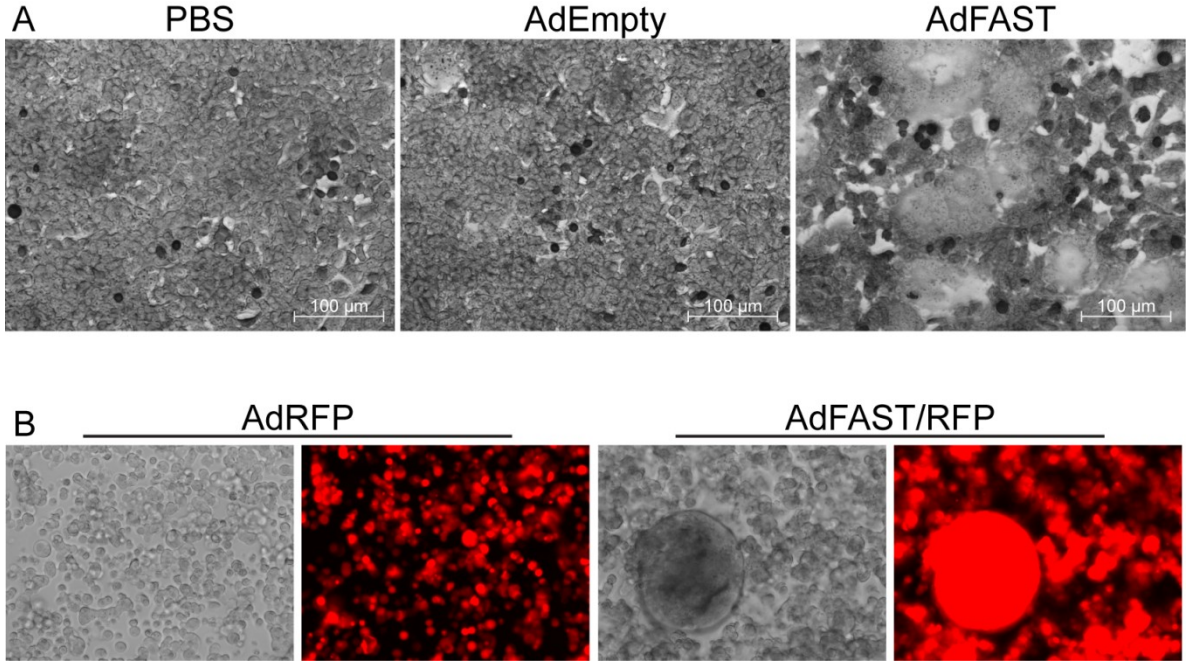

A
PBS
AdEmpty
AdFAST
100 µm
100 µm
100 µm
B
AdRFP
AdFAST/RFP

Figure 3.2: FAST protein expression induces extensive cell fusion in 293 cells. A) 293 cells were infected with AdEmpty or AdFAST at an MOI of 1 and stained with Giemsa stain 18 h later. Images were captured using bright field microscopy (20x objective). Representative images from two independent experiments are shown. **B)** 293 cells were infected with AdRFP or AdFAST/RFP at MOI 10 and observed using fluorescence microscopy at 48 h post infection. All images were taken using a 20x objective.

this in our system, we infected 293 cells with AdEmpty or AdFAST at varying MOI and assayed for lactate dyhydrogenase (LDH) release into the supernatant at 72 hpi (Figure 3.3A). Cells were infected with VSVΔ51 or lysed with 9% Triton X-100 as positive controls. We observed an increase in LDH release with increasing MOI for both viruses, suggesting induced loss of membrane integrity. However, at high MOI, LDH levels released from cells infected with AdFAST were significantly higher than from AdEmpty ($p<0.05$), indicating that FAST protein expression enhanced membrane permeability.

We next performed cell viability assays, to determine if expression of FAST protein from the replicating Ad altered the kinetics of cell death relative to the control vector. 293 cells were infected with varying MOI of AdFAST or AdEmpty and the cells were assessed for cell viability 48 h later using a fluorescence based live/dead assay. In this assay, live cells stain with calcein AM (green) whereas dead cells stain with ethidium-homodimer 1 dye (red). Mock infected cells did not show any signs of fusion and only a limited number of red cells (not shown). Cells infected with AdEmpty showed a mix of individual green and red cells due to cell death caused by active replication of the E1-deleted vector in 293 cells (Figure 3.3B). In contrast, AdFAST treated cells showed large, fused regions of red or green, suggesting that fusion did not necessarily lead to immediate cell death. The large, red cells likely represent a grouping of nuclei staining positive with the ethidium-homodimer dye within a single syncytium. At an MOI of 10, a majority of AdFAST infected cells fused, but were still "alive" (i.e. they fluoresced green), suggesting that syncytia formation precedes cell death.

We then quantitatively assessed whether AdFAST affected metabolic activity to a greater degree than the control virus using MTS assays. 293 cells were infected with AdEmpty or AdFAST at an MOI of 10 and the relative metabolic activity was monitored

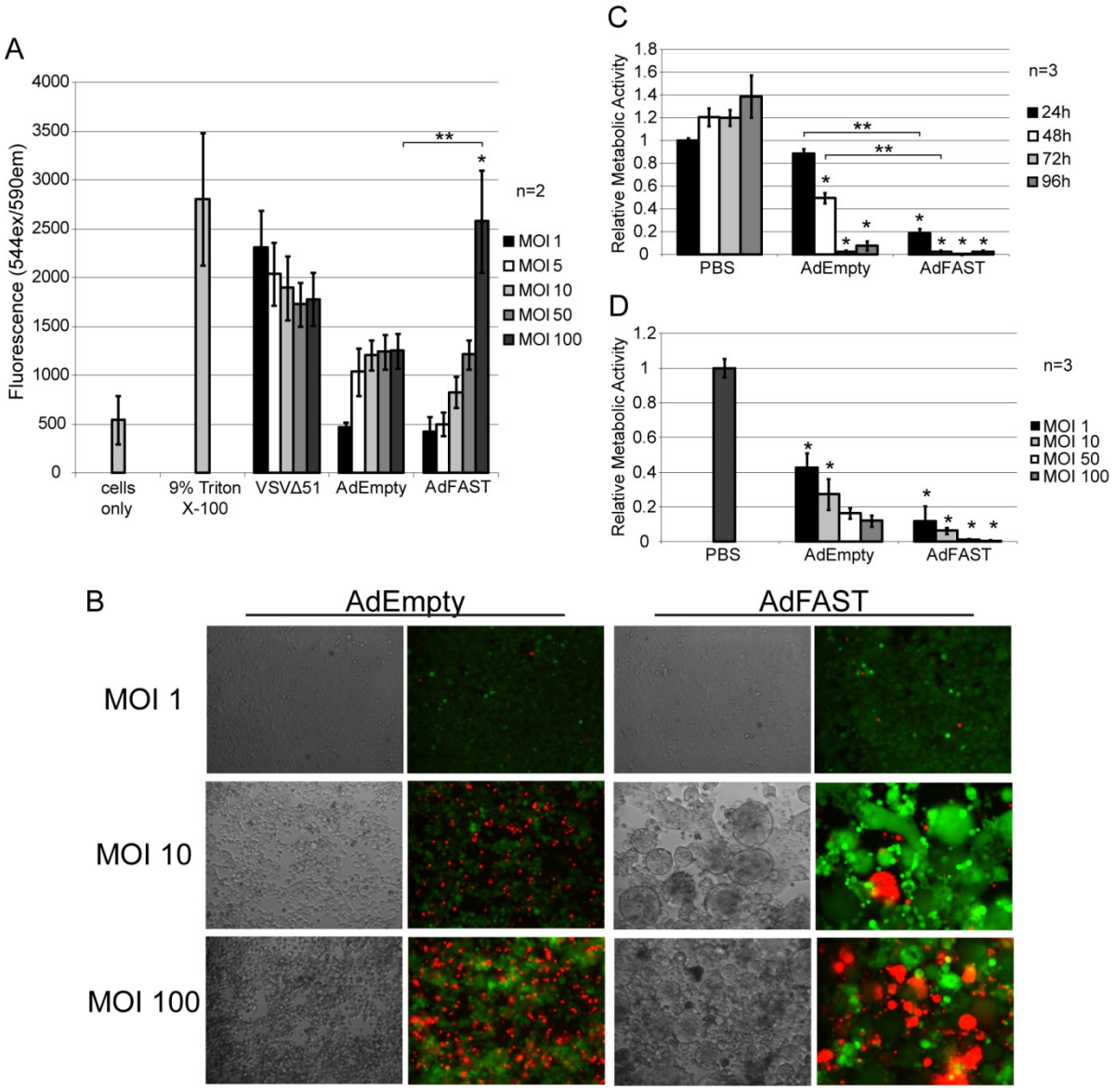

A
Fluorescence (544ex/590em)
4000
3500
3000
2500
2000
1500
1000
500
0
cells only
9% Triton X-100
VSVΔ51
AdEmpty
AdFAST
**
*
n=2
MOI 1
MOI 5
MOI 10
MOI 50
MOI 100
C
Relative Metabolic Activity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
AdEmpty
AdFAST
n=3
24h
48h
72h
96h
D
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
PBS
AdEmpty
AdFAST
n=3
MOI 1
MOI 10
MOI 50
MOI 100
B
AdEmpty
AdFAST
MOI 1
MOI 10
MOI 100

Figure 3.3: FAST protein expression in 293 cells affects metabolic activity and compromises cell membrane integrity. **A)** 293 cells were infected with AdEmpty, AdFAST or VSVΔ51 at varying MOI. Membrane permeability, measured through lactate dehydrogenase activity, was determined 72 hpi. Results shown are the average of two independent experiments (n=2) done in triplicate and the error bars show the standard error of the mean. *$p<0.05$ compared to cells only. **$p<0.05$ comparing AdFAST to AdEmpty infected cells. **B)** 293 cells were infected with AdEmpty or AdFAST at an MOI of 1, 10 and 100. Cells were treated with dyes from the LIVE/DEAD® Cell Viability Assay kit (Invitrogen) (live-green, dead-red) at 48 hpi and images were captured using fluorescence microscopy (20x objective). **C)** 293 cells were infected at a MOI of 10 with AdEmpty or AdFAST and relative metabolic activity was determined using an MTS assay over a 96 h interval every 24 h. Experiments were completed in triplicate and the average of three independent experiments is shown (n=3). Values were normalized to mock infected cells at 24 hpi. Error bars denote the standard error of the mean. *$p<0.05$ compared to mock infected cells. **$p<0.05$ when comparing AdFAST to AdEmpty infected cells. **D)** Cells were infected at an MOI of 1, 10, 50 or 100 with the two viruses in triplicate and metabolic activity was determined at 72 hpi using an MTS assay. Averages and the standard error of the mean of three independent experiments is depicted with normalization to mock infected cells (n=3). *$p<0.05$ compared to mock infected cells.

over 96 h using an MTS assay. Treatment with both AdEmpty and AdFAST decreased metabolic activity relative to PBS-treated controls, likely due to progressive replication of the viruses in this cell line (Figure 3.3C). However, a significant difference between the two viruses was observed during the first 48 h of infection, suggesting that expression of FAST decreased metabolic activity in this cell type. Both viruses showed a similar sequential decrease in metabolic activity with increasing MOI at 72 hpi, although this effect was again enhanced in the AdFAST-treated cells (Figure 3.3D). Taken together, these data suggest that high-level expression of FAST protein under replication permissive conditions in the 293 cell lines leads to extensive cell fusion and decreases metabolic activity.

3.2.3. Ad-mediated FAST protein expression in A549 cells induces cell-cell fusion

Infection of 293 cells with AdFAST led to high levels of protein expression (Figure 3.1A), resulting in extensive cell-cell fusion (Figure 3.2), significantly increased cell membrane permeability (Figure 3.3A) and decreased metabolic activity (Figure 3.3C, D). Under non-replicating conditions in A549 cells, Ad-mediated FAST protein expression was noticeably reduced (Figure 3.1A). We therefore examined whether AdFAST could mediate cell fusion in this cell line and whether cellular metabolic activity and membrane integrity were adversely affected. We used three assays to assess cell fusion. First, A549 cells were infected with AdRFP or AdFAST/RFP at varying MOI and the cells were simply visualized at 48 hpi using fluorescence microscopy. Only isolated regions of the AdFAST/RFP-treated monolayer showed large cells indicative of cell fusion (Figure 3.4A, white arrows). To confirm that cell-cell fusion was occurring in A549 cells, we next performed a cell coculture assay. AdFAST/RFP- or AdRFP-infected A549 cells were cocultured at a 1:1 ratio with A549 cells that constitutively expressed GFP (Figure 3.4B). After 72 h of coculture,

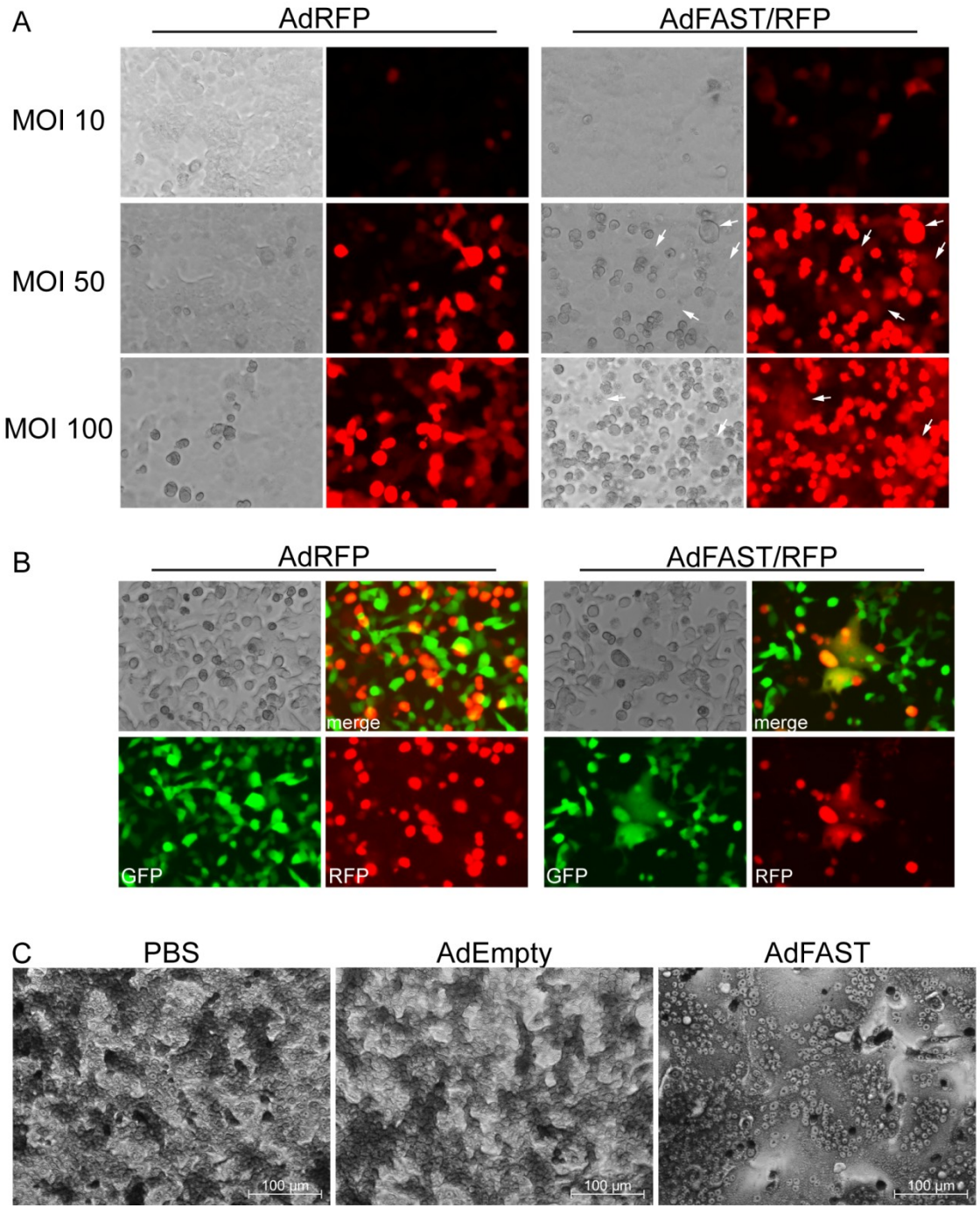

A
AdRFP
AdFAST/RFP
MOI 10
MOI 50
MOI 100
B
AdRFP
AdFAST/RFP
merge
merge
GFP
RFP
GFP
RFP
C
PBS
AdEmpty
AdFAST
100 µm
100 µm
100 µm

Figure 3.4: Ad-mediated FAST protein expression induces cell fusion in A549 cells. A) A549 cells were infected with AdRFP or AdFAST/RFP at an MOI of 10, 50 or 100. Fluorescence microscopy was used to visualize cells 48 hpi (20x objective). Fused cells are indicated by the white arrows. **B)** A549 cells infected with AdRFP or AdFAST/RFP at an MOI of 10 were cocultured 6 hpi at a 1:1 ratio with A549 cells constitutively expressing GFP. After 72 h of coculturing, cells were visualized using fluorescence microscopy with a 20x objective. The upper right image shows the merged image while the two bottom panels show GFP and RFP fluorescence. **C)** A549 cells were infected with AdEmpty or AdFAST at an MOI of 50 and stained with Giemsa stain after 48 hpi. Cells were visualized using bright field microscopy with a 20x objective. Two independent experiments were conducted with representative images depicted.

incubation of AdRFP-infected cells with A549::GFP cells showed some evidence of merged fluorescence signal (yellow), but this appeared to be exclusively due to cell overlap. In contrast, coculture with AdFAST/RFP-infected cells resulted in large areas of yellow signal, suggesting true fusion into multinucleated syncytium. Finally, A549 cells were treated at an MOI of 50 with AdEmpty or AdFAST and, 48 h later, the cells were stained with Giemsa (Figure 3.4C). Once again, cells infected with AdEmpty showed no discernible difference relative to PBS-treated control. In contrast, cells treated with AdFAST showed evidence of cell fusion, and the formation of multinucleated syncytia. Taken together, these data indicate that expression of FAST protein in A549 cells does result in cell-cell fusion. The effect was not as dramatic as observed in 293 cells, and required relatively high MOI, suggesting that high levels of FAST protein are required for efficient fusion.

3.2.4. Treatment of A549 cells with AdFAST affects cell membrane integrity and cellular metabolic activity

We previously showed that treatment of 293 cells with AdFAST resulted in increased cell membrane permeability at a high MOI of infection relative to cells infected with AdEmpty (Figure 3.3A). To determine if treatment of A549 cells with AdFAST also resulted in enhanced membrane permeability, we examined LDH release into the supernatant (Figure 3.5A). Treatment of cells with AdEmpty at an MOI up to 100 did not result in any significant release of LDH while treatment of A549 cells with AdFAST at as low an MOI as 5 led to extensive cell membrane permeability and release of LDH, which was further enhanced with increasing MOI. Thus, FAST protein expression directly compromises cell membrane integrity in A549 cells.

To more closely examine the effect of AdFAST on cell function, we evaluated cellular metabolic activity through MTS assays. A549 cells were infected with AdEmpty

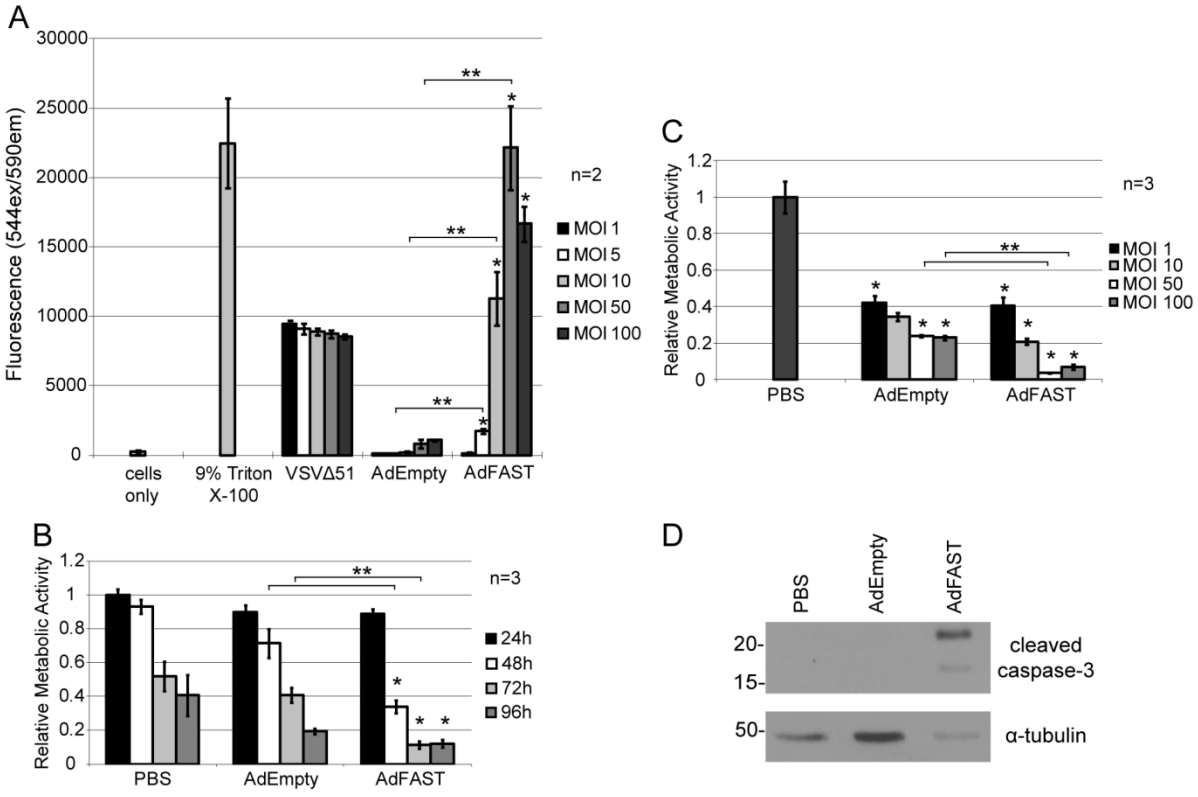

A
Fluorescence (544ex/590em)
30000
25000
20000
15000
10000
5000
0
cells only
9% Triton X-100
VSVΔ51
AdEmpty
AdFAST
n=2
MOI 1
MOI 5
MOI 10
MOI 50
MOI 100
B
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
PBS
AdEmpty
AdFAST
n=3
24h
48h
72h
96h
C
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
PBS
AdEmpty
AdFAST
n=3
MOI 1
MOI 10
MOI 50
MOI 100
D
PBS
AdEmpty
AdFAST
20-
15-
cleaved caspase-3
50-
α-tubulin

Figure 3.5: FAST protein expression in A549 cells decreases metabolic activity and promotes apoptosis. **A)** A549 cells were infected with AdEmpty, AdFAST or VSVΔ51 using a range of MOIs. Relative cell membrane integrity was measured based on the lactate dehydrogenase levels in the supernatant 72 hpi. The average of two independent experiments (n=2) is shown with each experiment done in triplicate. Error bars denote the standard error of the mean. *$p<0.05$ compared to cells only. **$p<0.05$ when comparing AdFAST to AdEmpty infected cells. **B)** A549 cells were infected at an MOI of 10 with AdEmpty or AdFAST. Cellular metabolic activity was assessed every 24 h until 96 hpi using an MTS assay. The average of three independent experiments (n=3) done in triplicate is plotted with the standard error of the mean. Values were normalized to mock infected cells at 24 hpi. *$p<0.05$ compared to mock infected cells at their corresponding timepoints. **$p<0.05$ comparing AdFAST to AdEmpty infected cells. **C)** A549 cells were infected with AdEmpty or AdFAST at an MOI of 1, 10, 50 or 100. An MTS assay was conducted 72 hpi. The average of three independent experiments (n=3) done in triplicate ± the standard error of the mean is shown with normalization to mock infected cells. *$p<0.05$ compared to mock infected cells. **$p<0.05$ comparing AdFAST to AdEmpty treated cells. **D)** A549 cells were infected with AdEmpty or AdFAST at an MOI of 10. Following a 72 h incubation, whole cell lysates were collected and samples were probed for expression of cleaved caspase-3. Alpha-tubulin served as a loading control. The blot is representative of three independent experiments. No cleaved caspase-3 expression was observed in AdEmpty-infected or mock-infected cells in all three experiments.

and AdFAST at an MOI of 10 and the metabolic activity was assessed every 24 h. For PBS-treated cells, we observed a decrease in metabolic activity over the 4-day course of the assay, likely reflecting reduced metabolic activity as the cells reached confluence and underwent contact inhibition. For cells treated with AdEmpty, no difference in metabolic activity, relative to PBS-treated control cells, was observed. In contrast, AdFAST-infected cells had significantly decreased metabolic activity at 48 hpi and 72 hpi compared to AdEmpty-infected cells ($p<0.05$) (Figure 3.5B). Moreover, with increasing MOI, AdFAST had a greater negative effect on metabolic activity ($p<0.05$) relative to AdEmpty (Figure 3.5C). To determine if decreased metabolic activity could lead to increased cell death, we assayed cells for an apoptotic marker, cleaved caspase-3. A549 cells were treated with PBS, AdEmpty or AdFAST at an MOI of 10. Crude protein samples were isolated 72 h later, and assayed for cleaved caspase-3 by immunoblot (Figure 3.5D). As expected, PBS-treated cells also did not show evidence of cleaved caspase-3. No evidence of cleaved caspase-3 was noted for AdEmpty-treated cells, indicating that although AdEmpty infection can reduce metabolic activity (Figure 3.5C), it does not necessarily promote apoptotic cell death. Even though the AdFAST lysate sample was slightly underloaded, as shown by the relative tubulin signal, a significant cleaved caspase-3 signal was observed for AdFAST-treated cells. Taken together, these results show that infection of A549 cells with AdFAST resulted in increased membrane permeability and enhanced apoptotic cell death, suggesting that AdFAST may be an effective sole anti-cancer therapeutic.

3.2.5. FAST protein expression from a replication defective Ad does not induce syncytia formation or improve survival in an A549 xenograft cancer model

Since we had observed significant fusion and cell death in A549 cells infected with AdFAST *in vitro*, we examined whether FAST protein expression would be beneficial in an

in vivo setting. CD-1 nude mice were injected with A549 cells in the right hind flank to establish subcutaneous tumors and were subsequently treated with intratumoral injections of PBS or virus. To determine whether AdFAST induced syncytia formation in the A549 subcutaneous tumors, tumors were isolated from mice intratumorally injected with PBS, AdEmpty or AdFAST 5 days post injection. H/E stains showed no obvious histological differences in virus-injected tumors (Figure 3.6A). As Ad normally localizes to the liver, liver samples were also subjected to H/E staining, and no differences in pathology was observed between the various treatments (not shown). At day 20 post injection, while AdFAST injected tumors were slightly smaller in size compared to AdEmpty and PBS injected tumors, this did not reach statistical significance (Figure 3.6B). Furthermore, AdFAST did not improve survival of treated mice (Figure 3.6C). Thus, *in vivo* studies suggest that expression of FAST protein from an E1-deleted, replication defective Ad vector is not sufficient to achieve a beneficial therapeutic effect in the A549 xenograft tumor model in immunocompromised mice.

3.3 Discussion

Ad has shown great promise as a delivery vehicle in many animal models of human disease. Unfortunately, when injected into a solid tumor, Ad does not disseminate far from the injection site, rarely infecting cells more than about 5 mm from the injection site (93). To overcome this problem, we investigated whether the cell-cell fusion abilities of the p14 FAST protein could enhance Ad distribution. Cell fusion would theoretically allow spread of Ad encoded gene products, or Ad itself, to neighbouring cells, potentially enhancing its therapeutic benefits. In addition, since fusogenic proteins can act as effective sole anti-cancer therapeutics (103, 113-115), we also examined the effect of Ad-mediated FAST protein expression on A549 adenocarcinoma cell line viability and in a xenograft nude

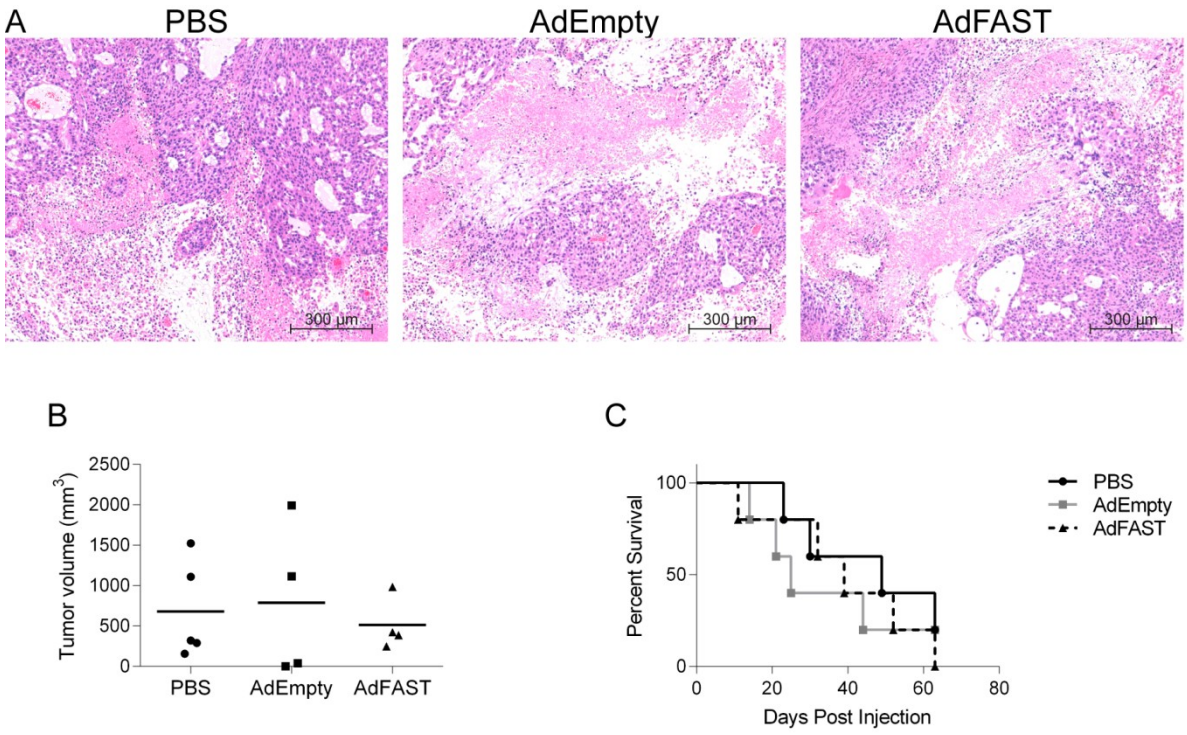

A
PBS
AdEmpty
AdFAST
300 µm
300 µm
300 µm
B
Tumor volume (mm3)
2500
2000
1500
1000
500
0
PBS
AdEmpty
AdFAST
C
Percent Survival
100
50
0
0
20
40
60
80
Days Post Injection
PBS
AdEmpty
AdFAST

Figure 3.6: AdFAST does not induce fusion or promote survival in immunodeficient CD-1 mice with subcutaneous A549 tumors. A) CD-1 nude mice harbouring subcutaneous A549 tumors were intratumorally injected with PBS, $5x10^8$ pfu AdEmpty or AdFAST. Five days post injection, tumors were excised, fixed, sectioned and subjected to hematoxylin and eosin staining. Results are representative of 2-3 mice (10x objective). **B)** Subcutaneous A549 tumors intratumorally injected with PBS, $5x10^8$ pfu AdEmpty or AdFAST were measured at day 20 post injection. The line in each column represents the average of the associated treatment group. **C)** A Kaplan-Meier survival curve shows the percentage survival of CD-1 mice with subcutaneous A549 tumors intratumorally injected with PBS, AdEmpty or AdFAST over time. Each treatment group consisted of 5 mice.

mouse tumor model.

After confirming FAST protein expression from our Ad constructs (Figure 3.1B), we examined the effects of FAST protein in 293 cells under conditions where the E1-deleted vector would replicate and yield very high levels of p14 FAST protein. FAST protein expression caused extensive cell fusion (Figure 3.2) and reduced metabolic activity within the cells (Figure 3.3C, D). Previous studies with the FAST protein has shown that extensive syncytium formation induces cell death, which itself may be responsible for the altered membrane integrity (134). As discussed in the introduction, the exact mechanism by which FAST protein mediates cell fusion has yet to be elucidated. For the GALV fusion protein, syncytia remain viable for up to 2 days after fusion, but then suffer from a collapse of mitochondrial membrane potential due to suspected GALV protein mislocalization and concomitant severe depletion of ATP leads to cell death (123). We also observed large syncytia that appear viable (Figure 3.3B), but with a reduced metabolic activity (Figure 3.3C), which could suggest that FAST protein also impacts upon organelle function prior to the onset of cell death.

In A549 cells, which are non-permissive for replication of these E1-deleted vectors, FAST protein expression caused cell-cell fusion only at relatively high MOI (Figure 3.4), suggesting that there may be a threshold of FAST protein expression required for fusion. It is suggested that the FAST protein oligomerizes, which could explain why a high level of FAST protein expression is required for its function (137).

We also observed a significant decrease in cell membrane integrity in AdFAST-treated A549 cells (Figure 3.5A) which correlated with decreased metabolic activity (Figure 3.5B, C) and enhanced apoptotic cell death (Figure 3.5D). Interestingly, compromised membrane integrity was noted at an MOI of 5 or greater, conditions that did not necessarily

exhibit extensive cell fusion. In a study by Salsman *et al.* (134), overexpression of FAST proteins, including the reptilian reovirus p14 protein, Avian reovirus p10 protein and Nelson Bay reovirus p10 protein in Vero and QM5 cells, caused decreased cell membrane integrity only after cell fusion and initiation of apoptosis. Moreover, membrane integrity could be rescued through use of an apoptotic inhibitor, suggesting decreased membrane integrity was a result of cell death rather than of FAST protein expression. However, our observations are similar to studies of the Avian reovirus p10 protein in monkey BSC-40 cells where the expression of a truncated p10 FAST protein compromised membrane integrity, but did not induce cell-cell fusion (162). These data suggest that effects on membrane leakiness and cell fusion are two independent events. Differences in cell lines and expression conditions may cause the FAST proteins to induce membrane permeabilization without extensive cell-cell fusion, as seen in our study. It is also possible that small syncytia were formed at low MOIs, leading to the membrane permeability detected.

In our nude mouse studies, we did not see any obvious signs of fusion in AdFAST injected tumors (Figure 3.6A). Furthermore, treatment with AdFAST did not lead to a significant decrease in tumor burden or better survival compared to control mice (Figure 3.6B, C). Our *in vitro* studies suggested that high level FAST protein expression is required to achieve significant fusion in A549 cells (Figure 3.4). It is possible that the difference between the tumor microenvironment and *in vitro* conditions affects gene expression from the Ad vector, resulting in lower relative expression of FAST protein *in vivo* compared to *in vitro*. Alternatively, the effective multiplicity of infection for cells contained in a tumor, and hence the level of FAST protein expression, may not reach the threshold level required for efficient fusion. Under conditions of active viral replication, FAST protein expression is higher (Figure 3.1B) and associated with enhanced fusion (Figure 3.2) and decreased

metabolic activity (Figure 3.3C, D). This observation suggests that with high FAST protein expression from a viral vector, we would observe enhanced syncytia formation, membrane permeability and cell death.

In this chapter, we showed that Ad vectors can be used to express the p14 FAST fusogenic protein in human cancer cells, resulting in direct cell-cell fusion, and compromised cell membrane integrity leading to enhanced cell death. Our data shows that the ability of the FAST protein to fuse cells, compromise membrane integrity and induce apoptotic cell death is directly related to the FAST protein expression level. Although a replication defective vector expressing the FAST protein did not provide a significant therapeutic benefit in a xenograft model of cancer, our study suggests that high levels of Ad-mediated FAST protein expression may improve vector efficacy and distribution.

CHAPTER 4: Investigation of Ad-mediated FAST protein expression in murine cell lines *in vitro* and in a syngeneic mouse tumor model

4.1 Introduction

The process of cell-cell fusion can promote release of tumor-antigen containing syncytiosomes, resulting in enhanced activation of the immune system (103). Furthermore, more syncytiosomes are released during the cell fusion process and associated death, compared to other mechanisms of cell death such as irradiation, freeze-thaw, osmotic-shock and HSV-1 TK/ganciclovir administration (103). In bilateral subcutaneous tumor mouse models, tumors injected with Ad expressing fusogenic proteins and uninjected contralateral tumors both showed a reduction in size, infiltration by immune cells and a T-cell specific response (113-115). Moreover, the therapeutic benefit was enhanced with immunostimulatory gene expression, indicating the important role of the immune system in promoting an anti-tumor response (113-115). Another study observed similar therapeutic efficacy in injected and uninjected tumors in a bilateral subcutaneous tumor model with Ad expressing the RSV fusion protein alone or in combination with the RSV G glycoprotein, a protein that enhances syncytia formation (129). A cytotoxic T cell response was observed with both viruses. Mice treated with Ad expressing a non-fusogenic soluble version of the F protein or G glycoprotein alone did not reduce tumor burden or elicit strong T-cell specific anti-tumor responses, highlighting the importance of fusion-associated cell death and subsequent immune system activation (129). In the previous chapter, we examined Ad-mediated FAST protein expression in human models of cancer, but did not see an enhanced effect in treated A549 xenograft nude mice. As we were using nude mice, the contribution of the immune system in mediating tumor regression could not be examined. Therefore, in

our next set of studies, we investigated the effects of FAST protein expression in murine cancer cell lines *in vitro* and in a subcutaneous syngeneic mouse model.

4.2 Results

4.2.1. 4T1 cells have lower Ad susceptibility to Ad infection, and support lower levels of transgene expression compared to A549 cells

We first examined a number of common murine cancer cell lines for their susceptibility to Ad infection and FAST protein mediated cell fusion. Mouse colon cancer CT26, melanoma B16F10, and mammary cancer 4T1 cells were infected with AdFAST/RFP or AdRFP, and examined for gene expression and evidence of fusion 48 h later. At an MOI of 50, the CT26 cell line did not show evidence of infection, as no red cells were observed (Figure 4.1). The 4T1 and B16F10 cell lines show a similar level of infection. However, neither cell line showed obvious signs of syncytia formation at this MOI.

We previously showed that FAST protein expression could compromise membrane integrity in the absence of cell fusion. As such, we examined the relative membrane permeability of these three cell lines when infected with AdEmpty or AdFAST, by determining the quantity of LDH released into the supernatant at 72 hpi. CT26 and B16F10 cells showed no apparent difference in membrane integrity after infection at an MOI as high as 100 (Figure 4.2A, B). AdFAST-infected 4T1 cells showed a modest increase in membrane permeability at MOI 100 compared to cells infected with AdEmpty (Figure 4.2C). We therefore chose the 4T1 cell line for further characterization.

We also previously showed that under conditions in which AdFAST could replicate (i.e. in E1-complementing 293 cells), rampant cell fusion was observed (Figure 3.2). However, under non-replicating conditions, high MOI of AdFAST was required to provide sufficient FAST protein expression to promote fusion in A549 cells. We therefore examined

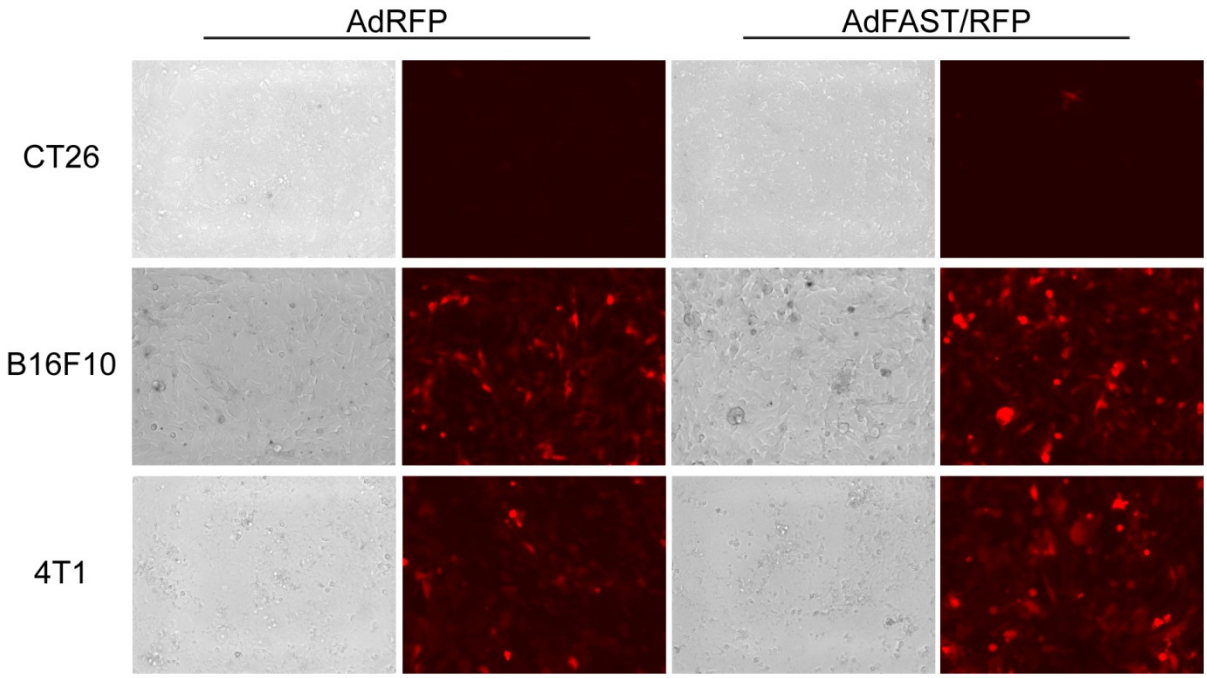
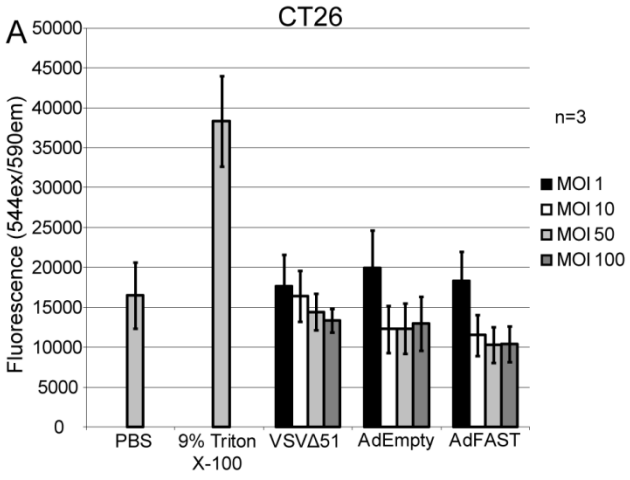
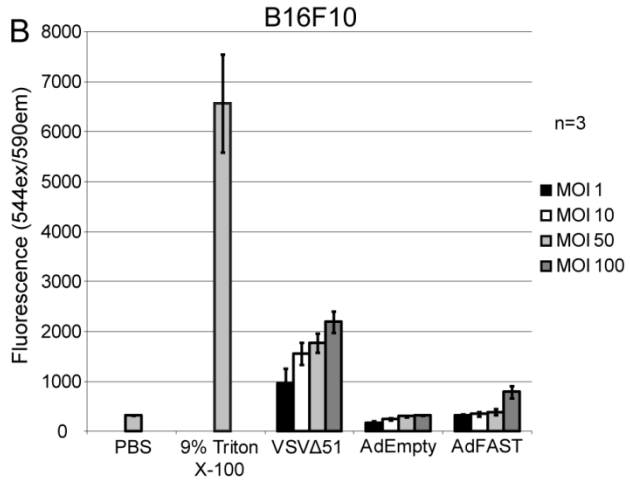

AdRFP
AdFAST/RFP
CT26
B16F10
4T1

Figure 4.1: **AdFAST/RFP does not induce extensive cell fusion in murine cell lines.** CT26, B16F10 and 4T1 cells were infected with AdRFP or AdFAST/RFP at an MOI of 50 and visualized using fluorescence microscopy 48 hpi.


A
CT26
Fluorescence (544ex/590em)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
PBS
9% Triton X-100
VSVΔ51
AdEmpty
AdFAST
n=3
MOI 1
MOI 10
MOI 50
MOI 100


B
B16F10
Fluorescence (544ex/590em)
8000
7000
6000
5000
4000
3000
2000
1000
0
PBS
9% Triton X-100
VSVΔ51
AdEmpty
AdFAST
n=3
MOI 1
MOI 10
MOI 50
MOI 100

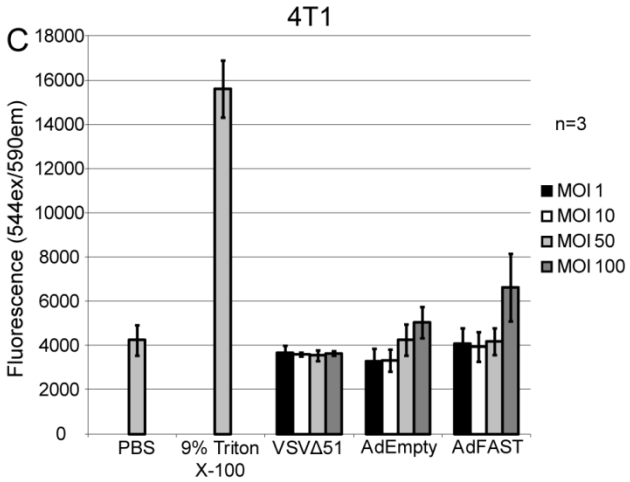

C
4T1
Fluorescence (544ex/590em)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
PBS
9% Triton X-100
VSVΔ51
AdEmpty
AdFAST
n=3
MOI 1
MOI 10
MOI 50
MOI 100

Figure 4.2: 4T1 cells infected with AdFAST show a modest decrease in membrane integrity. A) CT26 cells were infected with AdEmpty, AdFAST and VSVΔ51 at varying MOI from 1 to 100 for 72h. Membrane permeability was assessed by determining the lactate dehydrogenase levels in the cell supernatant. Three independent experiments (n=3) were conducted in triplicate. The values graphed represent the average and the standard error of the mean. Experiments were repeated using the same protocol for **B)** B16F10 and **C)** 4T1 cells.

both the relative infection level and human CMV promoter activity in 4T1 versus human A549 cells. We infected 4T1 and A549 cells at an MOI of 1 with Adβ-Gal and determined the percentage of infected cells using X-gal staining and promoter activity using a chemiluminescent β-gal activity assay 24 h later. Under these conditions, 1.2% of 4T1 cells stained blue, indicating infection by Adβ-Gal, while A549 cells were infected at an efficiency of 5.9% (Figure 4.3A). Hence, Ad was able to infect A549 cells approximately 5-fold more efficiently than 4T1 cells. An assessment of the promoter activity using the chemiluminescent assay showed that A549 cells had a 10.6-fold increase in activity compared to 4T1 cells (Figure 4.3B). When β-gal activity was normalized to the number of infected cells, A549 cells had ~2.1-fold higher level of β-gal expression per infected cell compared to 4T1 cells (Figure 4.3C). Although the level of protein expression driven from the CMV promoter in an Ad vector was not dramatically different between the two cell lines, a higher MOI was required to achieve the same degree of cell infection, and thus overall level of FAST protein expression, in 4T1 cells compared to A549 cells.

4.2.2. FAST protein expression in 4T1 cells causes cell fusion and modestly reduces metabolic activity

We next examined whether high level expression of the FAST protein can cause fusion of 4T1 cells. We previously showed that in E1-complementing 293 cells, AdFAST can replicate to increase the template copy number and generate very high levels of FAST protein, resulting in cell fusion readily observed with as low an MOI as 1 (Figure 3.1). However, in A549 cells, in which AdFAST cannot replicate, a significantly higher MOI (MOI 50) was required to induce obvious cell fusion (Figure 3.4). Given the slightly lower level of protein expression observed from an Ad vector in 4T1 cells and the 5-fold reduction in overall infection efficiency (Figure 4.3), we would predict that a higher MOI would be

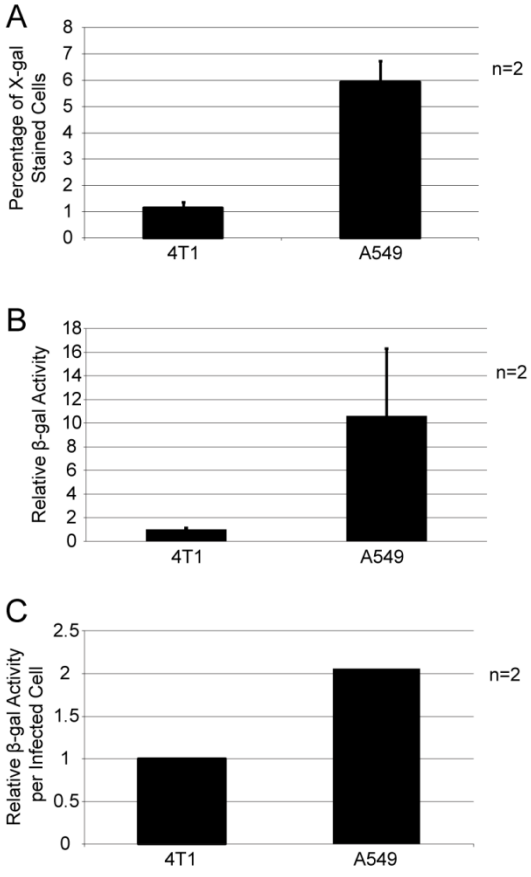

A
Percentage of X-gal Stained Cells
8
7
6
5
4
3
2
1
0
4T1
A549
n=2
B
Relative β-gal Activity
18
16
14
12
10
8
6
4
2
0
4T1
A549
n=2
C
Relative β-gal Activity per Infected Cell
2.5
2
1.5
1
0.5
0
4T1
A549
n=2

Figure 4.3: Adβ-Gal shows lower infection efficiency and promoter activity in murine 4T1 cells relative to human A549 cells. 4T1 and A549 cells were infected with Adβ-Gal at an MOI of 1 for 24h. Cells were either **A)** fixed and stained with X-gal or **B)** examined for β-galactosidase activity using a chemiluminescence assay. Values for the chemiluminescent assay were normalized to the average of the two 4T1 chemiluminescent readings. **C)** The relative β-galactosidase activity per infected cell was determined (n=2). The average is shown for all experiments, and the error bars represent the standard deviation.

needed to observe significant fusion in this cell line. 4T1 cells were infected at varying MOI and stained with Geimsa stain 48 hpi to visualize fused cells. As shown in Figure 4.4A, although we did not observe evidence of cell fusion in 4T1 cells at an MOI of 50 (data not shown), we did observe fusion at an MOI of 1000. No obvious fusion was observed for cells infected with the AdEmpty control virus. Thus, FAST protein expression from an Ad vector can cause 4T1 cell fusion.

We next examined whether high levels of FAST protein expression in 4T1 cells led to significantly enhanced cell membrane permeability. In A549 cells, there was a dramatic increase in cell membrane permeability even at low MOI, at which no obvious signs of cell fusion was observed (Figure 3.5). This compromised membrane integrity was accompanied by the increase of the apoptotic marker, cleaved caspase 3, indicating that the FAST protein could be an effective sole therapeutic, even in the absence of extensive syncytia formation. To examine cell membrane integrity in AdFAST-infected 4T1 cells, we infected the cells with AdEmpty or AdFAST at MOI ranging from 100 to 1000 and assessed membrane integrity using an LDH release assay. Cells infected with AdFAST at an MOI of 1000 showed a ~3-fold increase in membrane permeability compared to AdEmpty infected cells at the same MOI, but this effect did not reach statistical significance (Figure 4.4B). Furthermore, the positive control, VSVΔ51, only caused a ~4-fold decrease in membrane permeability compared to Triton X-100, which would ideally lyse all cells. In our previous study, A549 cells infected with VSVΔ51 was able to cause membrane permeability levels about ~66% of Triton X-100 cells (Figure 3.3A), suggesting that 4T1 cells may be more naturally resistant to virus-mediated cell death/membrane permeability.

Previous work with the GALV fusion protein suggested that protein-mediated cell death may be caused by the protein mislocalizing to the mitochondria, leading to the disrupt-

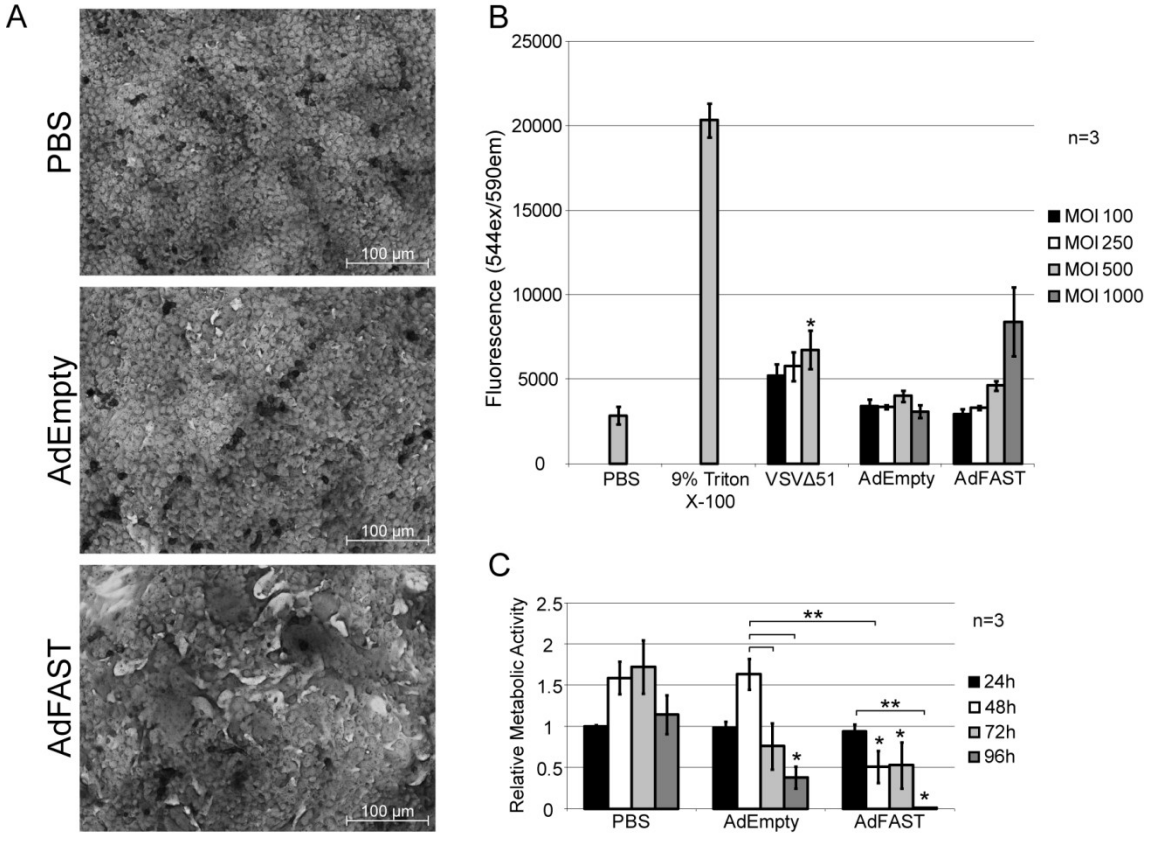

A
PBS
AdEmpty
AdFAST
100 µm
100 µm
100 µm
B
Fluorescence (544ex/590em)
25000
20000
15000
10000
5000
0
PBS
9% Triton X-100
VSVΔ51
AdEmpty
AdFAST
n=3
MOI 100
MOI 250
MOI 500
MOI 1000
C
Relative Metabolic Activity
2.5
2
1.5
1
0.5
0
PBS
AdEmpty
AdFAST
n=3
24h
48h
72h
96h

Figure 4.4: Infection of 4T1 cells with AdFAST promotes cell fusion and reduces cellular metabolic activity. A) 4T1 cells were infected with AdEmpty or AdFAST at an MOI of 1000, or mock infected with PBS. Cells were fixed and stained with Giemsa stain 48 hpi. Images depicted are representative of two replicates (n=2) (20x objective). **B)** Subconfluent 4T1 cells were infected with VSVΔ51, AdEmpty or AdFAST at varying MOIs. Seventy-two hours post infection, cellular membrane integrity was assessed by measuring the lactate dehydrogenase activity in collected supernatants. The average of three independent experiments (n=3) completed in triplicate is shown with the standard error of the mean. *$p<0.05$ compared to mock infected cells. **C)** Subconfluent 4T1 cells were infected at an MOI of 1000 and the relative metabolic activity was determined using an MTS assay over a timecourse. Results depict the average of three independent experiments (n=3) done in triplicate. Error bars show the standard error of the mean. *$p<0.05$ compared to mock infected cells. **$p<0.05$

-ion of the mitochondria membrane potential and loss of ATP production (123). Our studies also suggest that FAST protein expression may impact upon organelle function as indicated by a decrease in metabolic activity. FAST protein could induce changes in metabolic processes through uncharacterized mechanisms or possibly mislocalize to the mitochondria, leading to metabolic failure. To investigate whether treatment of 4T1 cells with AdFAST also adversely affected cellular metabolic activity, we infected cells at an MOI of 1000 with either AdEmpty or AdFAST and examined cellular metabolic activity over 96 h through an MTS assay. At 24 hpi, cells treated with these viruses showed metabolic activity similar to PBS-treated cells (Figure 4.4C). For both PBS- and AdEmpty-treated cells, there was an increase in apparent metabolic activity at 48 hpi relative to the earlier time point. In contrast, treatment of cells with AdFAST resulted in a statistically significant decline in metabolic activity to about one third of controls. At 72 and 96 hpi, metabolic activity was similar in AdEmpty and AdFAST-infected cells, likely due to vector itself as PBS-treated cells had higher metabolic activity at these timepoints. Thus, treatment of 4T1 cells with AdFAST results in cell fusion, a modest increase in cell membrane permeability, but a significant loss of metabolic activity. Taken together, these results suggest that AdFAST may provide a therapeutic effect in 4T1 tumors in mice.

4.2.3. Ad-mediated FAST protein expression does not reduce tumor burden or promote survival in subcutaneous 4T1 tumors of immunocompetent mice

We next examined whether AdFAST could provide a therapeutic effect in an immunocompetent 4T1 tumor model. Balb/C mice with 4T1 subcutaneous tumors were injected with PBS or $1.9x10^9$ pfu AdFAST and tumor volume and survival were monitored post-treatment. Treatment of tumor-bearing mice with AdFAST did not alter the rate of tumor growth nor did it enhance survival (Figure 4.5A, B). Tumors that were excised 3 days

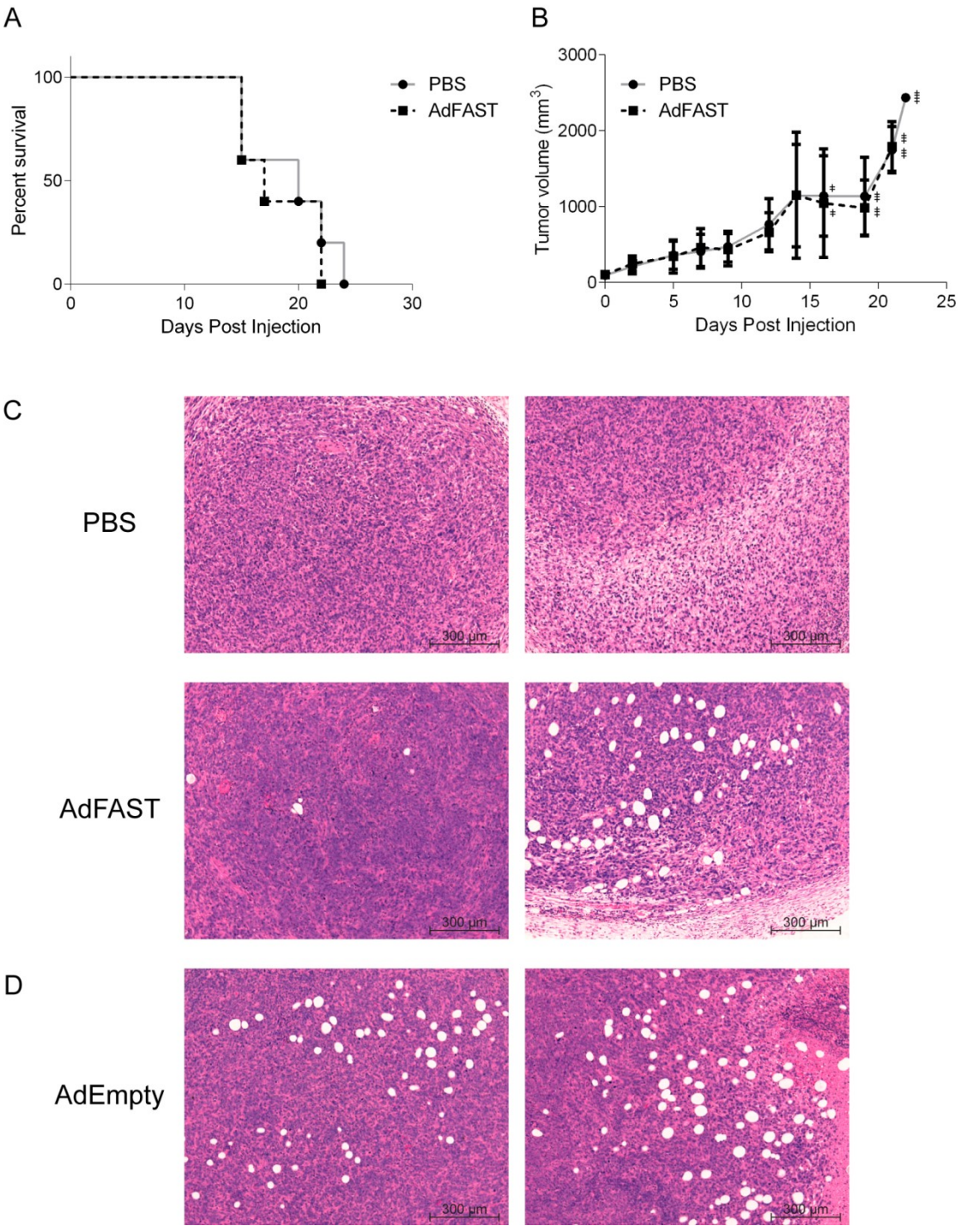

A
Percent survival
100
50
0
0
10
20
30
Days Post Injection
PBS
AdFAST
B
Tumor volume (mm3)
3000
2000
1000
0
0
5
10
15
20
25
Days Post Injection
PBS
AdFAST
C
PBS
AdFAST
D
AdEmpty
300 µm

Figure 4.5: AdFAST does not promote survival or reduce tumor growth in immunocompetent Balb/C mice with subcutaneous 4T1 tumors. **A)** Kaplan-Meier survival curve for 4T1 tumor-bearing mice treated with AdFAST. Balb/C mice with subcutaneous 4T1 tumors were intratumorally injected with PBS or $1.9x10^9$ pfu AdFAST. Each treatment group consisted of 5 mice. **B)** The average size of tumors treated with PBS or AdFAST as described in Panel A. The error bars represent the standard deviation. The + symbols show the number of mice that had reached endpoint by the time measurements were taken. **C)** Subcutaneous 4T1 tumors from Balb/C mice were removed, fixed, sectioned and stained with hematoxylin and eosin stain 3 days post injection. Images are representative from a group of 3 mice per treatment. **D)** Three Balb/C mice with subcutaneous 4T1 tumors were intratumorally injected with $1.4x10^8$ pfu AdEmpty. Three days post injection, tumors were excised, fixed, sectioned and subjected to hematoxylin and eosin stain.

post injection showed no obvious signs of syncytia formation (Figure 4.5C). However, in AdFAST treated tumors, we observed numerous lesions located near tumor margins, which appeared as holes in the tissue. As we were unsure whether this was due to FAST protein expression or the Ad vector itself, we injected 4T1 tumor bearing mice intratumorally with $1.4x10^8$ pfu AdEmpty and removed the tumors 3 days post injection for analyses. AdEmpty-treated tumors also had similar lesions, indicating the effects seen were due to the Ad vector and not expression of the FAST protein (Figure 4.5D). Because Ad normally accumulates in the liver (163), livers were also collected at the time of sacrifice. There were no obvious differences in pathology of livers from PBS or AdFAST treated mice (data not shown). Therefore, in the 4T1 immunocompetent mouse tumor model, AdFAST showed no obvious therapeutic benefit as assessed by tumor volume and mortality.

4.3 Discussion

In chapter 3, we examined the effects of FAST protein expression in the A549 lung adenocarcinoma model *in vitro* and in an A549 xenograft nude mouse model. In the A549 model, although we observed evidence of fusion, increased membrane permeability, and enhanced *in vitro* effects, treatment of established tumors in the immunocompromised mice with AdFAST did not provide a therapeutic benefit. Previous studies have shown that part of the efficacy of fusogenic proteins can be due to enhanced release of tumor associated antigen-containing syncytiosomes from the fused cell, which can subsequently act to stimulate immune-mediated rejection of the tumor (103). This effect would be absent in our A549 xenograft studies. We therefore investigated whether AdFAST would have a therapeutic effect in an immunocompetent mouse tumor model.

Of the three mouse cancer cell lines we investigated, we found that Ad-infected B16F10 and 4T1 tumor cells with a similar efficiency (Figure 4.1). However, infection of

these two cell lines with AdFAST showed that the 4T1 cells had a higher degree of FAST protein-mediated cell membrane permeability (Figure 4.2, 4.4), although a very high MOI was required for this effect. 4T1 cells showed an overall ~5 fold lower efficiency of infection relative to A549 cells (Figure 4.3A) and also approximately half the relative amount of expression per infected cell (Figure 4.3C). In our AdFAST vector, FAST expression is regulated by the human CMV immediate early enhancer/promoter (Figure 2.1). Previous studies have shown that the CMV enhancer/promoter is less active in murine cells than in human cells (164), consistent with our observations. Thus, although the 4T1 model may be adequate to evaluate the efficacy of AdFAST in an immunocompetent mouse model, higher amounts of virus may be required to see an effect.

Even though treatment of 4T1 cells with AdFAST led to modest membrane permeability, we observed some synyctia formation at a high MOI (MOI 1000) (Figure 4.4A, B). In our previous studies with A549 cells, we also observed a significant increase in FAST-mediated membrane permeability in the absence of extensive cell fusion (Figure 3.5A), suggesting either that increased membrane permeability may precede cell fusion, or the two events are independent events and not temporally connected. Studies by others using several FAST family members have shown that in some cell lines fusion and onset of apoptosis was required before significant membrane leakiness was observed (134). However, this is not always the case, and may in part reflect the cell line being studied (162). The absence of significant cell fusion is also perhaps not surprising, given that many fusogenic proteins have limited activity in mouse models (103). There may be fundamental differences between human and mouse cells that confer their ability to fuse. For example, the human Pit-1 receptor is required for GALV protein-mediated fusion, but murine cells express a homologue of Pit-1, resulting in limited or no fusion activity (125). As the complete

mechanism for FAST protein-mediated cell fusion has not been elucidated, there may be cofactors that are required for efficient FAST protein function that are absent in the cell lines we are using. Regardless, high MOI infection of 4T1 cells with AdFAST correlated with a decrease in metabolic activity (Figure 4.4C). It may be that a significant amount of FAST protein may mislocalize to the membrane of cellular organelles, such as the mitochondria, leading to disruption of metabolic processes and initiation of cell death (123). Taken together, these results suggested that AdFAST warranted testing for efficacy *in vivo.*

Treatment of 4T1 tumor-bearing mice with AdFAST did not result in enhanced survival (Figure 4.5A) or a reduction in tumor growth (Figure 4.5B). Previous studies with replication defective Ad vectors expressing RSV or MV fusion proteins showed that although these vectors mediated limited fusion in mouse cells *in vitro*, they were still able to act as effective sole therapeutics to reduce tumor burden in sygeneic mouse models (113, 115). Unfortunately, a similar effect was not observed for our experiments with the FAST protein. Work by Bateman *et al*. (103) demonstrated that fused, dying syncytia showed enhanced release of syncytiosomes, which are more effective at stimulating anti-tumor immunity than cells dying by other means. We had hoped that a similar mechanism would occur for FAST protein-expressing tumor cells. It is possible that the poor ability of the FAST protein to mediate fusion of the 4T1 cells prevented formation of syncytiosomes *in vivo* as we did not observe evidence of syncytia formation in tumor sections from AdFAST-treated mice (Figure 4.5C). However, we did observe a large number of pockets or holes in tumor sections from animals receiving treatment with either AdEmpty or AdFAST, but not in animals injected with PBS, suggesting these lesions were due to administration of the Ad vector. Although these vectors are deleted of the E1 and E3 region, and are thus largely replication defective, they still have the ability to express some viral proteins, such as

proteins encoded by E4 (165) One of the E4 open reading frames (orf), E4orf4, is capable of inducing p53-independent cell death through a non-classical apoptotic mechanism (166). The low level expression of some of these viral proteins may have contributed to the formation of the lesions, but the mechanism is unclear. Regardless, we did not observe a difference in rate of tumor growth between Ad- and PBS-treated animals, indicating that the lesions were not associated with tumor stasis or tumor regression.

In this chapter, we show that Ad-mediated p14 FAST protein expression did not induce extensive cell-cell fusion and had modest effects on membrane permeability and cellular metabolic activity in the mouse cancer cell lines examined. Moreover, AdFAST did not have an enhanced therapeutic benefit in the immunocompetent 4T1 mouse mammary cancer model compared to mock treated mice. Our results suggest that a modified treatment regime, vector modifications or additional immunostimulatory transgenes may be required to induce a therapeutic effect with AdFAST in immunocompetent *in vivo* models.

CHAPTER 5: Examining combinational therapies with Ad-mediated FAST protein expression for cancer treatment

5.1 Introduction

In chapter 3, we showed that FAST protein expression in human cancer cells can drastically increase membrane permeability. Decreased membrane integrity could promote the uptake of anti-cancer compounds such that lower quantities are needed to achieve a therapeutic effect. Moreover, FAST protein-mediated cell-cell fusion could release syncytiosomes containing metabolites that can be taken up by neighbouring cells and thereby, enhancing the bystander effect (103). The "bystander effect" is the ability to cause effects or cell death in non-transduced neighbouring cells (167). In this chapter, we investigated whether AdFAST in combination with Ad-mediated TK expression, SMC or chemotherapeutic drug administration can provide a more robust therapeutic outcome. Below is a brief summary of previous studies conducted on these therapeutics and their mode of action.

5.1.1. Thymidine kinase and ganciclovir system

Due to HSV-1 TK's ability to modify guanosine analogs, such as ganciclovir, the first application of HSV-1 TK was for fighting against viral infections (168-171). TK converts ganciclovir to deoxyguanosine monophosphate, followed by conversion of the intermediate product by cellular kinases to the active deoxyguanosine triphosphate analog that is incorporated into replicating DNA (168, 169, 172). When ganciclovir is first introduced to a population of dividing cells, most cells go through a complete cell cycle after initial drug exposure (i.e. divide once) as incorporated ganciclovir still contains a sugar ring hydroxyl group required for DNA elongation (173-175). In the second cell cycle, cells become arrested in the S and G_2/M phase, likely caused by attempting to replicate ganciclovir

incorporated DNA and causing double-stranded DNA breaks (173-176). The DNA breaks then initiate apoptosis through release of cytochrome c from the mitochondria and activation of caspase-9. Caspase-9 subsequently cleaves caspases 3, 7 and the anti-apoptotic protein, Bcl-2, to further promote cell death (174). The TK/ganciclovir system has been studied extensively for suicide gene therapy due to its strong bystander effect, a process that requires direct cell-cell contact (167, 177, 178). Ganciclovir can be transferred through gap junctions, resulting in death of neighbouring cells not expressing TK (177, 178). It has also been suggested that apoptotic bodies can be taken up by distant cells, leading to transfer of the phosphorylated compound (179-181).

The TK suicide gene system has been investigated in various clinical trials as a sole therapeutic and in combination with conventional cancer treatments or suicide genes (182-190). Clinical trials using TK/ganciclovir-based therapy have focused on the treatment of solid cancers such as glioma, prostate cancer, mesothelioma and hepatocellular carcinoma (183-192). In a recent phase III study by Westphal *et al.* (190), patients were treated with an E1 and E3 deleted Ad expressing HSV TK under the control of the CMV promoter (sitimagene ceradenovec) after glioma resection, radiotherapy, and administered ganciclovir. Even though the treatment group showed increased median time to re-intervention or mortality after treatment, there was no increase in overall survival rate compared to control patients. In another phase III study (187), injection of cells retrovirally transduced to express HSV TK into the tumor cavity after glioblastoma multiforme surgery, followed by radiotherapy and ganciclovir administration, did not improve survival rate or slow tumor progression compared to the control group. The authors attributed the treatment outcome to poor transgene delivery to cancer cells. Thus, improvements to vectors expressing TK can still be made to improve the bystander effect and efficacy.

5.1.2. Smac-mimetic compounds

SMCs are based on the intracellular Smac/DIABLO protein that is released from the mitochondria during apoptosis and inhibit inhibitors of apoptosis (IAPs) (193-195). SMCs can be designed to inhibit XIAP, and/or degrade cIAP1 and cIAP2, promoting intrinsic apoptosis and increased susceptibility to extrinsic apoptotic signalling (196-200). Normally, XIAP inhibits caspase-3, 7, 9 while cIAPs are typically inhibit the death inducing signalling complexes (194, 200). The absence of IAPs (i.e. cIAPs) limits RIP1 ubiquitination and promotes RIP1 disassociation from tumor necrosis factor-receptor 1 and formation of the apoptosis initiating-complex II composed of RIP1, Fas-Associated protein with Death Domain (FADD), and caspase-8 (200, 201). Downregulation of IAPs also promotes apoptosis through ripoptosome formation and TNFα and/or TNF-related apoptosis-inducing ligand (TRAIL) mediated formation of death-inducing complexes (199, 200-203). SMCs are classified as monovalent or bivalent, depending on the number of IAP binding motifs (194, 196). Bivalent SMCs are approximately 100-1000 times more effective compared to their monovalent counterparts (194). Five SMCs are currently undergoing clinical testing (204).

The efficacy of SMC as an anti-cancer therapeutic to mediate cell death is associated with the intracellular levels of the caspase-8 homologue, cellular FLICE-inhibitory protein (cFLIP) (202, 205, 206). cFLIP competitively binds to FADD that is present in several complexes involved in caspase-8 dependent apoptosis (202, 205, 207). Furthermore, silencing of cFLIP sensitizes SMC-resistant cells to SMCs (205). As such, SMCs are only naturally effective in a subset of cancers such as those defective or express low levels of cFLIP and can express TNFα (205).

Not only are SMCs effective as single anti-cancer agents *in vitro* and *in vivo* (204, 208-210), several studies have shown that SMCs are effective in combinational therapy to

promote cell death in both SMC susceptible and non-susceptible cancers. SMCs administered in combination with cytokines, protein inhibitors or chemotherapeutic drugs in cancer models showed enhanced cell death (205, 208-215, 216 , 217-219). A recent study by Beug *et al.* (197) showed that in murine cancer models, the combination of oncolytic VSV and SMC had a synergistic effect by promoting type I IFN, TNFα, and TRAIL production, and cancer cell killing via the extrinsic apoptotic pathway.

In our studies, we focused on the cIAP targeting monovalent SMCs LCL161 and bivalent OICR720. LCL161 was effective against various cancer cell lines *in vitro* and co-administration of protein inhibitors or the chemotherapeutic drug paclitaxel had synergistic effects (210, 211, 216). Furthermore, LCL161 administration sensitized esophageal squamous carcinoma cells to radiotherapy (220). LCL161 was tested in a phase I trial for treatment of patients with advanced solid tumors (221). Results from this trial showed that dosages below 1800 mg administered weekly were well tolerated by patients. Phase 1b and phase II trials combining LCl161 with paclitaxel are in progress. To date, no clinical studies have been published using OICR720 for cancer treatment.

5.1.3 Chemotherapeutic drugs

Chemotherapeutic drugs are used as part of conventional therapy for cancer treatment. Two commonly used chemotherapeutic drugs are etoposide and bleomycin. The epipodophyllotoxin derivative, etoposide, enters the cell through passive diffusion and inhibits activity of topoisomerase II, an enzyme that releases helical strain during DNA replication (222-225). This results in DNA breaks, cell cycle arrest and cell death. Etoposide is typically used in a chemotherapeutic regime and given orally or intravenously to patients with small cell lung cancer and testicular cancer (226). Its use with cisplatin or carboplatin has an overall response rate of 75% in small cell lung cancer patients (227). For

germ cell cancer patients with good prognosis, the cure rate are ≥90% with treatment regimes involving bleomycin/etoposide/cisplatin and etoposide/cisplatin (228). Common side effects of etoposide treatment include hair loss, hypotension and irritation of the gastrointestinal tract (226).

Bleomycin is isolated from *Streptomyces verticillus* and is a glycopeptide that creates DNA single stranded and double stranded breaks (229-233). Briefly, bleomycin is activated by binding to Fe(II) or Cu(I) , oxygen and a one-electron reductant (229). In its active form, it can break amide bonds in proteins by hydrolyzation and create hydroxyl radicals to oxidize lipids. Activated bleomycin removes the 4' hydrogen atom from the C4' deoxyribose pyrimidine, leading to creation of a 4' radical intermediate within the DNA strand. The affected pyrimidine is typically situated 3' to a guanine. The 4' radical binds to oxygen to create a 4'-peroxy radical, which is then reduced to a 4'-hydroperoxide. Multiple chemical transformations follow, resulting in a cleaved DNA strand with a 3'-phosphoglycolate, 5' phosphate and a pyrimidine propenal. A subsequent bleoymcin-mediated reaction can lead to a double-stranded DNA break (229).

Bleomycin is approved for use against squamous cell carcinoma, lymphoma and testicular carcinoma, and can be administrated through various routes (226, 229, 234). It is a hydrophilic chemotherapeutic drug as it cannot cross cell membranes through passive diffusion and enters the cell through an uncharacterized mechanism (224, 229). Bleomycin accumulates in and has adverse effects on the skin and lungs as they do not have the bleomycin inactivating enzyme, bleomycin hydrolase (230, 231, 235, 236). Bleomycin efficacy is limited as it can cause dose-dependent, adverse effects such as interstitial pneumonitis and pulmonary fibrosis (229, 230, 237). As mentioned above, etoposide and bleomycin are commonly used together with cisplatin in chemotherapy for germ cell cancers

(228), and they have also been tested with various other anti-cancer moieties and in modified regimes in germ cell cancer and Hodgkin lymphoma clinical trials (238-241).

5.2 Results

5.2.1. Combination of AdTK and ganciclovir reduces HeLa cell metabolic activity

We first examined the combination of FAST protein and the TK and ganciclovir suicide system, as cell fusion could promote transgene and metabolite transfer and FAST protein-mediated membrane permeability could enhance drug uptake. Even though AdTK was able to mediate high levels of TK protein expression (~41 kDa) (242) in A549 cells (Figure 5.1A), addition of ganciclovir up to a final concentration of 250 μM did not significantly decrease A549 metabolic activity compared to the AdEmpty controls (Figure 5.1B). Thus, A549 cells do not appear to be particularly susceptible to AdTK/ganciclovir-mediated killing. We therefore utilized a different cancer model, HeLa cells, for our AdTK studies.

Ad-mediated TK expression was confirmed by infecting 293 and HeLa cells with AdEmpty or AdTK at an MOI of 10 and collecting whole cell lysates 48 hpi. TK was expressed at similar levels in infected 293 and HeLa cells as seen by the immunoblot in Figure 5.2A. Fibre expression was only detected in E1-complementing 293 cells infected with Ad, as expected since these vectors can replicate in 293 cells, but not in HeLa cells. To examine the effects of AdTK/ganciclovir alone, we infected HeLa cells at an MOI of 10 with AdEmpty or AdTK and added increasing concentrations of ganciclovir after 1 h of infection (Figure 5.2B). At 24 hpi, a significant decrease in metabolic activity was observed in cells infected with AdTK and receiving final concentrations of 10 μM ganciclovir or higher compared to the AdEmpty or PBS-treated controls ($p<0.05$). The relative metabolic activity of AdEmpty and mock infected cells were comparable at all concentrations except for 25 μM.

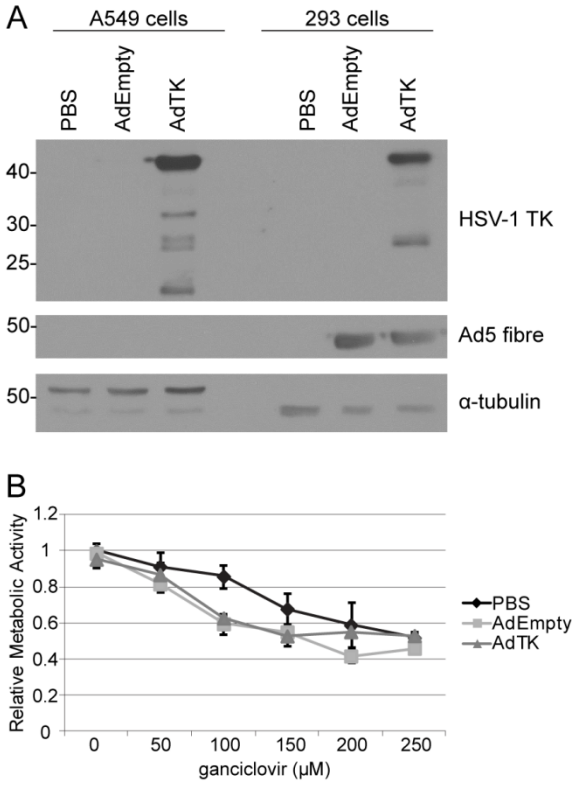

A
A549 cells
293 cells
PBS
AdEmpty
AdTK
PBS
AdEmpty
AdTK
40
30
25
HSV-1 TK
50
Ad5 fibre
50
α-tubulin
B
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
ganciclovir (μM)
PBS
AdEmpty
AdTK

Figure 5.1: A549 cells are not susceptible to TK/ganciclovir effects. A) A549 and 293 cells were infected with AdEmpty or AdFAST at an MOI of 10 for 48 h. Whole cell lysates were probed for HSV-1 TK, Ad5 fibre and tubulin. **B)** Subconfluent A549 cells were infected with AdEmpty or AdFAST at an MOI ~10. Following a 1 h infection, varying concentrations of ganciclovir was added and relative metabolic activity was assessed after 24 hpi. The average of three replicates is shown with the standard error of the mean.

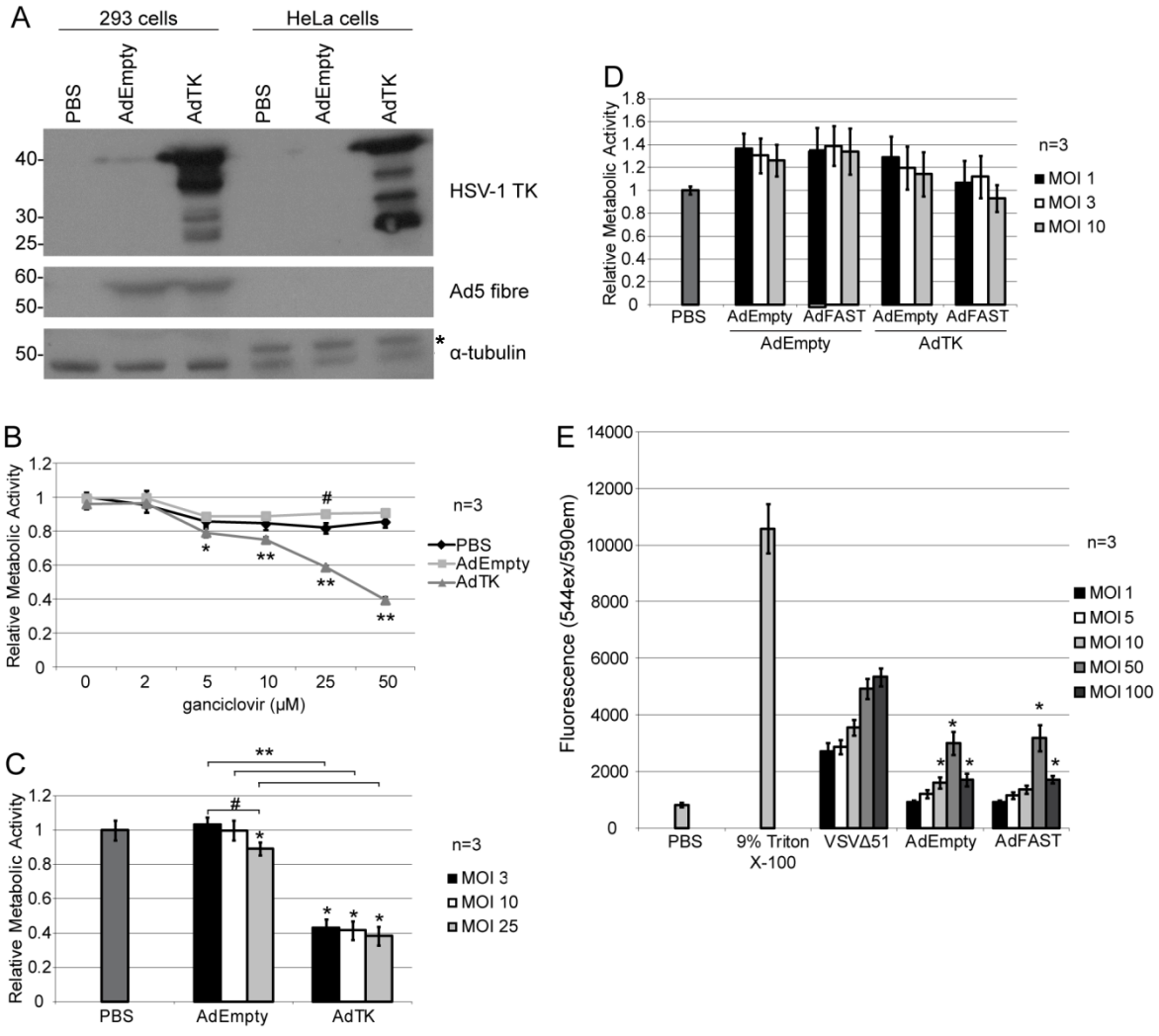

A
293 cells
HeLa cells
PBS
AdEmpty
AdTK
PBS
AdEmpty
AdTK
40
30
25
60
50
50
HSV-1 TK
Ad5 fibre
α-tubulin
*
B
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
0
2
5
10
25
50
ganciclovir (μM)
n=3
PBS
AdEmpty
AdTK
#
*
**
**
**
C
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
PBS
AdEmpty
AdTK
n=3
MOI 3
MOI 10
MOI 25
**
#
*
*
*
*
D
Relative Metabolic Activity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
AdEmpty
AdFAST
AdEmpty
AdFAST
AdEmpty
AdTK
n=3
MOI 1
MOI 3
MOI 10
E
Fluorescence (544ex/590em)
14000
12000
10000
8000
6000
4000
2000
0
PBS
9% Triton X-100
VSVΔ51
AdEmpty
AdFAST
n=3
MOI 1
MOI 5
MOI 10
MOI 50
MOI 100
*
*
*
*
*

Figure 5.2: Ad-mediated FAST protein expression does not enhance cellular metabolic effects caused by AdTK/ganciclovir in HeLa cells. A) HeLa and 293 cells were infected with AdEmpty or AdTK at an MOI of 10 and whole cell lysates were collected 48 hpi. Samples were probed for HSV-1 TK, Ad5 fibre, and α-tubulin was used as a loading control. In the HeLa cell lysate samples, * indicates the non-specific bands in HeLa cells. **B)** Subconfluent HeLa cells were infected at an MOI ~10 ($3.2x10^5$ pfu) with AdEmpty or AdTK. Ganciclovir was added at 1 hpi at varying final concentrations and the relative metabolic activity was determined 24 hpi. Results shown are the average of three independent experiments completed in triplicate and normalized to mock infected cells (n=3). The standard error of the mean is shown. *$p<0.05$ AdTK compared to AdEmpty, **$p<0.05$ AdTK compared to AdEmpty and mock infected cells, #$p<0.05$ AdEmpty compared to mock infected cells. **C)** Subconfluent HeLa cells were infected at varying MOIs. Ganciclovir was added to a final concentration of 10 μM 1 hpi. Relative metabolic activity was assessed 72 hpi. The average of three independent experiments with normalization to mock infected cells and standard error of the mean is shown (n=3). *$p<0.05$ compared to mock infected cells, **$p<0.05$ comparing AdTK and AdEmpty-infected cells. #$p<0.05$ comparing AdEmpty-infected cells at an MOI of 3 and 25. **D)** Subconfluent HeLa cells were co-infected at an MOI of 1 with either AdEmpty or AdTK and AdEmpty or AdFAST at varying MOIs. A final concentration of 10 μM ganciclovir was added to the wells infected with AdTK 48 hpi. Following a 24 h incubation, the relative metabolic activity was determined and results were normalized to mock infected cells. The average of three independent experiments conducted in triplicate is shown with the standard error of the mean (n=3). **E)** Subconfluent HeLa cells were infected with varying MOI of AdEmpty, AdFAST or VSVΔ51 for 72 hpi. The relative metabolic activity was assessed using an MTS assay. The average of three independent experiments (n=3) conducted in triplicate is shown with the standard error of the mean. *$p<0.05$ compared to mock-infected cells.

For subsequent experiments, we chose a fixed ganciclovir concentration of 10 μM, as this was the concentration we started to observe a significant metabolic activity decrease in AdTK-infected cells compared to the controls. We next determined the optimal virus concentration for our experiments. We infected HeLa cells at an MOI of 3, 10 or 25 with AdTK or AdEmpty and supplemented AdTK infected cells with a final concentration of 10 μM ganciclovir. The relative metabolic activity was determined at 72 hpi (Figure 5.2C). At all MOIs, cells infected with AdTK showed a significant decrease in metabolic activity compared to both controls ($p<0.05$). Increasing the MOI beyond 3 did not result in an enhanced effect. It is possible that the most of the cells are dividing (i.e. not in the S phase) and therefore, are not affected by activated ganciclovir.

5.2.2. Combined FAST and HSV-1 TK protein expression does not significantly decrease metabolic activity in HeLa cells

To determine if FAST protein and TK/ganciclovir could enhance the bystander effect compared to TK/ganciclovir alone, we co-infected HeLa cells with AdEmpty or AdTK at an MOI of 1 along with AdEmpty or AdFAST at an MOI of 1, 3 or 10 (Figure 5.2D). We used a low MOI of AdTK to ensure that we could observe bystander effect changes. A final concentration of 10 μM ganciclovir was added 48 hpi and cellular metabolic activity was assessed at 72 hpi. In AdTK-infected cells, co-infection with AdEmpty or AdFAST did not have a significant effect on cellular metabolic activity. Although there was a trend toward lower metabolic activity in AdTK and AdFAST-treated cells, it did not reach significance.

We previously showed that FAST protein expression causes extensive A549 cell membrane permeability, even in the absence of extensive cell fusion. An intact cellular membrane could explain why we did not see an enhanced effect in AdFAST and AdTK/gancicolvir combined therapy. Thus, we examined whether FAST protein could

induce membrane permeability in HeLa cells. HeLa cells were infected at varying MOIs and LDH release into the supernatant was determined at 72 hpi (Figure 5.2E). While both AdEmpty and AdFAST decreased membrane integrity at high MOIs compared to mock infected cells, FAST protein expression did not promote enhanced membrane permeability in comparison to the empty vector control. Therefore, FAST protein expression did not further promote TK-mediated bystander effects likely due to its limited ability to induce membrane permeability in HeLa cells.

5.2.3. AdFAST and SMC combinational therapy decreases metabolic activity in SMC susceptible SNB75 cells

As discussed in the introduction, SMC administration can promote cell death by increasing susceptibility to TNFα and TRAIL-mediated extrinsic apoptosis, but is only effective against certain cancers which is due, in part, to intracellular cFLIP levels (197-200, 205). Our Ad vectors are deleted of the E3 proteins that downregulate TNFα-mediated apoptosis and since administration of E1 and E3 deleted vectors can induce an innate response, leading to cytokine production (e.g. TNFα, IL-6). Thus, SMC administration should increase extrinsic apoptotic signalling in Ad-infected cells (243-246). Furthermore, FAST protein expression can enhance membrane permeability (134), which may promote SMC uptake and enhance cell death in cancer cells.

We first examined the effects of AdFAST and administration of the SMC, LCL161 or OICR720 on SMC-resistant A549 cells (205). A549 cells were infected with varying MOIs of AdEmpty or AdFAST for 24 h before treating the infected cells with the monovalent SMC LCL161. The relative metabolic activity was determined after a further 24 h incubation (Figure 5.3A). A significant decrease in metabolic activity was observed in cells treated with AdFAST at an MOI 100 compared to cells infected with AdEmpty at the same MOI. Since

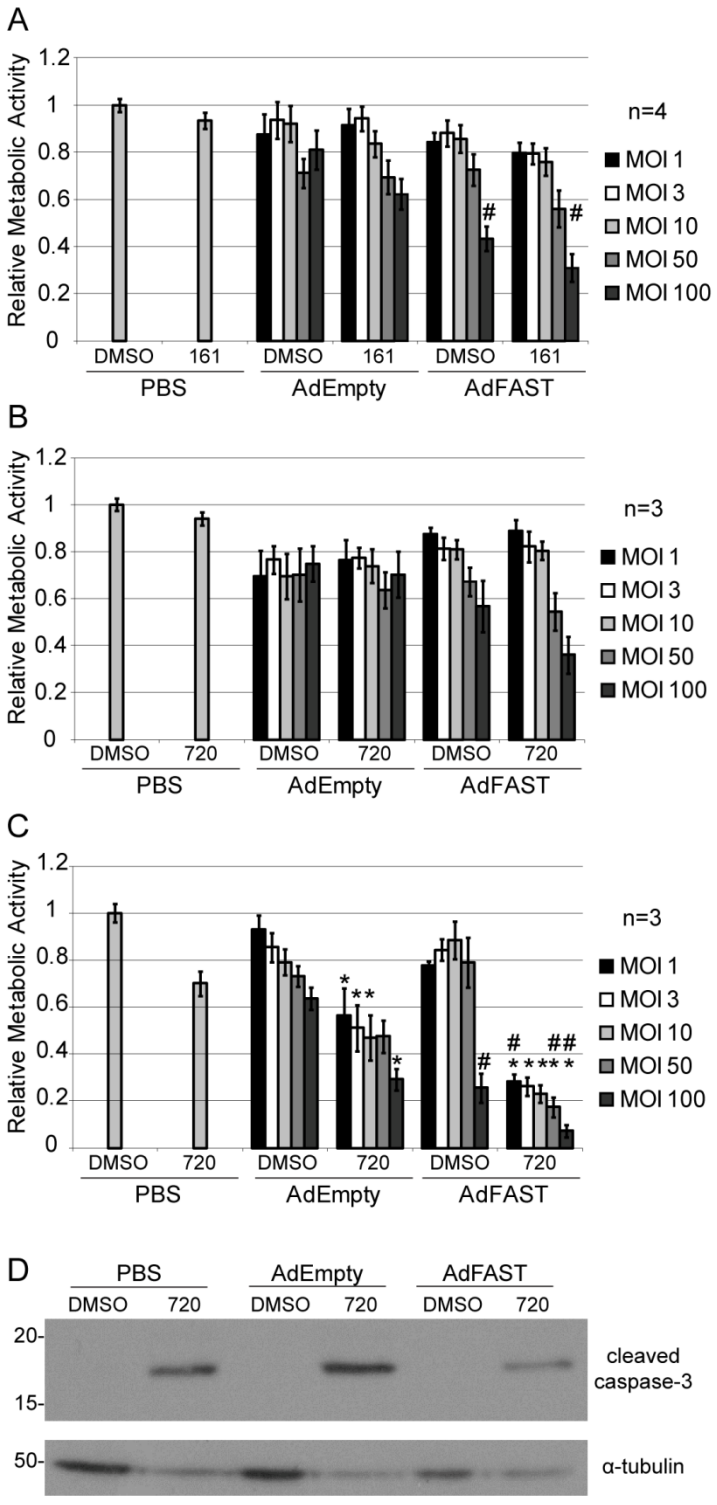

A
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
161
PBS
AdEmpty
AdFAST
n=4
MOI 1
MOI 3
MOI 10
MOI 50
MOI 100
B
720
n=3
C
D
cleaved caspase-3
α-tubulin
20-
15-
50-

Figure 5.3: A combination of FAST protein expression and OICR720 decreases metabolic activity in SMAC mimetic susceptible SNB75 cells. **A)** Subconfluent A549 cells were infected at varying MOI with AdEmpty or AdFAST 24 h. A final concentration of 0.05% DMSO or 5 μM LCL161 was added, followed by a 24 h incubation and assessment of metabolic activity. The averages of four experiments (n=4) completed in triplicate is shown with the standard error of the mean. #$p<0.05$ compared to cells infected with AdEmpty and given the same drug concentration. **B)** Subconfluent A549 cells were infected with AdEmpty or AdFAST. Cells were supplemented with 0.05% DMSO or 0.25 μM OICR720 24 hpi. Cells were incubated for another 24 h before an MTS assay was conducted. All experiments were done in triplicate. The graph depicts the average of three independent experiments (n=3) and the standard error of the mean. **C)** Subconfluent SNB75 cells were infected with AdEmpty or AdFAST in triplicate for 24 h. A final concentration of 0.05% DMSO or 0.25 μM OCIR720 was added and cells were incubated for another 24 h before determining the relative metabolic activity. The average of three independent experiments (n=3) is graphed and the error bars show the standard error of the mean. *$p<0.05$ compared to cells infected with the same virus and given a final concentration of 0.05% DMSO. #$p<0.05$ compared to cells infected with AdEmpty and administered the same drug concentration. **D)** Subconfluent SNB75 cells were infected with AdEmpty or AdFAST at an MOI of 50. Twenty four hours post infection, a final concentration of 0.05% DMSO or 0.25 μM of OICR720 was added to the cells. Following a 24 h incubation, whole cell lysates were collected and probed for cleaved caspase-3 and α-tubulin expression.

there was no significant difference between AdFAST-infected cells treated with DMSO or LCL161, the decrease in metabolic activity was due to FAST protein expression only. Similar results were observed with bivalent OICR720 (Figure 5.3B), where there was no significant decrease in metabolic activity between the AdFAST treatment groups comparing with and without OICR720 administration. When A549 cells were infected at an MOI of 50 with AdEmpty or AdFAST for 24 h and treated with DMSO or OICR720 for another 24 h, there was no evidence of cleaved caspase-3 in any of the treatments (data not shown), suggesting that apoptosis was not induced. These data indicate that FAST protein expression and SMC administration together did not promote cell death in A549 cells.

We next examined whether the combination of FAST protein expression and SMCs would have an enhanced effect in a SMC susceptible cell line. SNB75 were infected with AdEmpty or AdFAST for 24 h and incubated with OICR720 for another 24 h (Figure 5.3C). OICR720 significantly decreased metabolic activity in both AdEmpty and AdFAST infected cells. Cells infected with AdFAST at high MOI combined with OICR720 showed a significant decrease in metabolic activity compared to cells infected with AdEmpty and supplemented with OICR720 and AdFAST-infected cells given DMSO, indicating that FAST protein expression combined with SMC has an enhanced therapeutic effect. To determine if there was enhanced apoptotic signalling in the combinational treatments, SNB75 cells were infected with AdEmpty or AdFAST at MOI of 50 for 24 h, followed by 24 h incubation with OICR720. Immunoblots probing for cleaved caspase-3 showed that while OICR720 promoted apoptosis in SNB75 cells, there was no enhanced apoptotic signalling in AdFAST-infected cells (Figure 5.3D). Nevertheless, FAST protein expression and addition of SMCs show an enhanced effect in the SMC susceptible SNB75 cell line.

5.2.4. Infection of A549 cells with AdFAST enhances bleomycin-mediated cell killing

A FAST protein-mediated increase in membrane permeability suggests that cells may be more sensitive to certain chemotherapeutic drugs whose entry into the cell would normally be impeded by the cell membrane. We therefore tested whether cell killing mediated by two different chemotherapeutic drugs, etoposide and bleomycin, was enhanced by FAST protein expression. Etoposide can enter cells through passive diffusion while bleomycin, a hydrophilic chemotherapeutic drug, does not easily pass through the cell membrane (222, 224, 229). Thus, we predicted that FAST protein might not affect the efficacy of etoposide, but would enhance the sensitivity of cells to bleomycin. Subconfluent A549 cells were infected at an approximate MOI of 10 for 24 h, followed by a 48 h incubation with varying concentrations of etoposide (Figure 5.4A). Cells infected with AdFAST alone caused an 83% decrease in metabolic activity compared to AdEmpty infected cells. Addition of etoposide caused a 72% decrease in metabolic activity between AdEmpty- and AdFAST-treated cells at all drug concentrations, suggesting there was no enhanced therapeutic benefit achieved through expression of the FAST protein. This result was expected as etoposide can enter the cell freely (224), so enhanced membrane permeability mediated by the FAST protein would unlikely promote an enhanced therapeutic effect.

We next examined whether treatment of A549 cells with AdFAST could enhance the cell killing mediated by bleomycin. A549 cells were infected at an MOI of 10 for 24 h and incubated with different concentrations of bleomycin. Metabolic activity was assessed after a 48 h incubation with the chemotherapeutic drug. FAST protein expression caused a 23% decrease in metabolic activity alone (relative to AdEmpty-treated cells), while increasing bleomycin concentrations caused 29% (50 μM) and 55% (100 μM) decreases in metabolic activity compared to AdEmpty infected, bleomycin treated cells (Figure 5.4B). We observed

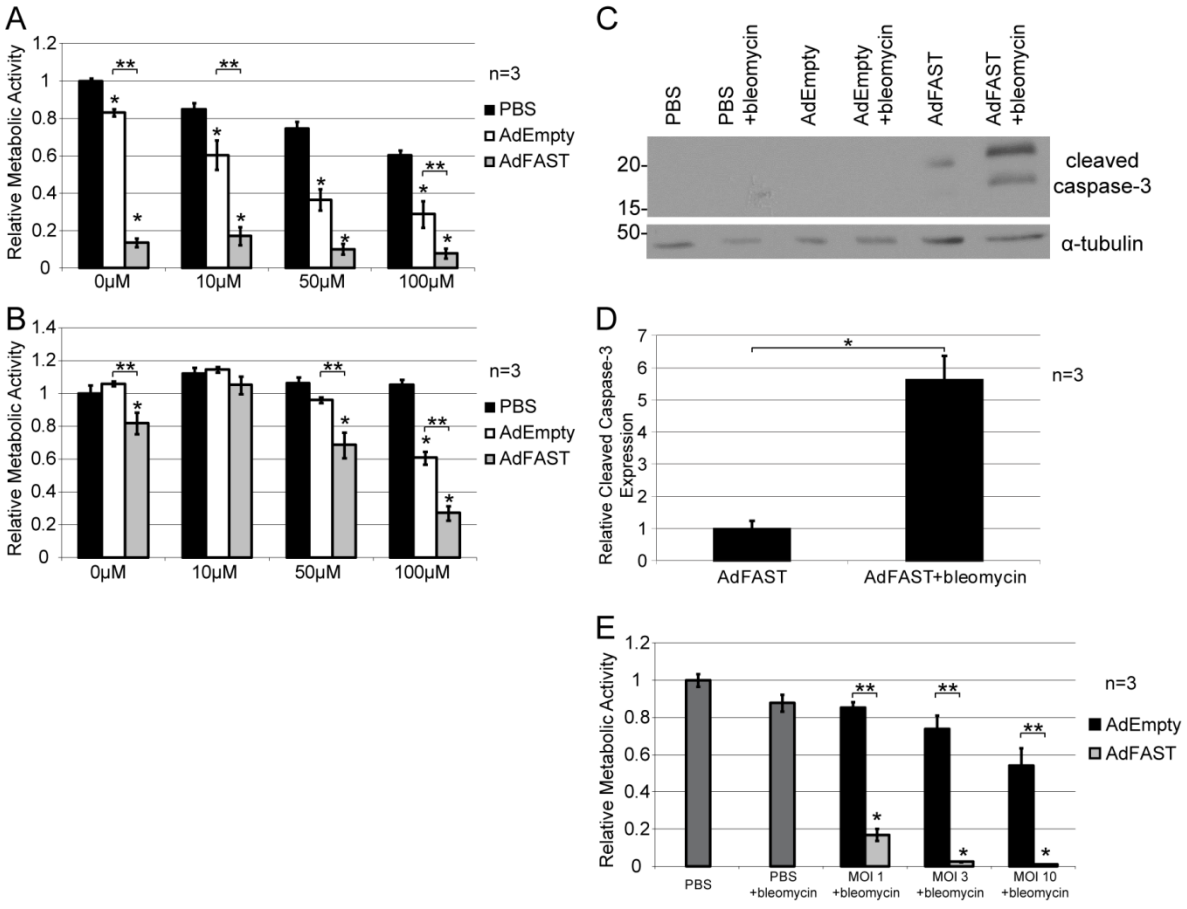

A
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
**
*
0μM
10μM
50μM
100μM
n=3
PBS
AdEmpty
AdFAST
B
Relative Metabolic Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0μM
10μM
50μM
100μM
n=3
PBS
AdEmpty
AdFAST
C
PBS
PBS +bleomycin
AdEmpty
AdEmpty +bleomycin
AdFAST
AdFAST +bleomycin
20-
15-
50-
cleaved caspase-3
α-tubulin
D
Relative Cleaved Caspase-3 Expression
7
6
5
4
3
2
1
0
AdFAST
AdFAST+bleomycin
n=3
E
Relative Metabolic Activity
1.2
1
0.8
0.6
0.4
0.2
0
PBS
PBS +bleomycin
MOI 1 +bleomycin
MOI 3 +bleomycin
MOI 10 +bleomycin
n=3
AdEmpty
AdFAST

Figure 5.4: Ad-mediated FAST protein expression enhances bleomycin-mediated cell killing. A) Subconfluent A549 cells were infected at an approximate MOI of 10 ($3.2x10^5$ pfu) with AdEmpty or AdFAST for 24 h. Etoposide was added at final concentrations of 0 μM, 10 μM, 50 μM or 100 μM and the cells incubated for a further 48 h. Relative metabolic activity was determined using an MTS assay. Three independent experiments (n=3) were completed in triplicate with values normalized to mock infected cells. The standard error of the mean is shown. *$p<0.05$ compared to mock infected cells at the corresponding drug concentrations. **$p<0.05$ comparing AdFAST to AdEmpty infected cells. Experiments were repeated using **B)** bleomycin. **C)** A549 cells were infected with AdEmpty or AdFAST at an MOI of 10. Bleomycin treated cells were given a final concentration of 100 μM bleomycin 24 hpi. Seventy two hours post infection, whole cell lysates were collected and samples were probed for cleaved caspase-3 and alpha-tubulin as a loading control. Three independent experiments were conducted and a representative blot is shown. **D)** Relative cleaved caspase-3 expression was determined based on three independent experiments conducted as described for panel C. Error bars depict the standard error. *$p<0.05$ **E)** Subconfluent A549 cells were infected with $3.2x10^4$ pfu (~MOI 1), $9.6x10^4$ pfu (~MOI 3) or $3.2x10^5$ pfu (~MOI 10) of AdEmpty or AdFAST in triplicate for 24 h. After 24 hpi, a final concentration of 100 μM bleomycin was added followed by a 48 h incubation. Relative cellular metabolic activity was determined using an MTS assay. The average ± standard error of the mean of three independent experiments is shown (n=3). Readings were normalized to mock infected cells. *$p<0.05$ compared to mock infected cells. **$p<0.05$ when comparing AdFAST to AdEmpty infected cells.

similar metabolic activity in virus-infected cells treated with 10 μM of bleomycin for reasons which are unclear. This effect, however, was consistent between several independent experiments. Thus, at high concentrations of bleomycin, FAST protein expression enhanced bleomycin-mediated effects on cellular metabolism, likely due to the ability of the FAST protein to increase cell membrane permeability and enhance bleomycin uptake.

To determine whether decreased metabolic activity was reflective of enhanced cell death, the levels of cleaved caspase-3 was examined in A549 cells that had been infected with AdEmpty or AdFAST at an MOI of 10 for 24 h and incubated with 100 μM bleomycin for 48 h (Figure 5.4C). An increase in cleaved caspase-3 was observed in AdFAST/bleomycin-treated cells compared to AdFAST-treated cells ($p<0.05$), while no cleaved caspase-3 expression was observed for either mock-infected or AdEmpty-infected cells (Figure 5.4C, D). Hence, while AdEmpty decreases metabolic activity compared to mock infected cells (Figure 5.4B), it does not induce apoptotic cell death at the timepoint examined. In contrast, FAST protein expression alone promotes apoptosis, and synergizes with bleomycin to dramatically increase cell death.

We also examined whether AdFAST could enhance the activity of bleomycin at low MOI, as might be observed after vector administration *in vivo*. A549 cells were infected at an MOI of 1, 3 or 10 for 24 h, treated with bleomycin for 48 h, and metabolic activity assessed by MTS assays. Significant differences in metabolic activity were observed between AdFAST infected and AdEmpty- or mock infected-cells ($p<0.05$) at all MOIs tested (Figure 5.4E). An MOI of 1 with AdFAST combined with bleomycin caused an 80% decrease in metabolic activity compared to bleomycin-treated, AdEmpty-infected cells. Increasing the MOI to 3 and 10 caused 96% and 98% decreases in metabolic activity respectively, when comparing AdEmpty- to AdFAST- treated cells. Thus, even at very low

MOI, AdFAST treatment can significantly enhance the ability of bleomycin to promote cell killing. Taken together, our data indicate that delivery of the FAST protein by an Ad vector can augment the activity of some anti-cancer drugs with poor cellular bioavailability.

5.3 Discussion

With an overall 5 yr relative survival rate of 63% in Canada (4), there is a need to improve conventional therapies and develop new therapeutics. Since the FAST protein is able to induce cell fusion and enhance membrane permeability in cancer cells, we examined whether FAST protein could complement the limitations of several cancer treatments to provide an enhanced therapeutic benefit. We focused on three anti-cancer treatments: TK/ganciclovir, SMCs and chemotherapeutic drugs.

To treat cancer, the TK/ganciclovir system and its potent bystander effect has been previously examined (167, 182-190). However, limited efficacy is observed with TK/ganciclovir in phase III clinical trials (187, 190), highlighting the need to improve transgene expression and spread of the vector. As such, we first examined the combinational effect of FAST and TK expression. There were three possible ways that FAST protein expression could promote the TK bystander effect: (i) FAST protein-mediated membrane permeability promotes uptake of ganciclovir/related metabolites, (ii) FAST protein expression allows for lateral transfer of ganciclovir/activated analog or TK through cell-cell fusion and/or (iii) FAST protein expression promotes release of syncytiosomes that contain activated ganciclovir, which are taken up by neighbouring cells. We first examined TK-mediated effects in A549 cells, a cell line used extensively in our previous studies, but we observed that TK/ganciclovir had limited effects on A549 cells (Figure 5.1). Hence, we used HeLa cells, which were susceptible to TK/ganciclovir effects (Figure 5.2B, C). However, Ad-mediated FAST and TK protein expression did not significantly decrease metabolic

activity compared to the controls (Figure 5.2D). As FAST protein expression did not cause significant membrane permeability in HeLa cells in comparison to our previous A549 experiments (Figure 3.5A, 5.2E), our results suggest that benefits of FAST protein expression may only be effective against a subset of cancers. Since the exact mechanism of how p14 FAST protein mediates cell-cell fusion and cell death is unclear, certain cofactors required for FAST protein function may be present in A549 cells and not in HeLa cells. In the context of combinational therapy, FAST protein expression did not provide an enhanced therapeutic benefit with AdTK in HeLa cells.

With multi-modal therapy using FAST protein expression and SMCs, we predicted that FAST protein-mediated membrane permeability and metabolic activity effects might improve SMC uptake in SMC resistant cells. Unfortunately, we did not observe a significant decrease in metabolic activity in SMC resistant A549 cells (Figure 5.3A, B). While FAST protein-mediated membrane permeability could have promoted SMC uptake, high levels of intracellular cFLIP likely prevented any SMC effects in this cell line (205). Interestingly, there was no evidence of apoptosis in AdFAST-treated A549 cells at 48 hpi. We had previously observed significant caspase-3 cleavage at 72 hpi (Figure 3.5D), suggesting that the cells remain viable for a few days before the onset of cell death, similar to what is observed for GALV fusogenic protein-mediated cell fusion (123).

Combinational treatment of FAST protein and SMC led to a significant decrease in metabolic activity in SMC susceptible SNB75 cells (Figure 5.3D), suggesting FAST protein could augment SMC uptake. It is also possible that extrinsic apoptotic signalling was enhanced through TNFα production from Ad-infected and FAST protein-expressing cells and subsequent, TNFα-mediated cell death (243-246). However, we did not observe increased caspase-3 cleavage in AdFAST-treated cells compared to AdEmpty-treated cells

given OICR720. We are unsure why we did not see increased cleaved caspase-3 in AdFAST/SMC treated cells. It is possible that we had administered the maximum effective SMC concentration, and therefore, saw high levels of cleaved caspase-3 in all SMC-treated cells. Because caspase-3 cleavage is one of the downstream events of apoptosis, analysis of cleaved caspase-8 may provide a more accurate analysis of SMC activity as caspase-8 is part of the death inducing protein complexes formed when SMC is first administered (199-203). Nevertheless, our results suggest Ad-mediated FAST protein expression could work synergistically with SMCs, which may allow for lower SMC doses in a potential therapeutic.

In regards to our studies with etoposide, we did not see an enhanced effect (Figure 5.4A). As previously mentioned, we did not expect etoposide to have a pronounced effect in combination with FAST protein expression since etoposide is readily taken up by cells (224). With our experiments examining whether AdFAST-treated cells were more sensitive to bleomycin, a hydrophilic chemotherapeutic drug that usually has a poor ability to enter cells (224, 247), AdFAST combined with a high concentration of bleomycin (100 μM) showed an enhanced effect compared to the controls (Figure 5.4B). Although bleomycin alone did not induce apoptosis in the A549 cells, and AdFAST caused some cell death, the combined treatment of AdFAST and bleomycin dramatically increased cell death (Figure 5.4C, D). There was a virus dependent decrease in metabolic activity and this appeared to be enhanced with FAST protein expression (Figure 5.4E), suggesting that FAST protein expression is essential in mediating the therapeutic benefit observed. This result supports the idea that FAST protein expression compromises membrane integrity to allow bleomycin to enter the cell more easily and promote apoptosis. Thus, AdFAST may provide a benefit in combinational therapies to enhance the bioavailability of certain chemotherapeutic drugs.

In our study, we investigated the effects of FAST protein expression in combination

with TK/ganciclovir, SMC and chemotherapeutic drug administration. FAST protein and TK expression did not significantly decrease cancer cell metabolic activity compared to controls. Combinational therapy of FAST protein expression and SMC showed enhanced therapeutic benefit, but only in SMC susceptible cells. AdFAST and etoposide administration did not have an enhanced therapeutic effect. FAST protein expression with administration of the membrane-impermeable drug bleomycin enhanced cell killing compared to FAST protein expression alone. Our results suggest that FAST protein expression in combination with certain anti-cancer molecules could be used as a cancer treatment regime.

CHAPTER 6: General Discussion

Given the complexity of cancer, development of cancer therapeutics has been an ongoing effort. While we have implemented strategies such as surgery, chemotherapy and radiation therapy to combat the disease, as of 2014, 2 in 5 Canadians will develop cancer and 1 in 4 Canadians will die because of this disease (3, 4). As discussed in the introduction, viruses, such as Ad, have been engineered to express fusogenic proteins in hopes of improving virus distribution (104, 105, 107-116). One of the limitations of using this approach is that fusogenic proteins are normally large in size, which makes them difficult to incorporate into viral vectors with other required regulatory elements (i.e. in oncolytic vectors) (112, 122, 130). Thus, we investigated whether the expression of the reovirus FAST protein (375 bp) from an Ad vector could improve cancer killing and/or virus distribution (131, 132).

6.1 Implications of intratumorally injected Ad expressing the FAST protein

In chapter 3, we examined the effect of FAST protein expression from a replication defective Ad under replication permissive (i.e. 293 cells) and non-permissive (i.e. A549 cells) conditions. In both cases, FAST protein expression caused syncytia formation, but the effect was more pronounced under replication permissive conditions (Figure 3.2, 3.4). Extensive cell fusion caused by high-level expression of the FAST protein from a replicating Ad vector suggests that the FAST protein may be appropriate for inclusion in oncolytic Ad vectors. In that context, FAST protein expression may help promote distribution of the virus throughout the tumor mass, thus circumventing limited distribution of Ad and many other vector systems when intratumorally administered. For example, tumorigenesis can be inhibited by infection of as little as 5% of tumor cells by oncolytic ONYX-015, provided infected cells are evenly distributed during inoculation (97). Consequently, distribution and efficacy can

be improved through multiple, sequential intratumoral injections of vector (97). FAST protein expression from an oncolytic Ad would not only allow the virus to spread through normal routes (i.e. tumor cell lysis and virus release), but an infected cell can "spread" its infectious payload through direct cell-cell fusion. Furthermore, in A549 cells, a high MOI was required to induce synyctia formation (Figure 3.4), suggesting that a threshold of protein expression is required for FAST protein-mediated fusion. The idea of a threshold may be beneficial in the context of an oncolytic Ad where selective replication will increase FAST protein expression to levels high enough to induce fusion only in cancer cells, whereas low level, spurious expression in normal cells would have little effect.

The small size of the FAST cDNA also means that large cancer-relevant regulator regions could be easily accommodated in the vector for tumor-specific regulation of the essential E1 region. Alternatively, immunostimulatory genes could be accommodated in an AdFAST oncolytic vector to enhance the therapeutic effect. Such a virus would function through multiple modalities to achieve tumor cell killing and would potentially be more effective than current oncolytic Ad. Development of an oncolytic Ad expressing the p14 FAST protein will be discussed in more detail in section 6.2.

Our studies also showed that Ad-mediated FAST protein expression caused an initial decrease in metabolic activity and formed syncytia were viable as fused cells stained with calcein AM (green) in the fluorescence based live/dead assay (Figure 3.3, 3.5). Expression of other fusogenic proteins also show that syncytia remained metabolically active the first few days after formation (103, 123). FAST protein expression induced membrane permeability independent of cell-cell fusion, as AdFAST-infected A549 cancer cells had decreased membrane integrity in the absence of synyctia formation (Figure 3.4, 3.5).

Eventually, FAST protein expression caused cell death as illustrated by cleavage of caspase-3 (Figure 3.5B).

In our studies of AdFAST infection in murine cancer cell lines *in vitro*, we observed that a high MOI of AdFAST was required to induce cell fusion and modestly reduce metabolic activity (Figure 4.4). However, AdFAST did not significantly decrease membrane integrity. The limited therapeutic effects can be partially explained by lower CMV enhancer/promoter activity (Figure 4.3) (164). As the complete mechanism of FAST protein-mediated fusion has not been elucidated, the FAST protein may require accessory proteins not expressed in certain cell types (i.e. murine cells, HeLa cells) required for its function. In the context of clinical application, the use of the FAST protein might require pre-screening of tumor type to determine whether the patient is suitable for treatment. This would require more in depth knowledge of the mechanism of FAST protein-mediated cell-cell fusion and development of a screen or test to determine whether FAST protein can mediate tumor cell fusion in the particular cancer type.

When we examined AdFAST efficacy *in vivo*, single intratumoral injections did not result in syncytia formation or tumor regression in xenograft or syngeneic models (Figure 3.6, 4.5). We believe that the major factor influencing the limited therapeutic effect in our studies was low transgene expression. Since we were using a replication defective Ad, the level of transgene expression in an *in vivo* tumor microenvironment may not be enough to induce efficient cell-cell fusion. Again, in the case of the syngeneic model, FAST protein function may be restricted due to absence of accessory proteins in murine cells.

Since we had observed increased membrane permeability with FAST protein expression in chapter 3 and fusing cells and dying syncytia can release syncytiosomes (103), we investigated whether FAST protein expression could enhance the bystander effect or

uptake of anti-cancer molecules. With combined administration of AdFAST and TK/ganciclovir, we did not see an enhanced effect in HeLa cells (Figure 5.2). We did not see an increase in LDH release in AdFAST-infected HeLa cells, suggesting that the FAST protein could not decrease membrane permeability in this cell line. Thus, FAST protein may only function in a subset of cancer cells. As previously mentioned, differences in protein expression levels, protein trafficking, and accessory protein expression could lead to limited FAST protein effects. In SMC resistant cell lines, combinational therapy involving AdFAST and SMCs did not cause a significant decrease metabolic activity (Figure 5.3). We also did not observe an enhanced effect with AdFAST and etoposide, but since etoposide can easily cross cell membranes (224), this result was not surprising. Decreased metabolic activity was enhanced with administration of AdFAST and bleomycin or SMCs in SMC-susceptible cancer cells (Figure 5.3, 5.4). We also observed increased cleaved caspase-3 expression in AdFAST and bleomycin treated cells. These *in vitro* studies suggest that FAST protein expression can be used against certain human cancers as a sole therapeutic and in combination with other anti-cancer treatments.

Our preliminary studies of FAST protein expression in human and murine models of cancer suggest that FAST protein expression could improve Ad efficacy. In a replication competent context (i.e. in 293 cells), Ad-mediated FAST protein expression led to a more pronounced effect on membrane permeability and metabolic activity, suggesting that an oncolytic Ad would be more efficient at cancer cell killing. FAST protein expression can also be combined with other anti-cancer treatments to provide an enhanced therapeutic benefit.

6.2 Development of oncolytic Ad expressing FAST protein

An oncolytic Ad expressing the FAST protein would allow for higher levels of FAST protein expression and production of viral progeny, which would contribute to efficient cancer cell killing and a more complete therapeutic effect. As such, we have attempted to construct an oncolytic Ad that expresses the FAST protein.

To confer replication selectivity in an oncolytic Ad, we had chosen the human telomerase reverse transcriptase promoter to drive E1A expression and express the FAST protein under the control of the MLP (i.e. after viral replication) using a splice acceptor (33, 248, 249). However, we did not observe replication selectivity in cancer cells with this promoter (data not shown) and we were unable to rescue any viruses with the FAST protein encoded in the late gene region.

We therefore decided to use a two component system in hopes of restricting virus replication. As previously discussed, expression of E1A with a 24 amino acid deletion can restrict virus replication to cancer cells with dysregulated or inactive Rb (21, 250-252). We also inserted one Sp1 site and two palindromic E2F binding sites before the E1A promoter to promote E2F binding and viral gene transcription (253). Sp1 is a transcription factor that works with E2F to promote transcription (254). E2F is normally complexed with Rb in the cell. Upon phosphorylation or inactivation by E1A, Rb dissociates from E2F, allowing E2F bind to its promoter composed of two imperfect palindromes (4 binding sites) and activate transcription and allow for G_1 to S phase progression (21, 255-257). Several Ad E4 proteins can complex with E2F to promote virus replication (258, 259). The E2F promoter alone or as an insulator element for the E1A promoter have been used to control Δ24 expression and confer selectivity in cancer cells (253, 259). Previous studies showed that an Ad with the E2F/Sp1 insulator element before the endogenous E1A promoter and Δ24 can increase virus

replication, anti-tumor effects and improve survival in xenograft mouse models of cancer (112, 253). Furthermore, an oncolytic Ad with these components and expressing the GALV fusogenic protein was able to restrict tumor growth, induce syncytia formation and improve Ad spread in xenograft cancer studies (112), indicating that all three components can be used together as a potential cancer therapeutic. As such, we created a virus with the E2F/Sp1 insulator element and Δ24 and found that virus replicated only in Rb-deficient cancer cells, as expected (data not shown).

Since we had inserted the FAST protein cDNA in the E3 region, we examined whether we could express a transgene under the control of the E3 promoter through removal of the splice acceptor from our original construct. Our preliminary experiments showed that a RFP transgene can be expressed from the E3 region of a non replicative Ad under replication competent conditions (i.e. in 293 cells). In contrast, infection of A549 cells, where Ad cannot replicate, did not result in obvious transgene expression (data not shown). Thus, if we created an oncolytic virus construct with the FAST protein under the control of the E3 promoter, the FAST protein would be expressed in cancer cells when Ad is replicating. We have constructed an oncolytic construct that has all three components: E2F/Sp1 insulator before the E1A promoter, Δ24 and FAST protein under the control of the E3 promoter. We have also created various control constructs with the individual components. Future studies can involve examining the selectivity and efficacy of the oncolytic Ad expressing FAST protein *in vitro* and in xenograft or syngeneic *in vivo* studies. In addition, because multiple consecutive injections in high volumes is more effective at preventing tumor growth and increased virus spread compared to a single intratumoral injection (97), studies can be conducted with multiple injections of the vector to improve virus distribution.

In terms of combinational therapy, it would be interesting to examine the therapeutic effect of an oncolytic Ad with TK/ganciclovir administration. A previous study suggested that since only a subset of tumor cells are in the S phase, the TK/ganciclovir system alone is not enough to provide a complete therapeutic effect (260). The authors showed using Ad E1A expression to drive cell cycle progression, the TK/ganciclovir system was able to inhibit growth of human and murine cancer cells. Furthermore, addition of the chemotherapeutic drug cisplatin decreased cell viability more drastically in E1A and TK/ganciclovir expressing cells in comparison to the controls. Hence, with an oncolytic Ad that would express E1A to promote cell division, an enhanced therapeutic benefit may be observed when used with the TK/ganciclovir system. The therapeutic potential can also be further enhanced with FAST protein-induced membrane permeability.

Since cancer cell susceptibility to SMCs is partially influenced by cFLIP expression (202, 205, 206) and we had observed no enhanced benefit with AdFAST and SMCs in SMC resistant A549 cells (Figure 5.3), decreasing cFLIP expression in SMC resistant cells could promote AdFAST/SMC activity. Interestingly, the Ad protein E1A downregulates cFLIP mRNA levels and promotes cFLIP degradation during TNFα-mediated signalling, allowing for cell death progression (261). As such, future studies using a replication competent Ad vector expressing FAST protein in combination with SMCs is warranted. We would also expect to see enhanced anti-cancer effects when we combine oncolytic Ad administration with bleomycin or SMCs in SMC susceptible cell lines, due to the increased FAST protein expression. Overall, we believe that an oncolytic Ad expressing the FAST protein would enhance the cytopathic effects we observed in our studies.

6.3 Application of FAST protein expressed from Ads administered systemically

Systemic administration is ideal for treatment of metastatic disease and potentially

allows for even virus distribution throughout the tumor via the bloodstream. However, systemic administration meets various limitations such as immune clearance and localization to the liver or spleen (92). A prominent obstacle includes getting the virus through the vasculature/blood stream to the tumor cells (92).

One group has attempted to use fusogenic protein expression to promote virus distribution into the tumor following systemic delivery. Chen *et al.* constructed a non-replicative Ad expressing the GALV fusogenic protein under the control of human endothelial receptor tyrosine kinase promoter and CMV enhancer (Ad-eTie1-GALV) (262). The endothelial receptor tyrosine kinase promoter is activated in endothelial cells during embryonic and adult vascular development, and angiogenesis. Thus, upon systemic Ad administration, infected tumor-associated endothelial cells would activate GALV protein expression. Ad-eTie1-GALV was able to selectively induce syncytia formation in human umbilical vein endothelial cells (HUVECs) and these infected cells were also able to fuse with cocultured human cancer epithelial cells *in vitro*. Coculture of infected HUVECs with Ad replication competent 293 cells allowed for enhanced Ad replication compared to the culture infected with the control virus expressing luciferase, suggesting that fusion allowed efficient transcomplementation of factors required for Ad replication. Tumors isolated from mice with 293 cell xenografts intraveneously injected with Ad-eTie1-GALV showed syncytia formation within the tumor and between murine endothelial and human 293 cells, indicating that fusion of the endothelial and xenograft cells allowed for Ad replication within the tumor. An increase in intratumoral virus production was also observed in mice treated with Ad-eTie1-GALV compared to the control virus. Thus, Ad-mediated fusogenic protein expression in endothelial-associated tumor cells could promote Ad distribution and replication during systemic delivery.

Even though our work has primarily focused on improving intratumoral Ad distribution, it is likely that the FAST protein expression can also improve Ad distribution through the tumor vasculature. We have shown efficient FAST protein-mediated cell-cell fusion and enhanced Ad progeny production in a replication competent context (Figure 3.2). We could also control FAST protein expression using endothelial specific promoters to achieve the same effect observed by Chen *et al*. (262). Given that the FAST protein is small in size, other regulatory elements or immunostimulatory genes may be introduced into the non-replicating or oncolytic vector to improve therapeutic efficacy.

6.4 Therapeutic potential of viroporins

Our results from chapter 3 and 5 suggest that protein-mediated cellular membrane permeability could promote cell death and enhance the uptake of anti-cancer molecules such as SMC and bleomycin. Thus, studies examining the therapeutic potential of viroporins in cancer treatment are a promising avenue. Like the FAST protein family, viroporins are small in size, typically ranging from 60-120 aa (263), which would allow for easy incorporation into a viral vector. While most viroporins are not required for virus replication, they can promote virus entry, spread and release. Their hydrophobic transmembrane domains allow for insertion into cellular membranes and oligermization allows for hydrophilic pore formation (263). Viroporin expression typically causes membrane permeability at late stages of infection and promotes cell death through caspase-dependent apoptosis (263, 264). Some viroporins that have be studied include poliovirus 2B, influenza A virus M2, HIV-1 vpu and Sindbis virus 6K (263, 265-269). Since FAST proteins and viroporins share similar properties, virus-mediated viroporin expression could promote cancer cell death and bioavailability of anti-cancer molecules, leading to an enhanced therapeutic effect.

It is important to note, however, that most viroporins localize to intracellular membranes such as the ER and Golgi complex, and less so to cellular membranes (263, 264). Modifications to viroporins of interest may be required to promote localization to the plasma membrane if they are to be used as a cancer therapeutic. Viroporins also do not have cell-cell fusion capabilities, which suggest they may be less effective in promoting virus distribution compared to FAST proteins.

Interestingly, insertion of an adenine at position 445 in E3-19K protein of wild-type Ad5 leads to viroporin activity and enhanced Ad release (270). The E3-19K protein normally binds to the MHC class I in the ER, preventing MHC class I presentation at the plasma membrane (27, 270). In this case, the mutation causes a premature stop codon, causing the loss of the ER retention signal and protein localization to the plasma membrane (270). Localization to the plasma membrane caused membrane permeability at earlier timepoints compared to the wildtype protein. Expression of the mutant protein also caused an increase in calcium influx, enhanced cytotoxicity, and viral progeny release. In a NP-9 xenograft mouse model, Ad expressing E3 19K-445A, administered intraveneously or intratumorally, were more effective at inhibiting tumor growth compared to wildtype Ad. In a Syrian hamster model, where Ad can replicate, intratumoral injections of an Ad containing E3 19K-445A into syngeneic subcutaneous HP-1 tumors increased intratumoral Ad distribution compared to wildtype Ad. Thus, this simple mutation in the E3 protein could promote cellular membrane permeability and Ad distribution, similar to what we see with FAST protein expression. Further study on the 445A mutation effect on uptake of SMC and hydrophilic drugs in combinational therapy would likely yield promising results.

6.5 Conclusion

In our series of studies, we showed that Ad-mediated FAST protein promotes syncytia formation, leading to decreased membrane integrity and cellular metabolic activity, resulting in cell death. However, AdFAST did not have enhanced effects in a xenograft model. In the 4T1 murine model, high MOI of AdFAST was able to induce cell fusion and decrease metabolic activity, but had modest effects on membrane permeability and did not appear to have a therapeutic benefit *in vivo*. AdFAST in combination with TK/ganciclovir or etoposide did not reduce metabolic activity in human cancer cells. However, AdFAST and SMCs in SMC susceptible cells or the membrane impermeable chemotherapeutic drug bleomycin combinational therapy did have an enhanced therapeutic effect. Our results suggest that an oncolytic Ad expressing the FAST protein would be advantageous and could be potentially used in combinational therapy with certain anti-cancer moieties. Our results also suggest that the FAST protein could be utilized to enhance the therapeutic potential of systemically administered Ad. Furthermore, our studies suggest that other membrane permeability inducing proteins, such as viroporins, could be developed as a cancer therapeutic.

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CONTRIBUTION OF COLLABORATORS

Some of the plasmids listed in the appendix were cloned by Dr. Robin Parks. The A549::WPI cell line was developed by Dr. Mike Kennedy during his tenure in the Parks lab.

The plasmid containing FAST protein cDNA (pVSV-FAST) was provided by Dr. John Bell (Ottawa Hospital Research Institute, Ottawa, ON). Theresa Falls of Dr. Bell's laboratory conducted the subcutaneous and intratumoral injections, tumor and liver excision for the *in vivo* studies.

Dr. Robert Korneluk (Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON) provided the SMCs LCL161 and OICR720.

APPENDIX: Plasmids

Name	Promoters/Transgenes	Plasmid Backbone/Cloning Details
pCW100	FAST-HA	pPoly
pCW101	FAST-HA in E1 region	Shuttle plasmid (pRP2645)
pCW102	FAST-HA in E3 region	pDC5
pCW103	FAST-HA in E1 region	pRP2014
pCW104	CMV-RFP in E1region FAST in E3 region	pRP2697
pCW105	FAST-HA	pDC9
pCW106	CMV-RFP in E1 region FAST-HA in E3 region	Cloning used pCW105 and pRP2697
pCW107	Full hTERT promoter-E1A FAST-HA in E3 region	Cloning used pCW105 and pGT119
pCW108	CMV-TK	pRP2483
pCW109	TK	pRP2870
pCW110	CMV-TK	Cloning used pCW108 and pRP2014
pCW111	Main hTERT(mhTERT) promoter	
pCW112	mhTERT-E1A	Cloning used pCW111 and pRP2075
pCW114	mhTERT-E1A FAST-HA in E3 region	Cloning used pCW112 and pCW105
pCW115	mhTERT-E1A FAST in E3 region	Cloning used pCW112 and pGT106
pCW116	FAST	pBluescript II KS(+) no stop codon after FAST protein cDNA
pCW117	FAST	pDC9
pCW118	CMV-RFP in E1 region FAST in E3 region (no splice acceptor)	Cloning using pCW117 and pRP2697
pCW119	mhTERT-E1A FAST in E3 region (no splice acceptor)	Cloning used pCW112 and pCW117
pCW120	RFP	pBluescript II KS(+)
pCW121	mhTERT-E1A no Ad5 fibre	Cloning used pCW111 and pRP2648
pCW122	mhTERT-E1A FAST in E3 region	Cloning used pCW121 and pGT106
pCW123	RFP in E3 region	pDC9
pCW124	mhTERT-E1A	Cloning used pCW111 and pDC9
pCW125	RFP in E3 region (no splice acceptor)	Cloning used pCW123 and pRP2468
pCW126	CMV-GFP in E1 region RFP in E3 region (no splice acceptor)	Cloning used pAVH6-2 and pCW125

Name	Promoters/Transgenes	Plasmid Backbone/Cloning Details
pCW127	E2F/Sp1 sites inserted infront of E1A promoter	pXCI
pCW128	E1AΔ24	pBluescript II KS(+)
pCW129	E1AΔ24	pXCI
pCW130	E1AΔ24	Cloning used pCW129 and pRP2014
pCW131	E2F/Sp1	Cloning used pCW127 and pRP2014
pCW132	E2F/Sp1 E1AΔ24	Cloning used pCW127 and pCW129
pCW133	E2F/Sp1 E1AΔ24	Cloning used pCW132 and pRP2014
pCW134	E2F/Sp1 E1AΔ24 FAST in E3 region (no splice acceptor)	Cloning used pCW133 and pCW118
pCW135	E1AΔ24 FAST in E3 region (no splice acceptor)	Cloning used pCW130 and pCW118
pCW136	E1AΔ24	pCW130 without AscI fragment
pCW137	E2F/Sp1 E1AΔ24	pCW133 without AscI fragment
pCW138	E1AΔ24	Cloning used pCW136 and pCW117
pCW139	E2F/Sp1 E1AΔ24	Cloning used pCW137 and pCW117
pCW140	E1AΔ24 FAST in E3 region (no splice acceptor)	Cloning used pCW138 and pRP2014 no stop codon after FAST protein cDNA
pCW141	E2F/Sp1 E1AΔ24 FAST in E3 region (no splice acceptor)	Cloning used pCW139 and pRP2014 no stop codon after FAST protein cDNA

Carmen Wong

SUMMARY

- Four years of graduate research in biologic therapeutic development
- Strong biochemical and molecular biology techniques
- Experience writing grants, literature reviews, primary manuscripts
- Proficient in using Microsoft Office and Adobe Creative Suite to create graphs, diagrams, oral and poster presentations
- Can analyze data using Excel, SigmaStat, Graphpad and FlowJo

EXPERIENCE

Graduate Researcher, Sept 2011-Present
Ottawa Hospital Research Institute, Ottawa, ON

- Using traditional and recombineering cloning strategies to clone adenoviral constructs
- Examining effects of adenovirus-mediated protein expression *in vitro* using biochemical and molecular biology techniques
- Evaluating the biologic in combination with chemotherapeutic drugs, Smac-mimetic compounds and HSV-1 thymidine kinase
- Developing and assisting with virus efficacy studies in xenograft and syngeneic models of cancer
- Generated results for three first-author manuscripts and secured a two year operating grant from the Cancer Research Society

Undergraduate Research Student, Research Assistant
Jan 2010-Apr 2011
McMaster University, Hamilton, ON

- Cloned retroviral constructs and examined chimeric antigen receptor expression and activity in transduced murine splenocytes
- Developed ELISA protocols to detect cytokines for chimeric antigen receptor projects
- Assessed viability in using cell lines instead of murine splenocytes for transduction experiments
- Utilized a fd filamentous phage display library to screen for

peptides that bind to antibodies specific to HIV-1 long term non-progressors

- Identified high-affinity mimotopes through ELISA, PCR and determined cross-reactivity using clinical samples

Teaching Assistant, May-June 2010
Department of Mathematics and Statistics, McMaster University Hamilton, ON

- Reinforced important concepts and applications in weekly tutorial sessions for an undergraduate statistics course
- Addressed concerns with class material through online discussion board and marked assignments

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SKILLS

- Cloning (traditional and recombineering), PCR
- Mammalian cell and virus culture, transfection, transduction
- SDS-PAGE, Western blotting, Giemsa and crystal violet staining, agarose gel electrophoresis
- Cell viability assays, LDH release and MTS assays
- Fluorescence microscopy, flow cytometry, ELISA

EDUCATION

Doctor of Philosophy, Sept 2011-Apr 2015
Microbiology and Immunology Program, University of Ottawa
Ottawa, ON

Honours Bachelor of Science, Sept 2007-Apr 2011
Honours Biochemistry
Biotechnology and Genetic Engineering Specialization
McMaster University, Hamilton, ON

SCHOLARSHIPS & AWARDS

Awarded during graduate studies:

- Queen Elizabeth II Graduate Scholarships in Science and Technology (Sept 2013-Apr 2015)
- University of Ottawa Excellence Scholarship (Sept 2013-Apr 2015)

- University of Ottawa Doctorate Admission Scholarship (Jan-Aug 2013)
- University of Ottawa Dean's Scholarship (Jan 2013)
- Faculty of Medicine Award of Excellence (Dec 2012)
- DNA Tumor Virus Meeting Travel Award (July 2012)
- Tenth International Adenovirus Meeting Best Gene Therapy Poster Award (June 2012)
- Faculty of Graduate and Postdoctoral Studies Conference Travel Grant (June 2012)
- Department of Biochemistry, Microbiology and Immunology Travel Award (June 2012)
- University of Ottawa Master's Admission Scholarship (Sept 2011-Dec 2012)

Awarded during undergraduate studies:

- Dean's Honour List (June 2011)
- Faculty of Science University Senate Scholarship (July 2009)
- McMaster Honour Award (July 2007-2010)

PUBLICATIONS

Examination of combinational therapies with adenovirus-mediated fusion associated small transmembrane protein expression for cancer treatment. Manuscript in progress.
Wong, C.M., Beug, S., Korneluk, R., Bell, J.C. and Parks, R.J. (2015)

p14 fusion associated small transmembrane protein expression from a replication-defective adenovirus vector does not show a therapeutic benefit in an immunocompetent mouse model. Manuscript in progress.
Wong, C.M., Falls, T.J., Bell, J.C. and Parks, R.J. (2015)

Adenovirus-mediated expression of the fusion-associated small transmembrane protein promotes cell fusion and enhances cancer cell killing. Submitted.
Wong, C.M., Tong, G., Christou, C., Kennedy, M.A., Falls, T.J., Bell, J.C. and Parks, R.J. (2015)

The adenovirus genome contributes to structural stability of the virion. *Viruses* **6**(9): 3563-3583.
Saha, B., Wong, C.M. and Parks, R.J. (2014)

Supraphysiological expression of Survival Motor Neuron protein from an adenovirus vector does not adversely affect cell function. *Biochem Cell Biol* **91**(4): 252-264.
Goulet, B.B., McFall, E.R., Wong, C.M., Kothary, R. and Parks, R.J. (2013)

The role of chromatin in adenoviral vector function. *Viruses* **5**(6):1500-1515.
Wong, C.M., McFall, E.R., Burns, J.K. and Parks, R.J. (2013)

PRESENTATIONS & SEMINARS

Improving adenovirus efficacy with p14 fusion-associated small transmembrane protein expression for cancer treatment
Wong, C.M.

- PhD seminar, University of Ottawa, Ottawa, ON (2014)

Adenovirus-mediated expression of the fusion-associated small transmembrane protein enhances cell killing and sensitivity to chemotherapeutic drugs
Wong, C.M., Tong, G., Christou, C., Kennedy, M.A., Bell, J.C. and Parks, R.J.

- Biochemistry, Microbiology and Immunology Poster Day, Ottawa, ON (2014)
- Canadian Cancer Research Conference, Toronto, ON (2013)
- Ottawa Hospital Research Institute Research Day, Ottawa, ON (2013)
- International Oncolytic Viruses Meeting, Quebec City, QC (2013)

Screening of small molecules inhibiting adenovirus replication
Saha, B., Wong, C.M. and Parks, R.J.

- Ottawa Hospital Research Institute Research Day, Ottawa, ON (2013)

Improving adenovirus efficacy with p14 FAST protein expression for cancer treatment
Wong, C.M., Tong, G., Christou, C., Kennedy, M.A., Bell, J.C. and Parks, R.J.

- Biochemistry, Microbiology and Immunology Seminar Day, Ottawa, ON (2013)

Treatment of cancer cells with an adenovirus vector encoding the p14 FAST protein causes cell fusion and enhances sensitivity to the chemotherapeutic drug bleomycin
Wong,C.M., Tong, G., Christou, C., Kennedy, M.A., Bell, J.C. and Parks, R.J.

- Ottawa Hospital Research Institute Research Day, Ottawa, ON (2012)

Enhanced spread of adenovirus through expression of the FAST cell fusion protein
Wong, C.M., Tong, G., Christou, C., Bell, J.C. and Parks, R.J.

- Biochemistry, Microbiology and Immunology Poster Day, Ottawa, ON (2012)
- DNA Tumor Virus Meeting, Montreal, QC (2012)
- Tenth International Adenovirus Meeting, Umeå, Sweden (2012)

Improving the efficacy of oncolytic adenovirus through expression of p14 fusion-associated small transmembrane (FAST) protein
Wong, C.M., Christou, C., Bell, J.C. and Parks, R.J.

- Ottawa Hospital Research Institute Research Day, Ottawa, ON (2011)