

**Assessing the oncolytic capacity of conditionally replicating adenovirus armed with p14
Fusion Associated Small Transmembrane protein and the adenovirus death protein.**

Joshua Del Papa

A thesis submitted in partial fulfillment of the requirements for the
Doctorate in Philosophy degree in Biochemistry

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

Abstract

Intratumoral injection of oncolytic viruses provides a direct means of tumor cell elimination for inoperable tumors. Unfortunately, oncolytic vectors based on human adenovirus (HAdV) typically do not spread efficiently throughout the tumor mass, reducing the efficacy of treatment. In this thesis, I explore the efficacy of conditionally replicating HAdV vectors expressing either the p14 Fusion Associated Small Transmembrane (FAST) protein (CRAdFAST) or p14 FAST protein in combination with the adenovirus death protein (CRAdFAST-ADP). The p14 FAST protein mediates cell-cell fusion, which may enhance spread of the virus-mediated, tumor cell-killing effect, while ADP aids in cell lysis and HAdV spread at late times in infection. I first explored the efficacy of CRAdFAST in the 4T1 immune competent mouse model of cancer. Treatment with CRAdFAST resulted in enhanced cell death compared to vector lacking the p14 FAST gene in vitro, but did not reduce the tumor growth rate in vivo. The 4T1 model was significantly resistant to HAdV infection and propagation, so I next explored CRAdFAST efficacy in human A549 cell culture and a xenograft mouse model of cancer. In the human A549 lung adenocarcinoma model of cancer, CRAdFAST showed significantly improved oncolytic efficacy in vitro and in vivo. In an A549 xenograft tumor model in vivo, CRAdFAST induced tumor cell fusion which led to the formation of large acellular regions within the tumor, and significantly reduced the tumor growth rate compared to control vector. Finally, to assess the use of a newly constructed CRAdFAST vector co-expressing the adenovirus death protein (ADP), a new model was explored comprised of CMT-64.6 mouse lung carcinoma cells which are syngeneic with Balb/C mice. This model was significantly more sensitive to HAdV infection and CRAdFAST induced fusion than the 4T1 cell line. In this model, expression of ADP and p14 FAST from a CRAdFAST-like vector (CRAdFAST-ADP) resulted in significant oncolytic synergy in vitro but

not in vivo. My results indicate that expression of p14 FAST protein, and potentially ADP, from an oncolytic HAdV can improve vector efficacy for the treatment of cancer, but improved in vivo models will be required to analyze the full preclinical potential of these oncolytic HAdV vectors.

Acknowledgements

First, I would like to thank my graduate supervisor Dr. Robin Parks, for providing thoughtful guidance, the freedom to explore my ideas, and continuous support. I would also like to thank my thesis advisory committee, Dr. Ian Lorimer, Dr. Jean-Simon Diallo and Dr. Carolina Ilkow for their pertinent advice and excellent questions. To Kathy Poulin, thank you for the excellent training you provided, for your technical expertise, and for the inside scoop on all of the latest drama. Thank you to the past and present students of the Parks lab, in particular, Briti, Leslie, Ryan and Charlie, for insightful discussion and companionship on countless coffee breaks. Finally, thank you to Mom, Dad, Dylan, Dante, Kyle, and especially, Justine, for being my foundation and providing an enormous amount of support in good times and bad. A special thank you is reserved for Sophie, without whom I would have surely lost my marbles while writing this thesis.

Funding was provided by grants from the Cancer Research Society, Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council. Josh Del Papa is a recipient of a Queen Elizabeth II Graduate Scholarship in Science and Technology and an Ontario Graduate Scholarship.

Table of Contents

Abstract	ii
Acknowledgements	iv
List of Abbreviations	viii
List of Figures	x
Chapter 1: Literature Review	1
1.1 Introduction	1
1.2 Adenovirus Biology and Application	5
Ad Biology	5
HAdV Vectors	8
1.3 Enveloped Virus Membrane Fusion	10
1.4 Antitumor Properties of Fusion Proteins	11
1.5 Expression of Fusogenic Proteins from Replication-Defective HAdV Vectors	15
Gibbon-Ape Leukemia Virus Fusogenic Membrane Glycoprotein	17
Measles Virus Fusion Proteins	20
Other Fusogenic Envelope Glycoproteins	22
1.6 Expression of Fusogenic Proteins from Replication Competent Ad	23
1.7 Fusion proteins from Non-Enveloped Viruses	25
p14 FAST	26
1.8 Expression of p14 Fusion Associated Small Transmembrane Protein in Ad	29
Vesicular Stomatitis Virus and p14 FAST	30
Adenovirus and p14 FAST	31
1.9 The Adenovirus Death Protein and Viral Oncolysis	32
Function of ADP in natural infection	32
ADP over-expression in CRAds	33
ADP and p14 FAST	34
1.10 Hypothesis	36
1.11 Objectives	36
Chapter 2: Materials and Methods	37
Cell culture	37
Adenoviral constructs	37

Primer Design, PCR and Sequencing.	39
Analysis of CRAdFAST growth kinetics.	40
Immunoblot analysis.	41
Microscopy.	42
Metabolic activity assay.....	42
Extracellular vesicle isolation.....	42
β -Galactosidase assay	43
Luciferase Assay.....	43
4T1 syngeneic cancer model.....	43
A549 xenograft tumor model.....	44
CMT-64.6 syngeneic cancer model.	44
Tumor Histology and Immunohistochemistry.	45
Statistical Analysis.....	45
Chapter 3: p14 FAST improves oncolytic efficacy of CRAd in vitro but not in vivo in a mouse cancer cell line	47
3.1 Introduction.....	47
3.2 Results.....	48
CRAd and CRAdFAST retain the $\Delta 24$ deletion in E1A following viral amplification	48
Characterization of CRAdFAST p14 FAST expression patterns	51
CRAdFAST can replicate and express viral genes but does not produce progeny virions in mouse 4T1 cells	54
Expression of p14 FAST protein from CRAd enhances 4T1 cell killing in vitro	62
Expression of p14 FAST protein from CRAd does not improve therapeutic efficacy in the 4T1 murine model of cancer in immune competent mice	68
3.3 Discussion	73
Chapter 4: CRAdFAST infection results in replication-dependent p14 FAST mediated fusion and cellular destruction in human cell culture and xenograft models of cancer	76
4.1 Introduction.....	76
4.2 Results.....	77
CRAdFAST shows improved replication characteristics in a human cancer cell line in vitro	77
CRAdFAST results in enhanced cancer cell killing in vitro in human A549 cells.	82
p14 FAST induces marked upregulation of extracellular vesicle production in CRAdFAST-infected cells	87

CRAdFAST shows enhanced cancer cell killing in vivo in human A549 cells	88
4.3 Discussion	104
Chapter 5: Adenovirus death protein (ADP) and p14 FAST synergize to improve oncolytic capacity of CRAd expressing p14 FAST in vitro but not in vivo in an HAdV semi-permissive mouse cell line.	107
5.1 Introduction.....	107
5.2 Results.....	108
E3 and p14 FAST synergize to promote cell death in CRAd infected A549 cells.....	108
CMT-64.6 cells show enhanced susceptibility to CRAd and CRAdFAST infection versus the resistant 4T1 cell line	114
ADP expression improves oncolytic efficacy of CRAdFAST in vitro in mouse CMT-64.6 cells	129
CRAdFAST and CRAdFAST-ADP delay tumor growth to a similar degree in an immune competent, semi-permissive mouse model of cancer	134
5.3 Discussion	137
Chapter 6: General Discussion.....	140
Reaching more tumor tissue: implications of improvements in viral spread.....	140
Implications of poor mouse model quality in pre-clinical evaluation of oncolytic HAdV	144
Conclusions.....	147
References	149
Contributions of Collaborators	162
Appendix 1: Published review article	163
Appendix 2: Published Manuscript.....	180
Curriculum Vitae	194

List of Abbreviations

$\Delta 24$: 24 base pair deletion in E1A

B-Gal: β -galactosidase

ADP: adenovirus death protein

ANOVA: analysis of variance

BGHpA: bovine growth hormone polyadenylation sequence

CAR: Coxsackie-adenovirus receptor

cDNA: complementary DNA

CHO: chinese hamster ovary

CMV: cytomegalovirus

CRAd: conditionally replicating adenovirus

DDR: DNA damage repair

DMEM: Dulbecco's Modified Eagle Medium

E1: early region 1

E3: early region 3

EGFR: epithelial growth factor receptor

ER: endoplasmic reticulum

EV: extracellular vesicle

FAST: fusion-associated small transmembrane protein

FMG: fusogenic membrane glycoprotein

FBS: fetal bovine serum

GaLV: Gibbon-ape leukemia virus

GFP: green fluorescent protein

GM-CSF: granulocyte macrophage colony-stimulating factor

HA: hemagglutinin

HAdV: human adenovirus (serotype 5 unless otherwise noted)

HAdV-II: human adenovirus serotype 2

HAdV-III: human adenovirus serotype 3
HIV: human immunodeficiency virus
hpi: hours post infection
HRP: horseradish peroxidase
IFN: interferon
IL: interleukin
ITR: inverted terminal repeat
MEM: Minimum Essential Media
MHC: major histocompatibility complex
MLP: major late promoter
MMP: matrix metalloproteinase
MOI: multiplicity of infection
MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium
MeV: Measles virus
PBS: phosphate buffered saline
PFA: paraformaldehyde
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
SV5: simian Virus 5
SV40: Simian virus 40
VA: viral associated
VV: vaccinia virus
VSV: vesicular stomatitis virus

List of Figures

Figure 1. Schematic diagrams of HAdV vectors used in this study	7
Figure 2. Schematic of mechanism of enhanced viral spread through expression of fusogenic protein.	13
Figure 3. Schematic diagram of p14 FAST protein.....	28
Figure 4. CRAd and CRAdFAST retain 24bp deletion following expansion in 293 cells.	50
Figure 5. Transgenes placed in the HAdV E3 region require HAdV replication and must be preceded by a splice acceptor for detectable protein expression.	53
Figure 6. Expression of p14 FAST does not inhibit CRAd growth in A549 cells.	56
Figure 7. CRAdFAST replicates but does not produce progeny virions in 4T1 cells.	59
Figure 8. Expression of p14 FAST is not tied to virus replication in CRAdFAST infected 4T1 cells.	61
Figure 9. CRAdFAST infection leads to monolayer fusion and destruction in 4T1 cells.	64
Figure 10. CRAdFAST reduces metabolic activity and leads to apoptosis of 4T1 cells.....	67
Figure 11. Expression of p14 FAST protein does not improve oncolytic efficacy of CRAd in the murine 4T1 model of cancer.	70
Figure 12. CRAdFAST induces syncytium formation in subcutaneous 4T1 tumors in vivo.	72
Figure 13. CRAdFAST expresses fiber and p14 FAST more efficiently in A549 cells than 4T1 cells.	79
Figure 14. p14 FAST expression is replication dependent in A549 cells infected with CRAdFAST.....	81
Figure 15. CRAdFAST infection leads to efficient monolayer destruction in A549 cells.	84
Figure 16. CRAdFAST reduces metabolic activity and leads to apoptosis of A549 cells.	86
Figure 17. Expression of p14 FAST enhances extracellular vesicle release in CRAd infected A549 cells.	90
Figure 18. Vesicles from CRAdFAST infected cells have similar size distributions to mock and CRAd treated cells.	92
Figure 19. Vesicles from CRAdFAST infected cells contain exosome marker proteins.	94
Figure 20. Expression of p14 FAST protein significantly improves oncolytic efficacy of CRAd following intratumoral injection in a human A549 xenograft model of cancer.	96
Figure 21. Intratumoral injection of CRAdFAST leads to improved spread relative to CRAd. ..	99
Figure 22. Treatment with CRAdFAST induces syncytium formation in A549 tumors in vivo.	101
Figure 23. Intratumoral injection of CRAdFAST induces p14 FAST associated fusion in mouse livers.....	103
Figure 24. E3 and p14 FAST synergize to promote cell death in CRAd infected A549 cells. ..	110

Figure 25. CRAdFAST-ADP co-expresses p14 FAST and HAdV ADP.	113
Figure 26. HAdV infectivity is significantly higher in CMT-64.6 cells than in 4T1 cells.	117
Figure 27. CMT-64.6 cells support CRAdFAST fiber and p14 FAST expression.	120
Figure 28. CRAd infected CMT-64.6 cells do not support a significant viral burst.....	122
Figure 29. Experimental schematic.....	125
Figure 30. CRAd produces progeny virions upon infection of CMT-64.6 cells.	128
Figure 31. CRAdFAST-ADP induces monolayer destruction at low MOI.	131
Figure 32. CRAdFAST-ADP reduces metabolic activity of infected CMT-64.6 cells.	133
Figure 33. CRAdFAST delays tumor growth in an immune competent, semi-permissive mouse model of cancer, but ADP does not offer further benefit.	136

Chapter 1: Literature Review

1.1 Introduction

Despite continuing efforts to curb the immense burden of cancer through treatment, research and prevention, approximately 30% of the human population will be diagnosed with some form of cancer during their lifetime [1]. Approximately half of these patients will succumb to the disease, underscoring the need for new therapeutic options. In Canada, the US, much of South America, Europe and Australia, cancer represents the most frequent cause of premature death (death prior to age 70) [2]. These numbers are expected to rise with global population and lifespan increase. Of all cancer cases discovered, 80% are classified as solid tumors [3]. When these tumors form in or near critical tissues, have diffuse borders, or occur in patients unable to tolerate surgery, they can be deemed inoperable. In such cases, chemotherapy (or other drug-based treatment) and radiation are the main modes of treatment. Although these treatments can often reduce disease burden and increase lifespan, prognoses for inoperable cancers are often grim [4].

Over 100 major types of cancer affecting humans have been identified, each of which can have a diverse array of founding mutations, which influences pathology and treatment effectiveness [3]. A major barrier to the success of classical therapeutics is that the survival of a single cancerous cell can lead to re-emergence of the tumor and, similar to the evolution of antibiotic resistance, these cells often have or gain additional mutations that allow for resistance to future treatments [5]. This, and other mechanisms, can lead to the proliferation of cancer cells that are resistant to traditional chemotherapies, highlighting the need for more potent and specific cancer treatment options for inoperable solid tumors [6].

One promising candidate therapy under development, viral therapy, has the capacity to offer highly cancer specific, broad-spectrum treatments to counteract the exceptional diversity that exists within this disease group. Two basic strategies exist when using engineered viral vectors against cancer: use of the virus to deliver genes encoding therapeutic proteins to cancer cells, or engineering of the virus such that it selectively replicates in and kills cancer cells [7, 8]. A wide array of viruses have been explored in preclinical and clinical studies, including vaccinia virus, herpes simplex virus, vesicular stomatitis virus, and adenovirus (HAdV) [9-12]. Indeed, an oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor (GM-CSF) has been approved recently for clinical use in North America [13].

For one of the most commonly used gene therapy vectors, HAdV, two vectors have been approved for cancer treatment in China—a non-replicating HAdV encoding p53, termed Gendicine, and an oncolytic HAdV that selectively replicates in cancer cells, termed Oncorine [14, 15]. In phase II/III clinical trials, intratumoral treatment with Gendicine with radiation treatment resulted in significant increases in both partial and complete responses, with a three-fold increase in complete responses versus radiation alone [14]. Oncorine combination treatment with chemotherapy resulted in similar success rates with a 72.7% response rate versus 40.4% with chemotherapy alone in a pilot phase II trial [15, 16]. These successful trials resulted in the approval of both candidates, which have shown a remarkable safety profile while boosting efficacy of first line chemotherapeutics [17, 18].

Ad vectors have been used extensively in molecular biology applications for many decades to achieve high-level gene expression of a desired transgene in mammalian cells or induce cancer cell death through conditional replication. As of December 2018, 18.5% (n =

541) of all human gene therapy clinical trials used Ad-based vectors to deliver a therapeutic gene of interest, with the vast majority of these trials directed towards treatment of cancer [19]. HAdV efficiently infects a wide variety of human cell types, regardless of cell cycle status, and has a relatively large cloning capacity [7]. These desirable qualities have led to a vast amount of research into their use as oncolytic vectors or as delivery vehicles for anti-cancer therapeutic genes. Unfortunately, many preclinical and clinical studies have shown that Ad-based therapeutics frequently mediate only a partial response [20, 21], partly due to limitations of the vector system combined with complexities of the target tissue. A major barrier to anti-cancer efficacy for HAdV, and many other therapeutic viruses, is efficient spread or distribution of virus throughout the tumor mass [22].

Like many oncolytic viruses, HAdV has a poor ability to home to the tumor mass following systemic delivery and, instead, is primarily sequestered in the liver [23, 24]. Although the amount of virus delivered to the tumor can be increased through direct injection of the vector into the tumor, HAdV typically does not transit far from the injection tract, leaving much of the tumor mass unaffected by the virus [22, 25, 26]. Naturally, HAdV infection results in relatively inefficient virus release from the host cell, and much of the virus remains cell-associated until very late times post-infection[27]. Furthermore, tumors typically exhibit a relatively high internal pressure, which inhibits effective spread of virus when injected directly into the tumor mass [22]. Tumors also contain a large amount of connective tissue, further impeding virus spread [28]. Many approaches have been investigated to overcome this barrier, including the expression of junction opening peptides or enzymes capable of dissociating the extracellular matrix, or increasing the rate of viral lysis and spread by overexpression of native viral components such as the HAdV death protein [29-31]. In

addition to mechanical barriers to intratumoral spread, there are also several biochemical barriers. Tumors often express low levels of the main HAdV receptor, the Coxsackievirus and Adenovirus Receptor (CAR), which can reduce the ability of HAdV to bind to and enter tumor cells [32, 33]. Several methods of structural modification of HAdV to increase CAR independent cellular uptake have been used, such as alterations to the fiber domain to increase efficiency of viral entry and fusion of single-domain antibodies to various capsid proteins [34-36]. Finally, pre-existing neutralizing HAdV antibodies are detectable in 30% to 60% of the US population, with higher rates in developing countries [37, 38]. The existence of these neutralizing antibodies in combination with the efficiency of complement recognition of HAdV can in theory cause rapid elimination of released virus following tumor cell lysis, further impeding spread throughout the tumor mass [39]. Interestingly, some studies suggest that the presence or absence of pre-existing neutralizing HAdV antibodies does not appear to affect treatment efficacy when HAdV is delivered intratumorally [12, 20]. This effect is likely a result of the immune privileged microenvironment of the tumor, suggesting intratumoral injections could be used in a wide array of patients, regardless of pre-existing HAdV neutralizing antibodies [40]. An encouraging approach under investigation to enhance viral spread through a tumor involves heterologous expression of fusion-inducing proteins from the therapeutic vector. This approach has shown promise in not only increasing spread of the oncolytic effect throughout the tumor mass, but also in facilitating activation of the host anti-tumor immune response.

Below, I summarize the research and techniques discovered to improve anti-cancer vector spread, with a focus on HAdV and fusogenic proteins.

1.2 Adenovirus Biology and Application

Ad Biology

Although there are over 60 distinct serotypes of human Adenoviridae, HAdV serotypes 2 and 5, both of subclass C, are the most well-studied[41]. HAdVs display a naturally diverse tropism for tissue types, which varies between viral subtypes. In nature, HAdV type 5 (HAdV-5) most often infects cells of the upper respiratory tract [42]. HAdV are icosahedral, non-enveloped, double stranded DNA viruses of approximately 70–100 nm in diameter for the main “body” of the virus, with varying lengths of the viral attachment protein, fiber, protruding from the vertices of the icosahedron [43]. The HAdV-5 genome is roughly 36 kb and is transcribed in essentially two stages upon entry into the host cell (Figure 1) [44]. Soon after the HAdV genome reaches the nucleus, the early genes are expressed, including early region 1 (E1), E3 and E4, which function to activate other transcription units on the HAdV genome as well as modulate the host cell cycle and immune responses [45]. Interestingly, one of the most late-acting proteins in the HAdV viral life cycle, the adenovirus death protein (ADP) is encoded within the E3 transcription unit [27]. Cells infected by HAdV lacking functional ADP show large quantities of viral particles within nuclei at late times post-infection, indicating ADP plays a crucial role in nuclear membrane lysis [46]. The E2 region encodes proteins directly involved in HAdV DNA replication, such as the virally-encoded DNA polymerase. Prior to replication, low levels of late gene expression occur, however, only after the onset of DNA replication is the major late promoter (MLP) activated [47]. Activation of the MLP results in the

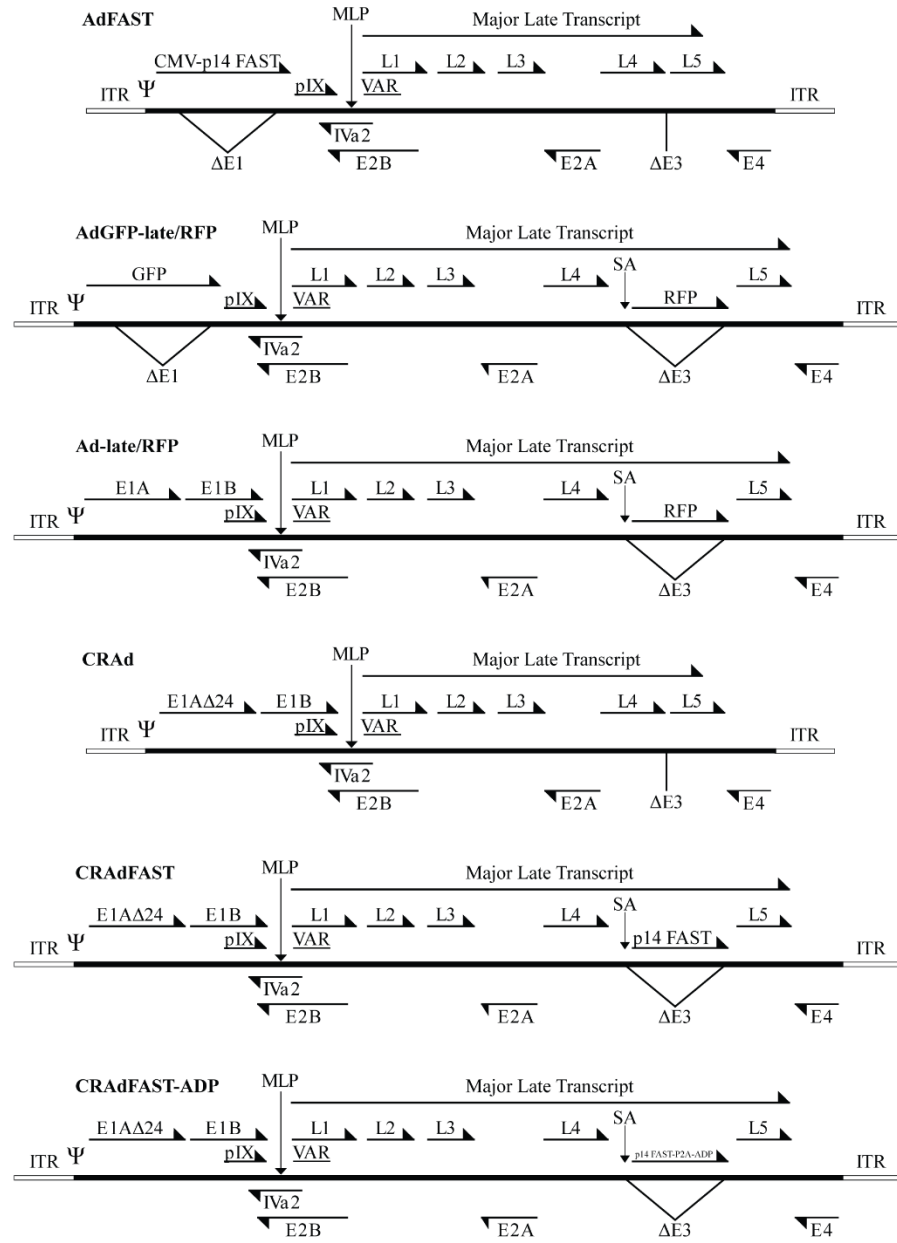


Figure 1. Schematic diagrams of HAdV vectors used in this study. AdFAST, an E1 and E3 deleted non-replicating HAdV vector expressing p14 FAST from the cytomegalovirus immediate early enhancer/promoter in the E1 region, AdGFP-late/RFP contains a CMV-GFP expression cassette in the E1 region, and the RFP cDNA in the E3 region preceded by a splice acceptor. Ad-late/RFP is identical to AdGFP-late/RFP but contains the wild-type E1 region. CRAd is an E1 Δ 24, E3-deleted conditionally replicating vector, and CRAdFAST, an E1 Δ 24, E3-deleted conditionally replicating vector expressing p14 FAST from the E3 region through the inclusion of a major late promoter splice acceptor (SA). CRAdFAST-ADP is identical to CRAdFAST but contains a P2A sequencing separating the p14 FAST cDNA from the ADP cDNA in the E3 region.

production of a large (roughly 28 kb) transcript which subsequently is processed through alternative splicing to produce the late transcripts late region 1 (L1) to L5[47]. These late regions encode the structural components necessary for HAdV assembly, including the HAdV capsid proteins [48]. There are also four small products produced at intermediate/late times of infection, including the structural protein IX (pIX), and the IVa2 protein that helps package viral DNA into immature virions [49]. The other intermediate/late products, the viral associated (VA) RNA I and II, inhibit activation of the interferon response, impede cellular micro-RNA processing, and may influence expression of host genes [50, 51]. The viral DNA also contains the origins of replication, the inverted terminal repeats (ITR), which comprise ~100 bp at each end of the genome and the packaging sequence, which is ~150 bp in length and is located immediately adjacent to the left ITR [52].

HAdV Vectors

The most extensively utilized class of HAdV vector is the replication-defective E1-deleted Ad. The E1A and E1B proteins encoded within the E1 region are essential for virus replication, and thus E1-deleted vectors are incapable of replicating in most cells [53], but can efficiently deliver their DNA to the nucleus of cells and express an encoded transgene placed under the control of a heterologous promoter. E1-deleted HAdV have been explored extensively as delivery vehicles for genes encoding therapeutic proteins with cancer-mitigating effects such as, for example, p53 or interleukin (IL)-12 [54, 55].

For oncolytic, or conditionally replicating HAdV (CRAd), there are two major approaches to achieve cancer-specific replication. When viral genes encoding proteins essential for viral replication, (e.g., E1) are placed under the control of a tissue (cancer)-specific promoter (e.g., the prostate specific antigen promoter), some selectivity can be

achieved in viral replication (e.g., only in prostate cancer cells) [56-58]. Alternatively, the virus can be attenuated or mutated such that it can only replicate in cancer cells with mutations in cellular pathways required to promote virus replication. For example, one of the functions of the HAdV E1A protein is to interact with the retinoblastoma (Rb) family of pocket proteins, to promote cell cycle progression [59]. Deletion of a 24 base-pair segment of conserved region 2 of the E1A protein creates a molecule that can no longer interact with the pocket proteins, and virus with this mutation can no longer replicate efficiently in normal cells [60]. However, since the Rb pathway is mutated in many cancers, HAdV vectors containing the E1A Δ 24 mutation can selectively replicate in these cells but not normal tissue [61]. As stated above, two Ad-based cancer treatments are approved for use in China, one from each class of the above vector types—Gendicine and Oncorine (H101) [14, 16]. Gendicine is a replication-deficient HAdV expressing wild-type p53, which would reestablish expression of p53 in p53-deficient cancer cells, and Oncorine is a conditionally replicating HAdV deleted of the E1B-55kD, which may also show specificity for cancers deficient in p53 [14, 16].

As mentioned, a common problem with HAdV is their restricted ability to disperse throughout a tumor, particularly following direct intratumoral injection [25, 62, 63]. Typically, HAdV infection is limited to a 5 mm area surrounding the injection tract, which prevents efficient transgene product delivery to all cells of the tumor [21]. While this may not be a significant issue when HAdV is used to deliver a gene for secreted proteins, such as cytokines, it may limit the efficacy of other types of therapeutic genes and proteins which require expression directly within the cell in order to exert their efficacy, such as, for example, the tumor suppressor p53. For oncolytic HAdV vectors, preclinical studies have shown that complete tumor regression can be achieved with the oncolytic HAdV ONYX-015 (a

conditionally replicating HAdV restricted to cells that do not express a functional p53 gene) with initial infection of as little as 5% of the tumor cells, but these cells must be equally distributed throughout the tumor [25]. Consequently, distribution and efficacy can be improved through use of multiple, sequential injections of vector [25]. However, better, more effective approaches are required to enhance virus dispersion and spread within the tumor in order to achieve better efficacy.

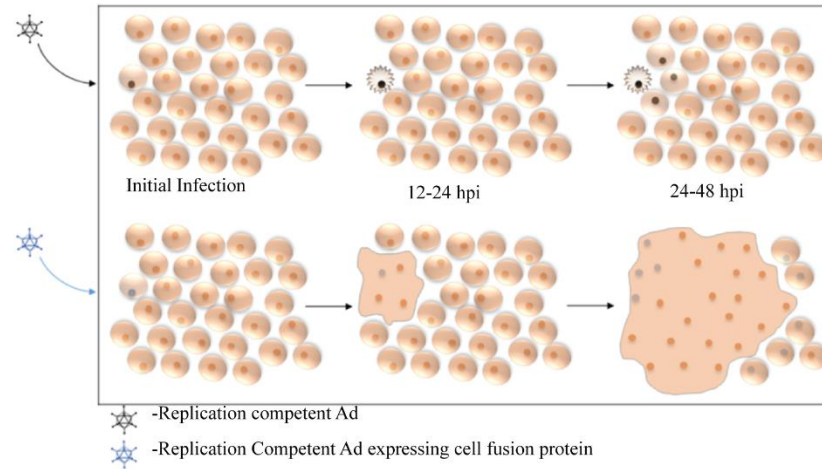
1.3 Enveloped Virus Membrane Fusion

One method to improve virus dispersion and spread of the anti-cancer effect may be to co-opt the membrane fusion machinery used by many enveloped viruses. A large number of processes occur naturally that require the fusion of adjacent lipid bilayers. Intracellular membrane fusion events occur frequently and include endoplasmic reticulum and Golgi reorganization as well as mitochondrial remodeling [64]. Lipids with larger polar head groups relative to their acyl chains tend to favor positive membrane curvature, whereas those with smaller polar head groups relative to their acyl chains tend to favor negative membrane curvature [65]. There exists an asymmetrical distribution of lipids within the cellular bilayer, with a higher proportion of lipids favoring positive curvature on the extracellular face of the cellular lipid bilayer. In order for fusion to occur, both lipid bilayers must undergo significant negative membrane curvature, a highly energetically unfavorable conformation [66]. There are multiple steps to membrane fusion, including initiation of membrane contact, pore formation, pore expansion and finally membrane fusion [67]. In order to perform these fusion events, there is recruitment of significant native machinery within the cell [68]. The process of membrane fusion and pore expansion requires dynamic and coordinated reorganization of the cellular cytoskeletal networks [69].

Enveloped viruses perform the task of host entry through the use of viral fusogenic membrane glycoproteins (FMG). Examples of common enveloped viruses include the influenza virus, the measles virus, Dengue virus and the *Herpesviridae* family of viruses. FMGs within each of the three families of viral fusion proteins exhibit similar structural properties [70]. Most FMGs are classified as type I integral membrane proteins, with a single pass through the lipid bilayer and a cytosolic C-terminus [71]. Most of the mass of these relatively large proteins is contained on the extracellular portion of the protein, which is often significantly glycosylated and can be fatty acylated. All known FMG function as oligomers, with trimers and tetramers being the most common conformation [72]. A common property of type I FMG is the existence of a fusion peptide; a small (roughly 20-40 amino acids) portion of the protein identified as necessary for fusion [71, 73]. Despite their structural similarities, FMG can have a wide array of mechanistic approaches to elicit membrane fusion. For example, viral fusion peptides can integrate into the host cellular membrane or the recipient cellular membrane and function as anchors for membrane fusion [66]. Expression and overexpression of FMG can lead to the formation of syncytium; a multinucleated structure formed when two or more cellular membranes fuse together [76].

1.4 Antitumor Properties of Fusion Proteins

In an attempt to improve HAdV vector or transgene distribution within the tumor, several research groups have explored whether inclusion of genes encoding fusogenic proteins can enhance therapeutic efficacy. Fusogenic proteins have the ability to promote cell–cell fusion, and thus their expression can promote lateral transfer of the virus or a co-expressed therapeutic gene product (Figure 2). The fusogenic proteins most often investigated for their oncolytic activity tend to originate from enveloped viruses.



Initial Infection: Virus particle enters cell followed by entry of viral DNA into nucleus
 12-24 hpi: Viral protein expression and progeny production
 24-48 hpi: Viral progeny released from initially infected cell, enters nearby cells

Figure 2. Schematic of mechanism of enhanced viral spread through expression of fusogenic protein. Upon infection, replication-competent oncolytic adenovirus (HAdV) must undergo productive replication and significantly damage the host cell in order to affect neighboring cells. Cell-fusion proteins allow Ad to spread through densely packed cells with much greater efficiency, before replication-associated cell death or progeny release. Initial Infection: Virus enters host cell and initiates gene expression and early phase of replication. 12–24 hpi: Viral protein expression and progeny production. 24–48 hpi: Viral progeny released from initially infected cell, enter nearby cells. hpi: hours post-infection.

Regardless of the virus of origin, the basic mechanism by which all fusogenic proteins function involves reducing the energy barrier required for lipid bilayer fusion to occur [77]. Despite the similarity of the task, the biochemical mechanism by which cell fusion occurs differs drastically between classes of viral fusogenic proteins. A few characteristics important for their anti-cancer activity are shared by all fusogenic proteins. Syncytia, multinucleated cells caused by the fusion of multiple cellular membranes, are initially viable, and remain active both metabolically and transcriptionally [78]. In tissue culture studies *in vitro*, initially viable syncytia eventually lift from the dish and undergo a type of cell death that has not been definitively characterized [79]. This mechanism is exceedingly important, as a single cell expressing a fusogenic protein can induce syncytium formation with many affected nuclei, thus rapidly increasing antitumor efficiency. Multiple conflicting studies have provided evidence in support of either apoptotic or non-apoptotic cell death following syncytium formation [78, 80, 81]. Some studies point to a necrotic cell death mechanism, with expression of heat shock protein 70, heat shock protein 90 kDa beta member 1 (gp96) and retention of inhibitor of kappa B ($\text{I}\kappa\text{B}\alpha$) in the cytoplasm, while others show elevation of cleaved caspase-3 and clear evidence of apoptotic DNA fragmentation [81, 82]. However, the actual mechanism of cell death may be unique to fused cells, or even vary between fusogenic proteins.

During syncytium formation and associated cell death, blebbing at the cell surface results in the enhanced release of exosome-like particles referred to as syncytiosomes. These particles were shown to contain high levels of immune-stimulatory molecules, and lower levels of major histocompatibility complex I (MHC I) than exosomes naturally released from tumor cells [79]. The presence of these syncytiosomes promoted dendritic cell activation and resulted in activation of an anti-tumor T-cell response and tumor cell killing. Thus, fusogenic protein-

induced formation of syncytia, and subsequent release of syncytiosomes, may enhance formation of anti-tumor immune responses, ultimately enhancing immune-mediated tumor rejection.

1.5 Expression of Fusogenic Proteins from Replication-Defective HAdV Vectors

The ability of viral fusogenic proteins to efficiently reduce cell viability while inciting a host anti-tumor response makes them excellent candidates as potential sole therapeutic molecules in non-replicating HAdV vectors. A number of viral fusogenic proteins have been investigated for their anti-cancer effects in this context (Table 1), as discussed below.

Table 1. HAdV expressing fusogenic proteins.

Origin of Fusion Protein		Replication-Defective HAdV Vector	Replication-Competent HAdV Vector	Reference
Gibbon-Ape Leukemia Virus		✓	-	[83, 84]
			✓	[85] ¹ , [86] ² , [87, 88]
Measles Virus		✓	-	[89-91]
		-	✓	[92-94] ³
Respiratory Syncytial Virus		✓	-	[95]
Vesicular Stomatitis Virus		✓	-	[96]
Simian Virus 5		✓	-	[97]
Reptilian Reovirus		✓	-	[80, 98]
Human Immunodeficiency Virus		-	✓	[99, 100]

¹Early region 1 (E1)-deleted viruses used in E1-expressing 293 tumors; ² Plasmid expressing fusion protein combined with E1-competent Ad; ³ E1-deleted HAdV trans-complemented with E1-competent Ad.

Gibbon-Ape Leukemia Virus Fusogenic Membrane Glycoprotein

Gibbon ape leukemia virus (GaLV) is an enveloped C-type retrovirus, whose cellular target for attachment, PiT-1, is ubiquitously expressed in human tissues [101, 102]. Through the examination of retroviral packaging cell lines, the GaLV fusion protein was shown to cause efficient human cell–cell fusion after truncation of the C-terminal R-peptide (a negative regulatory element) of the protein [103]. Bateman et al. showed that the GaLV FMG was able to significantly reduce viability of cancer cells *in vitro* and *in vivo* upon *in situ* transfection with a GaLV FMG expression plasmid [78]. Interestingly, mice vaccinated with tumor cells that had been pre-transfected with the GaLV FMG expression plasmid showed significant immune protection, compared to mice treated with cells which had been transfected with a plasmid encoding a non-fusogenic form of the protein, when subsequently challenged with subcutaneous tumors. As mice and hamsters do not express the PiT-1 receptor necessary for GaLV binding and fusion, these models could not adequately examine the safety profile of this therapeutic approach.

One concern with the use of fusion proteins is that fusion can occur in any cell type, including cancer and normal cells. To counteract this safety concern with the GaLV fusion protein, several groups attempted to restrict its fusogenic capacity specifically to cancer cells. One such proof-of-concept effort involved fusing Epidermal growth factor (EGF) to the N-terminal targeting domain of the GaLV FMG via a factor Xa cleavage/linker domain, which was designated EXGaLV (EGF-X-GaLV) [104]. Incorporation of EXGaLV FMG into a retroviral vector (murine leukemia virus) limited infection in cells expressing the EGF receptor, and protease-mediated cleavage of the factor Xa domain resulted in rescue of viral infectivity [104]. Following this success, Johnson et al. sought to use a similar construct to

target gliomas, which often overexpress the matrix metalloproteinase (MMP) enzymes MMP-2, MMP-9, MT-1 and MT-2, in stark contrast to the complete absence of MMPs in normal brain tissues [105]. The group used broad spectrum MMP-cleavable linkers to fuse the C-terminal domain of the cluster of differentiation 40 (CD40) ligand, to the N-terminus of the GaLV FMG (GaLV M40). Addition of the fragment of the CD40 ligand prevents attachment through steric restriction, but would be cleaved from GaLV FMG in tumors expressing MMPs, thus permitting syncytium formation. Transfection of a plasmid expressing this fusion protein into glioma cell lines in culture resulted in rampant cell fusion in cells with the highest expression of MMP, while no fusion was observed for normal human astrocyte (NHA) cells. When U87 glioblastoma cells were pre-transfected with a plasmid expressing the modified fusion protein and subsequently introduced into mice, MMP-mediated cleavage of the modified fusion protein was observed, along with fusion of the cancer cells and a concomitant significant reduction in tumor burden compared to animals receiving unmodified U87 cells. Unfortunately, the reduction of tumor volume was greatest in wild-type GaLV-transfected cells, compared to the GaLV M40-transfected cells, suggesting an overall reduction in fusogenic capacity of the engineered protein. All of the above studies involved plasmid-mediated expression of the fusion protein; however, in general, plasmid delivery *in vivo* is relatively inefficient, resulting in low overall efficacy.

To improve the delivery efficiency of the targeted GaLV genes *in vivo*, multiple groups have utilized non-replicating HAdV vectors. An HAdV vector expressing the MMP-dependent GaLV construct (AdM40) was compared to a vector expressing a non-cleavable version of the protein (AdN40) in *in vitro* and *in vivo* models of glioma [83]. Studies in tissue culture showed that AdN40 was able to efficiently infect cells, but was unable to induce fusion. However,

AdM40 induced significant fusion which was dependent on the expression of MMP, as co-administration of MMP inhibitors eliminated fusion. Upon injection of the viruses into subcutaneous U87 glioma xenograft tumors in BALB/c nude mice, a significant reduction in tumor volume was observed in AdM40-treated mice relative to untreated or AdN40-treated mice. Treatment with AdM40 also significantly improved survival, with 30% of treated mice showing complete eradication of tumors and survival at the end of the study period (50 days), compared to AdN40 which showed no improvement relative to untreated mice.

Another study used a non-replicating HAdV expressing GaLV FMG under the control of a modified human heat-shock protein HSP70b promoter (HSE.70b), for thermal-mediated regulation of expression [84]. Hyperthermia can be easily induced in localized tissues, such as tumors, and has shown significant potential when used in conjunction with chemotherapy or radiotherapy [106]. The ability of this HSE.70b promoter to increase transcript levels 950-fold in response to heating enables this construct to have impressive potential to restrict transgene expression both temporally and spatially [84]. A stably transfected HT-1080 fibrosarcoma cell line expressing the heat-inducible transgene grew unimpeded until challenged with hyperthermia, which induced syncytium formation and a 70% reduction in cell viability. The median survival of mice bearing tumors composed of this cell line increased from 4.3 to 52 days when tumors were treated with mild hyperthermia (44 °C for 30 min), compared to an increase from 7.9 to 12.5 days for mice bearing tumors established using the parental HT-1080 cell line which were also treated with the same hyperthermia regime. Treatment of a variety of cell lines with a replication-deficient HAdV containing the same expression cassette (Ad.HSE70b.GALV) showed no evidence of fusion until mild hyperthermia (43 °C for 30 min) was applied. Incorporation of the expression cassette into conditionally replicating HAdV

was suggested as the next step for follow-up on these encouraging results but, this has not yet been reported.

Measles Virus Fusion Proteins

Measles virus (MeV) is an enveloped, single-stranded negative-sense RNA human pathogenic virus from the Paramyxoviridae family [107]. A hallmark of many Paramyxovirus infections is the formation of syncytia in tissues harboring high-levels of virus [108]. Studies done to develop antivirals have elucidated the proteins responsible for virus-host and host-host fusion observed in MeV infection. The MeV FMG is a hetero-oligomeric protein composed of a tetramer of the Hn protein (receptor binding) complexed with a trimer of the F protein (fusion activity) [108]. Once associated in the endoplasmic reticulum, this complex of proteins is trafficked to the cell surface where it mediates both virus–host and host–host cell membrane fusion. The H protein is responsible for the MeV FMG cell specificity, and has two cellular protein targets: signaling lymphocyte activation molecule (SLAM, also known as CD150), or Nectin-4 (N4) [109]. Once the H protein binds to either of these targets, it mediates an irreversible conformational change in the F protein, which induces membrane curvature and ultimately membrane fusion [108].

In 1990, Alkhatib et al. published a study on their efforts to express the F protein of MeV from a non-replicating HAdV [89]. The goal of the study was to explore the biochemical mechanism for fusion of the H/F FMG, however this was also the first proof-of-concept that heterologous expression of a viral FMG could generate functional fusogenic units and initiate cell–cell fusion in treated cells. Although the vector encoded only the F protein, expression of this protein induced syncytium formation in 293 and HeLa cell lines. Studies using plasmid-mediated expression of both the H and F proteins showed that fusion could be achieved in a

variety of glioma cell lines, and was most potent when the plasmids were administered at a 1:1 ratio [90]. To translate this work *in vivo*, two HAdV vectors were constructed, AdF and AdH, which were subsequently tested in a U87 xenograft glioma tumor model in nude mice [90]. Virus was administered once daily for three days and, at the end of the study period (70 days), 100% of the AdH/AdF-treated mice were alive, compared to only 20% of the mice treated with Ad-green fluorescent protein (AdGFP). Interestingly, these researchers also tested the efficacy of a lentiviral vector expressing the GaLV FMG, and saw no significant reduction in tumor burden or increase in lifespan. However, of note, the dose of lentiviral vector was much lower than that of the HAdV, due to the difficulty in generating high-titer lentivirus stocks. This observation highlights one of the advantages of HAdV vectors—the ability to easily generate high-titer stocks of vector.

Due to the ubiquitous expression of SLAM and CD40 throughout the human body, several research groups have attempted to target the MeV FMG H protein to cells expressing particular cell surface markers. Single chain antibodies (scFvs) targeting carcinoembryonic antigen (CEA) or the myeloma marker CD38 were fused to the H protein of MeV FMG [110, 111]. These mutant H proteins conferred specificity upon ablation of the native H receptor binding domain, and MeV expressing the mutant proteins were able to selectively fuse cells expressing the targeted binding proteins. In a follow-up study, the group fused a scFv recognizing EGF receptor (EGFR) to the H protein, and expressed this protein from a non-replicating HAdV vector also expressing the F protein [109]. Upon infection in EGFR(–) Chinese Hamster Ovary (CHO) cells, this virus did not induce cell fusion. However, infection of genetically engineered EGFR(+) CHO cells, resulted in widespread fusion. Taken together,

these studies clearly show that some fusogenic proteins can be altered to decrease “generalized” fusion, and achieve cell selective fusion using hybrid proteins.

To enhance immune responses to the treated tumor cells, and essentially promote *in situ* vaccination, an HAdV vector expressing the MeV H/F FMGs (Ad.MV-H/F) was tested alone and in combination with HAdV vectors expressing various cytokines IL-2, IL-12, IL-18, IL-21 or granulocyte-macrophage colony-stimulating factor (GM-CSF) [91]. Previous studies had shown that expression of an FMG could improve the immunogenicity of a weakly allogenic melanoma vaccine [112], possibly due to the enhanced release of syncytiosomes from the fused cells, and subsequent cross-presentation of tumor-associated antigens to dendritic cells [79]. Mice bearing MC38 syngeneic colorectal tumors in both left and right hind flanks received intratumoral injection of Ad.MV-H/F in combination with the various cytokines in only one of the two tumors [91]. Combination treatment led to an 87%–98% reduction in injected tumor size at the end of the study, compared to treatment with Ad.MV-H/F (51% reduction) or Ad-cytokines alone (1%–40% reduction). Impressively, contralateral, uninjected tumors showed similar response rates, with a significantly greater response in mice treated with Ad.MV-H/F in combination with cytokines (85% reduction in tumor size) compared with treated with either Ad.MV/H-F (54% reduction) or HAdV expressing cytokines alone (8% to 46%). Both the injected and non-injected tumors showed high levels of macrophage infiltration when Ad.MV-H/F was combined with the cytokine-expressing vectors.

Other Fusogenic Envelope Glycoproteins

In 2007, Hoffman et al. examined the therapeutic efficacy of HAdV vectors expressing the RSV fusion peptide RSV-F or vesicular stomatitis virus (VSV) fusion peptide VSV-G [95, 96]. Reduction in tumor growth in mice treated with Ad.RSV-F was similar to that observed for Ad.MV-H/F noted above. Although Ad.VSV-G also mediated a significant anti-tumor effect, it was less effective than HAdV vector expressing either of the other two fusogenic proteins. Another potential FMG for immune stimulation is the simian virus SV5-F protein. The SV5-F protein is a potent antigen, and displayed promise in early cell-culture models of cancer [97]; however, no follow up experiments were performed to further explore its anticancer capacity *in vivo*. Considering these results, the measles virus fusion proteins, as well as other similar fusogenic glycoproteins, offer considerable promise in arming oncolytic HAdV, but this remains to be tested experimentally.

1.6 Expression of Fusogenic Proteins from Replication Competent Ad

To date, few groups have successfully shown expression of a fusogenic protein from a replication competent HAdV, mainly due to two barriers to vector propagation. First DNA packaging limitations of the HAdV capsid limits the amount of “extra” DNA that can be included in the vector, and genes for fusogenic proteins tend to be large. Second, high-level expression of the fusogenic protein during vector propagation may lead to premature cell death, resulting in an inability to effectively grow stocks of the virus. In 1989, Dewar et al. used an E1-competent, E3-deleted HAdV to express the human immunodeficiency virus (HIV) envelope glycoproteins (Ad5env) [99], which was the first evidence that HAdV replication was compatible with cell–cell fusion. Twelve years later, Li et al. showed that expression of the HIV envelope glycoprotein from this same virus increased viral release, spread, and

replication, compared to wildtype HAdV-5, in a HeLa-CD4⁺ cell culture model, which is permissive for HIV envelope protein-mediated fusion [100].

Depending on the vector and the FMG in question, incorporation of FMG into conditionally replicating HAdV has proven difficult, as FMG can cause cell death prior to generation of high titers of virus. Some evidence suggests that tetracycline regulation of toxic transgenes could help to bypass this problem by restricting expression of the transgene during viral propagation *in vitro* [113]. Another strategy to circumvent this issue is to place the FMG under regulation by the HAdV MLP, thus allowing the virus to begin replication prior to expression of the lethal fusogenic protein [87]. This strategy was used to generate a conditionally replicating virus containing the GaLV FMG under regulation of the MLP, with E1 expression controlled by a tumor-selective promoter containing eight E2F-binding sites and one specificity protein 1 (Sp1)-binding site, designated ICOVIR16. This virus showed potent anti-tumor activity in mouse xenograft subcutaneous models of pancreatic cancer (NP18) and melanoma (SkMe128) [88]. ICOVIR16 was significantly better than its parent virus, ICOVIR15, which contains only the regulated E1 expression and lacks an FMG, at reducing tumor volume following intratumoral injection. In addition, the virus also showed anti-tumor efficacy following systemic administration, which would be important for potentially reaching undetected metastases. As mouse cells are non-permissive for HAdV replication, a Syrian hamster model was used to assess toxicity of the armed virus. Importantly, arming of ICOVIR16 did not increase the systemic toxicity of the virus, suggesting that the presence of the fusogenic protein did not affect tumor-specific replication, thus offering a promising platform for future studies.

1.7 Fusion proteins from Non-Enveloped Viruses

Despite the fact that non-enveloped viruses, such as HAdV, enter the host cell through a fusion-independent mechanism, there exists a class of fusogenic, non-enveloped reoviruses that express non-structural fusion proteins (i.e. not included as part of the infectious virion) [114]. Reoviridae are a diverse family of non-enveloped double-stranded RNA viruses that infect a wide range of hosts from mammals to fungi to plants [115]. There are four fusion-associated small transmembrane (FAST) proteins expressed in orthoreoviridae and two expressed in avian reoviridae [116]. Like FMG, the FAST proteins are type I integral membrane proteins with an extracellular N-terminal domain. Unlike viral envelope FMG, FAST proteins are relatively small, ranging in size from 10 to 22 kDa. The most striking difference between FAST proteins and their FMG counterparts is the extracellular domain. FAST proteins all have exceedingly small extracellular domains, which suggests they likely have a different mechanism of action compared to other FMG [116]. Lipid modification is important for all FAST proteins, whether they contain myristoylation of the N-terminus or palmitoylation of cysteine proximal to the cytoplasmic surface of the cellular membrane [116]. Interestingly, despite their high degree of structural similarity, the FAST proteins appear to have significantly diverse evolutionary histories [117].

The FAST proteins are highly modular, meaning they are made up of distinct subdomains which can be exchanged between different family members, to some degree [118]. For example, the avian reovirus p10 and reptilian reovirus p14 proteins contain hydrophobic sections of amino acids on their extracellular domains, whereas the Broome reovirus p13, baboon reovirus p15 and aquareovirus p16 and p22 FAST proteins contain a hydrophobic patch on the intracellular domain [116]. It is possible that the hydrophobic extracellular

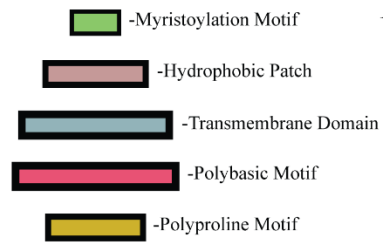
domains of p10 and p14 insert into donor or target membranes to induce negative curvature in order to reduce the energy barrier required for pore formation [116]. Conversely, the cytoplasmic hydrophobic patches of p13, p15, p16 and p22 could internally mediate membrane curvature, thus performing a similar task.

p14 FAST

A significant amount of work has been done to elucidate the mechanistic details of the p14 FAST fusion process. The p14 FAST protein, isolated from the reptilian reovirus, is approximately 14 kDa in size. p14 FAST contains a myristoylated N-terminus, followed by an extracellular hydrophobic patch, the transmembrane domain, a membrane proximal polybasic motif and finally a polyproline domain on the C-terminus (Figure 3) [116, 119]. All domains except for the polyproline patch are crucial for p14 FAST fusion activity, as shown by a number of substitution and deletion experiments [116, 120-122]. p14 FAST protein, like all FAST proteins, is shuttled to the plasma membrane through the ER-Golgi system by the membrane proximal polybasic motif. Interestingly, when specific p14 FAST protein extracellular mutants lacking fusogenic capacity are co-expressed with native p14 FAST protein, the fusogenic capacity of the native protein was completely disrupted [123]. This dominant-negative effect suggests p14 FAST protein undergoes multimerization mediated by the extracellular domain, and inclusion of a single damaged copy abrogates the fusogenic capacity of the multimer. Corcoran et al. (2011) showed through the co-expression of a hemagglutinin (HA) tagged p14 FAST protein with an untagged version that p14 indeed undergoes homomultimerization [123]. Furthermore, disruption of the Golgi-complex led to a lack of p14 protein multimers, as shown by co-immunoprecipitation of HA-tagged and native p14 FAST protein, suggesting Golgi-trafficking is required for p14 FAST multimerization.

A.

MGSGPSN FVNHPAGEAIVTGLK GADK VAGTISHITL WEVIAGLVALLTFLAFGFWL R KYLQKRERRRRQLTEFQKRYL R NSYRLSEIQRPISQHEYED PYEPPSRKRKPPPPV STYVNIIDNVSAI



B.

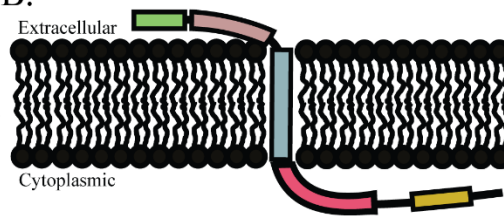


Figure 3. Schematic diagram of p14 FAST protein. A) The primary amino acid sequence of p14 FAST overlayed with functional units. The N-Terminal Extracellular domain contains an N-terminal myristoylation motif followed by a hydrophobic patch of amino acids. The C-terminal cytosolic domain contains a polybasic motif followed by a polyproline motif. B) Schematic of the conformation of p14 FAST relative to the lipid bilayer.

To initiate membrane fusion, p14 FAST must first induce hemifusion (the fusion of the outer leaflets of the donor and target cells), which requires very close membrane apposition (~1nm) and energetically unfavorable negative membrane curvature [66]. Due to its location on the extracellular leaflet, the N-terminal myristate moiety of p14 FAST protein likely acts as a fusion peptide, similar to enveloped virus fusion proteins [120]. The myristate moiety may interact with donor or target extracellular membranes to induce membrane curvature or to act as an anchor, reducing the distance between opposing membranes. The extracellular hydrophobic patch of p14 FAST could potentially synergize with adjacent myristate moieties to enhance the induced membrane curvature. Additionally, p14 FAST protein aggregates in membrane microdomains [124]. Due to the small size of the extracellular domain of p14 FAST protein, homomultimerization and aggregation within membrane domains may be key events in overcoming energetic barriers in membrane fusion.

1.8 Expression of p14 Fusion Associated Small Transmembrane Protein in VSV and HAdV

Non-enveloped viruses do not enter the cell through fusion, therefore the primary function of the non-structural FAST proteins is believed to be to aid in lateral transmission of the virus. Furthermore, the small size of the FAST proteins makes them excellent candidates for use in oncolytic viruses, since they readily fall within the cloning capacity of most vector systems. In the context of HAdV, use of such small fusogenic proteins means that there would be sufficient remaining cloning capacity to accommodate a tissue-specific promoter to confer tumor selectivity or a second transgene (e.g., a cytokine to enhance immune stimulation) in the same vector. Early studies showed that transfection of a plasmid expressing the p14 FAST

protein induced extensive syncytium formation and apoptosis, once again suggesting that these proteins may be ideal as anti-cancer therapeutics [81].

Vesicular Stomatitis Virus and p14 FAST

To evaluate the potential role for p14 FAST as a virulence factor, Brown et al. examined expression of the p14 FAST protein from an otherwise wild-type VSV [125]. In cell culture, this VSV/FAST recombinant virus induced the formation of large syncytia with no deleterious effect on VSV replication. After intravenous injection, the VSV/FAST virus entered the central nervous system of BALB/c mice more rapidly and replicated to a higher titer than VSV/GFP (1 to 4 logs higher at all timepoints between 24 and 96 h post infection). When delivered intranasally, VSV/FAST also caused clinical pathogenesis (hind-limb paralysis) at a much lower dose than VSV/GFP (1×10^7 plaque-forming unit (PFU) vs 5×10^8 PFU per mouse, respectively). These results indicate that the p14 FAST protein can act as a virulence factor in non-endogenous viruses. The efficacy of FAST was also examined in a VSV derivative that is selective for replication in cancer cells, VSV Δ 51 [126-128]. VSV Δ 51/FAST, was tested for oncolytic potency alone and in combination with VVDD, a double-deleted vaccinia virus which is replication restricted to cells that overexpress the E2F transcription factor [127, 128]. In the combination therapy trial, the two viruses were chosen for their complementary effects; VVDD naturally inhibits production of type I interferons (IFNs), while VSV Δ 51 is sensitive to this antiviral pathway [126, 129]. Compared to treatment with VVDD/VSV Δ 51, VVDD/VSV Δ 51FAST caused increased cell killing (shown by crystal violet assay) and a 100-fold increase in VVDD virus production. In a separate study, alone, intratumoral injection of VSV-p14 (VSV Δ 51/FAST) increased survival of Balb/c mice bearing subcutaneous 4T1 tumors, increased the number of tumor infiltrating lymphocytes and

increased the frequency of activated T-cells (CD4⁺ and CD8⁺) in tumors and draining lymph node[128]. These results suggest that the p14 FAST protein can be used in oncolytic viruses to enhance direct efficiency and antitumor immune activity.

Adenovirus and p14 FAST

Due to the attractive properties of p14 FAST, our lab created a non-replicating, E1-deleted HAdV, AdFAST, expressing the p14 FAST protein from the high-activity cytomegalovirus immediate early enhancer/promoter (CMV) replacing the E1 region [80]. In A549 lung adenocarcinoma cells in culture, AdFAST efficiently caused formation of syncytia at high multiplicity of infection (MOI). Syncytium formation was shown to reduce metabolic activity compared to a control HAdV vector lacking a transgene. AdFAST-treated cells showed a significant increase in cleaved caspase-3 compared to cells treated with control virus, suggesting the AdFAST induced apoptosis. Unfortunately, these promising *in vitro* results did not translate *in vivo*, as immune deficient mice bearing subcutaneous A549 tumors did not show improved tumor regression or prolonged lifespan when treated with AdFAST compared to animals treated with control virus or vehicle. AdFAST was also tested in an immune competent, syngeneic mouse model of cancer and similar results were observed[98]. Studies *in vitro* in 293 cells, in which the AdFAST virus can replicate due to complementation of the E1-deletion in this cell line, showed that extensive fusion and a decrease in metabolic activity could be achieved at a very low MOI [80]. These observations suggest that the ineffectiveness of AdFAST in the A549 and 4T1 models may be due to insufficient levels of FAST protein expression, and that FAST may be more effective when included in an oncolytic HAdV vector:

replication of the oncolytic HAdV would dramatically increase the template copy number of the FAST gene within the tumor cell, resulting in enhanced FAST protein expression and, likely, improved tumor regression. Therefore, equipping selectively-replicating HAdV with fusogenic proteins may be a powerful strategy to increase antitumor activity.

1.9 The Adenovirus Death Protein and Viral Oncolysis

The HAdV E3 region codes for 7 different integral membrane proteins [130]. These proteins are 14.7K, 14.5K (RID β), 10.4K (RID α), 11.6K (adenovirus death protein, ADP), gp19K, 6.7K and 12.5K, and they mainly function as immune modulators of cellular innate and adaptive immune responses to HAdV [130, 131]. Overall, aside from ADP, the E3 proteins act to remove “death” receptors from the cell surface, inhibit apoptosis and sequester MHC class I molecules in the ER, all actions which allow the HAdV life-cycle to survive the cellular antiviral response systems [130]. The E3-11.6K protein, also known as the adenovirus death protein or ADP, functions at very late times post-infection. Unlike other early-region proteins, ADP is primarily expressed at late-times in infection from the MLP [27]. Indeed, given that the ADP transcript is spliced to the MLP tripartite leader, similar to all other late transcripts, ADP should likely be considered a late gene [132]. This observation illustrates the sheer complexity of the HAdV transcriptional machinery.

Function of ADP in natural infection

The exact mechanistic function of ADP is unknown, however, a collection of observations by Scaria et al. shed the first insight into the function of ADP in HAdV-2, which is highly homologous to the ADP of HAdV-5 [131]. ADP contains a hydrophobic transmembrane domain at amino acids 41-62, and is synthesized in the ER before ultimately

trafficking to the nuclear membrane [131]. Using a series of E3 mutants, it was shown that HAdV deleted of ADP exhibit a small-plaque phenotype, which can be attributed to inefficient release of progeny viruses from infected cells [46]. Strikingly, at late times post-infection (7 and 15 days), A549 cells infected with HAdV lacking functional ADP showed normal nuclear morphology with positive DAPI staining, and as HAdV is assembled in the nucleus, high numbers of intra-nuclear HAdV particles. Importantly, most nuclei infected with wild-type HAdV lacked a positive DAPI stain and HAdV particles were not present at these late times post-infection [46]. Taken together, these data suggest that ADP functions through disruption of the nuclear membrane with subsequent release of viral particles at late times in infection.

ADP over-expression in CRAds

In the first generation of non-replicating HAdV vectors, as well as subsequent conditionally replicating vectors, the E3 region is typically removed [133]. In a tissue culture setting where immunological barriers do not exist, this region is non-essential, and removal of the E3 region allows for increased cloning capacity for foreign DNA within the vector. However, inclusion of E3 in an oncolytic HAdV may benefit its antitumor capacity in two ways [134]. First, with the capacity to evade immune detection in early stages of infection, HAdV may have an increased opportunity to replicate and spread within the tumor mass. Furthermore, ADP may allow increased cell lysis and HAdV spread within the infected tumor [134]. Suzuki et al. explored the use of two CRAd based on the E1A Δ 24 mutation, one expressing the wild-type E3 region, and a second in which the E3 region was replaced by the GFP cDNA (Ad5- Δ 24E3 and Ad5- Δ 24GFP, respectively)[134]. Infection of cells in culture with Ad5- Δ 24E3 led to significantly improved monolayer destruction *in vitro* compared to Ad5- Δ 24GFP. Furthermore, Ad5- Δ 24E3 significantly reduced tumor growth rates compared

to the E3- control when A549 tumors were injected intratumorally. In this study, the relative contribution of the seven E3 proteins was not assessed, and therefore, it is unclear whether ADP or other E3 proteins were responsible for the observed effect. A subsequent study in immune-competent mice revealed intricate details regarding the relative contributions of the E3 immune-regulatory proteins in CRAd efficacy [135]. CRAd deleted of RID α , RID β and 14.7K was more rapidly cleared from tumors, but resulted in significantly more macrophage infiltration and expression of TNF α and interferon- γ , indicating that, despite better immune surveillance of the infection, this CRAd may have an increased capacity to elicit an anti-tumor immune response.

The effect of overexpression of ADP from an HAdV vector, in the absence of other E3 proteins, has also been investigated. Overexpression of ADP alone in an E1A Δ 24 CRAd was more effective than a wild-type E3 region in inducing virus spread and reducing tumor burden in tumor bearing mice [136-139]. Importantly, ADP appears to specifically induce an apoptosis-like cell death [139], which may increase the immunogenicity of CRAd-induced tumor cell death. Taken together, these studies indicate that retention and over-expression of ADP with removal of other E3 coding regions improves CRAd spread and may improve the development of anti-tumor immunity.

ADP and p14 FAST

Following attachment and internalization of HAdV into the cell, the virion undergoes partial disassembly in the endosome and further controlled disassembly as the virion traffics through the cytoplasm on route to the nucleus [140]. Within a syncytium, HAdV progeny generated in one nuclei are unable to infect other nuclei within the same syncytia, likely due to the absence of controlled disassembly that normally occurs during infection [87]. Horn et

al. showed that an HAdV expressing the measles fusion glycoprotein induced syncytium formation, but failed to spread to new nuclei within the syncytium [141]. Fluorescence *in situ* hybridization (FISH) staining showed that only one or two nuclei within large syncytium (containing greater than ten nuclei) was positive for viral DNA. Interestingly, if the syncytia were re-challenged with virus, most nuclei stained positive for viral DNA, suggesting the effect is not due to defective or damaged nuclei, but an inability to spread between individual nuclei within a syncytium. Due to the ability of HAdV ADP to efficiently lyse nuclei and release HAdV particles, this protein may synergize effectively with p14 FAST to promote maximal virus spread and cellular destruction within the injected tumor.

1.10 Hypothesis

We hypothesize that the oncolytic efficacy of CRAd can be enhanced through expression of proteins designed to enhance viral spread (p14 FAST) and release (ADP).

1.11 Objectives

1. To construct, validate and characterize a conditionally replicating HAdV expressing the p14 FAST protein, and later, the adenovirus death protein, during viral replication.
2. To test the efficacy of this virus (CRAdFAST) in cell culture models of cancer.
3. To examine whether CRAdFAST and CRAdFAST-ADP will provide significant improvement over CRAd in mouse models of cancer.

Chapter 2: Materials and Methods

Cell culture.

Human A549 (ATCC CRM-CCL-185)[142] and 293 cells (ATCC CRL-1573)[143] were maintained in Minimal Essential Media (MEM) (Sigma Aldrich, Oakville, ON, Canada) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich), 2mM GlutaMAX (Invitrogen, Waltham, MA, USA) and 1X Antibiotic-Antimycotic (Invitrogen). Mouse 4T1 (ATCC CRL-2539)[144] and CMT-64 (ECACC 10032301)[145] cells were maintained in Dulbecco's Modified Eagles Medium-high glucose (DMEM) (Sigma Aldrich) with identical supplements.

Adenoviral constructs.

Schematic representations of select HAdV vectors are shown in Figure 1. All HAdV vectors are based on serotype 5 (HAdV-5), and were generated by a combination of traditional and RecA-mediated cloning [146]. AdFAST-HA is an early region 1 (E1) and E3 deleted HAdV vector, and contains the p14 FAST gene under regulation by the human cytomegalovirus (CMV) immediate early enhancer/promoter and bovine growth hormone polyadenylation sequence (BGHpA) replacing the E1 region, and has been described previously [80]. For simplicity, this vector will be referred to as AdFAST. HAdV vectors CRAd and CRAdFAST used in this study contain the E1A Δ 24 mutation and are also deleted of the E3 region. E1A Δ 24 prevents the E1A protein from binding the retinoblastoma (Rb) protein, which is required for efficient replication in normal tissues, thus conferring selective replication in cancer cells defective in the Rb pathway [147]. To link p14 FAST protein

expression to virus replication, we placed an expression cassette comprised of a splice acceptor (SA) site-p14 FAST coding sequence replacing the E3 deletion, which places p14 FAST expression under regulation by the viral major late promoter (MLP) [35, 148, 149]. Since the MLP is only active at appreciable levels following active virus DNA replication [150], p14 FAST protein is only expressed in cells in which the virus can replicate. The p14 FAST proteins expressed from AdFAST and CRAdFAST contain a C-terminal HA epitope tag, as previously described [80]. CRAdFAST/-SA is identical to CRAdFAST but lacks the splice acceptor preceding the p14 FAST cDNA. CRAdFAST-ADP is identical to CRAdFAST but contains the P2A ribosomal skipping motif immediately following the p14-FAST cDNA, followed by the HAdV ADP cDNA, and was constructed by Ryan Clarkin [151]. The P2A motif allows equi-molar expression of the gene proceeding and following a defined recognition amino acid sequence (GSGATNFSLLKQAGDVEENPGP)[152, 153]. Ad-late/RFP is similar in structure to CRAdFAST, but contains a wildtype E1 region, and encodes a SA-monomeric red fluorescent protein (RFP) expression cassette within the E3 region, rather than SA-p14 FAST, and has been described previously[149]. AdGFP-late/RFP is similar to Ad-late/RFP but is deleted of E1 and contains a cDNA for green fluorescent protein (GFP) under regulation by the human CMV immediate early enhancer/promoter and bovine growth hormone polyadenylation sequence (BGHpA) replacing the E1 region. AdRP3200 is an E1+ virus but is deleted of the E3 region, specifically a BglII-BglII fragment located at 28134 and 30819 bp of the conventional HAdV-5 genome, similar to pBHG10 [154]. AdRP3201 is identical to AdRP3200 but contains an intact E3 region. Both AdRP3200 and AdRP3201 also encode a FLAG-epitope tag on the C-terminus on the viral pIX, which has no discernible effect on virus growth and viability [35, 36]. AdRP2014 is an early region 1 (E1) and E3 deleted HAdV vector which has been previously described as AdEmpty [80]. AdCA35 is an E1/E3-deleted HAdV

vector encoding β -galactosidase under regulation by the murine CMV enhancer/promoter and simian virus 40 (SV40) polyadenylation sequence, and was generously provided by Dr. Christina Addison (Ottawa Hospital Research Institute, CAN) [155]. AdMA26 is an E1/E3-deleted HAdV vector encoding the Cre recombinase under regulation by the murine CMV enhancer/promoter and simian virus 40 (SV40) polyadenylation sequence, and was generously provided by Dr. Frank Graham (McMaster University, CAN). AdMA26 is similar in structure to AdCre [156], and will be referred to as AdCre in this study. AdMA19 is an E1/E3-deleted HAdV vector encoding a luciferase gene under regulation by the human CMV enhancer/promoter and SV40 polyadenylation sequence, but also contains a lox-stop-lox element between the promoter and luciferase gene [156]. Thus, AdMA19 expresses minimal luciferase until it is activated through Cre-mediated excision of the stop element, and is referred to as AdFloxLuc in this study. AdMA19 was a kind gift of Dr. Eric Kremer (Institut de Génétique Moléculaire de Montpellier, France). HAdV stocks were amplified in 293N3S cells, which were a kind gift of Dr Frank Graham (McMaster University) [157], with the exception that CRAdFAST was amplified in A549 cells in suspension culture. Viruses were purified and titered as described previously [158]. No evidence of abnormal particle to PFU ratio was observed for any of the above described viruses.

Primer Design, PCR and Sequencing.

To ensure CRAd and CRAdFAST viruses maintained the 24 base pair deletion in E1A following rescue in 293 cells, primers were designed flanking the deleted region within the CR2 domain of E1A. Primers were designed using Primer3 software to target a roughly 200bp region in the wild-type HAdV E1A sequence flanking the 24 base-pair deletions in CRAd and

CRAdFAST (Forward primer: GCAGGAAGGGATTGACTTACTC (Sense), Reverse primer: CAAACTCCTCACCCCTCTTCATC (AntiSense)) [159]. PCR conditions were as follows; 90 sec at 95°C for initial denaturation followed by 30 cycles of 30 sec at 95°C, 30 sec at 62°C for annealing and 20 sec at 68°C for extension, with a final extension at 68°C for 5 min. PCR products were separated on 2% agarose gels and visualized with ethidium bromide stain. The CRAdFAST PCR product was sequenced by the Ottawa Hospital Research Institute Stemcore Laboratories DNA Sequencing Facility. Sequence alignment was performed using Nucleotide Blast [160].

Analysis of CRAdFAST growth kinetics.

To determine whether expression of p14 FAST protein altered the growth kinetics of the vector, 35 mm dishes of A549, 4T1 and CMT-64.6 cells were infected at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell with either CRAd, CRAdFAST or AdRP2014 as a control. At 2, 24 and 72 hours post-infection (hpi), the cells were collected into the medium and subjected to two rounds of freeze-thaw to release progeny virions. Viral titers in the initial inoculum and at the various time points were determined by plaque assay [158]. To quantify HAdV genome replication in 4T1 cells, 35 mm dishes of 4T1 cells were infected at an MOI of 3 in 200µL of PBS with either AdFAST or CRAdFAST for 1 hr, washed with PBS, and medium was replaced. At 4, 24, 48 and 72 hpi, medium was removed and the cells were incubated with 500µL of SDS-Proteinase K buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, 1% SDS (w/v), 1 mg/ml proteinase K) overnight at 37°C. DNA extraction was performed as described previously [158], and qPCR were performed using 200 ng of isolated DNA and the following primer sets: Hexon (5'-CTTACCCCCAACGAGTTTGA and 5'-

GGAGTACATGCGGTCCTTGT) and actin (5'- CCGTCAGGCAGCTCATAGCTCTTC and 5'-CTGAACCCTAAGGCCAACCGT). qPCR for the actin gene was performed to verify that equal quantities of DNA were present in the samples (data not shown). In parallel, a standard curve of known viral genome quantities was prepared, and used to calculate genome concentrations in each sample. Copy numbers were calculated by the method previously described by Whelan et al. [161].

Immunoblot analysis.

Cells were seeded in 35 mm culture dishes at a density of 6.0×10^5 cells per well. Next day, the cells were infected with virus at varying MOI for 1 hr, and the medium replaced. For experiments including cytarabine arabinoside (Ara-C), medium containing 20 $\mu\text{g/mL}$ of drug was placed onto the cells after the 1 hr infection. At varying times post-infection, the medium was removed, the cells were harvested in 2X Laemmli buffer (62.5mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 5% 2-mercaptoethanol) and the samples stored at -20°C . Harvested samples were boiled for 5 min at 100°C , separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred using a semi-dry transfer protocol onto polyvinylidene difluoride (PVDF) membrane (Millipore, Etobicoke, ON, Canada). Membranes were probed for β -actin (1:10 000, Clone AC-15 A1978 Sigma Aldrich), HAdV-5 fiber (1/10,000 dilution; MS-1027-P0, Neomarkers (Fremont, CA, USA), caspase-3 (1:10 000, #9662 Cell Signaling (Beverly, MA, USA)), cleaved caspase-3 (1:1000, #9664 Cell Signaling), RFP (1:5,000, ab62341; Abcam) or HA epitope (1:3000, #2367 Cell Signaling). Immunoblots were developed using Luminata Classico HRP substrate (Millipore) and visualized by autoradiography.

Microscopy.

Phase contrast images were acquired using a Zeiss Axio Observer Microscope (Carl Zeiss Canada, North York, ON, Canada) and analyzed using Zen 2 (Blue Edition) software (Carl Zeiss Canada). Images were processed using GIMP [162] and compiled using Adobe Illustrator software.

Metabolic activity assay.

Metabolic activity was quantified using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). 4T1, A549 and CMT-64.6 cells were seeded at 2×10^4 cells per well in flat-bottom 96-well cell culture dishes (Thermo Scientific, Waltham, MA, USA). Next day, the cells were infected with varying MOI of virus in 25 μ L of PBS for 1 hr, and fresh medium replaced. Metabolic activity was evaluated at 24, 48 and 72 hpi. Twenty μ L of the MTS substrate was added to each well and incubated in the dark for 1 hr. Colour development was analyzed in the 96-well plate using a SpectraMax 190 plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 490nm.

Extracellular vesicle isolation

Whole populations of EVs (exosomes and microvesicles) were isolated by differential centrifugation from media. At various times post-infection, media was removed from cells and underwent an initial centrifugation at 300xG for 20 minutes to remove any suspended cells. Next, media underwent a subsequent centrifugation step of 2,000xG for 10 minutes to remove cellular debris. A final spin of 100,000xG for one hour was done to pellet remaining extracellular vesicles, which were suspended in PBS for downstream analysis. To isolate exosomes, media was spun at 20,000xG for 20 minutes to pellet microvesicles prior to the

100,000xG centrifugation step. Zeta-View particle counting (Particlenetrix, Germany) was performed according to the manufacturer's instructions.

β -Galactosidase assay

In order to identify cells which had been successfully infected by AdCA35, 4T1, CMT-64 and CMT-64.6 cells were infected at MOI 0.01 in 6-well plates. 24 hours later, cells were fixed in 4% paraformaldehyde (PFA) and stained with 50 μ l/ml of X-gal overnight [163]. Cells positive for β -Gal activity were counted by compound microscope, and the total number of infected cells per well was calculated using the ratio of the microscope field of view to the surface area of the well.

Luciferase Assay

The Promega luciferase assay system was used to quantify luciferase expression in cells infected with AdFloxLuc in the triple-infection experiments (Promega, Madison, WI, USA). 24 hpi, A549 cells were washed once with PBS, harvested in 400 μ L of reporter lysis buffer and subjected to one freeze-thaw according to the manufacturer's instructions. Debris were pelleted by centrifugation and supernatant was transferred to a new tube. 10 μ L of supernatant was added to 100 μ L of luciferase assay reagent, briefly vortexed and immediately read in a 20/20ⁿ luminometer (Turner Biosystems, Sunnyvale, CA, USA).

4T1 syngeneic cancer model.

All mouse experiments were approved by the Animal Care Committee at the University of Ottawa. For the 4T1 mouse mammary carcinoma tumor model, 30 Balb/c (Charles River Laboratories, Wilmington, MA, USA) mice were injected subcutaneously with 1×10^5 cells in the right hind flank. When tumors reached approximately 5x5mm (5 days post injection), the

mice received an intratumoral injection of 50 μ L PBS, or 7×10^9 PFU of CRAd or CRAdFAST diluted in PBS to a total volume of 50 μ L. Three days after vector delivery, two mice from each group were euthanized for histological analysis of tumor and liver samples. For the remainder of the animals, tumor size was measured three times weekly via electronic calipers: two measurements were taken, one at the largest diameter and another perpendicular to the first, and volumes were calculated as $(\text{length} \times \text{width}^2)/2$. The experiment achieved endpoint when any tumor reached 15x15mm, had a volume of approximately 1687.5 mm³, or the mice showed signs of ulceration.

A549 xenograft tumor model.

For the A549 human lung adenocarcinoma tumor model, 21 CD1/nude mice (Charles River Laboratories) were injected subcutaneously with 1×10^6 A549 cells in the right hind flank. Fifteen days later, when the tumors reached approximately 5x5mm, the mice received an intratumoral injection of 50 μ L PBS, or 1.0×10^9 PFU of CRAd or CRAdFAST diluted to a volume of 50 μ L in PBS. Mice were monitored for tumor growth and survival as described above.

CMT-64.6 syngeneic cancer model.

For the CMT-64.6 mouse lung carcinoma model, 16 C57BL/6J mice (Charles River Laboratories) were injected subcutaneously with 1×10^5 CMT-64.6 cells in the right hind flank. Fifteen days later, when the tumors reached approximately 5x5mm, the mice received an intratumoral injection of 50 μ L PBS, or 1.0×10^9 PFU of CRAdFAST or CRAdFAST-ADP diluted to a volume of 50 μ L in PBS. Mice were monitored for tumor growth and survival as described above.

Tumor Histology and Immunohistochemistry.

Immediately upon removal, tumors and livers were placed in 4% PFA solution. Forty-eight hours later, the samples were transferred to 70% ethanol, and further processed, embedded, sectioned and stained with hematoxylin and eosin at the University of Ottawa Histology Core Facility. Formalin-fixed, paraffin-embedded slides were deparaffinized in xylene and rehydrated through a 100-70% ethanol gradient. Heat-induced epitope retrieval was performed at 110°C for 12 minutes with citrate buffer (pH 6.0), and 3% H₂O₂ was used to block endogenous peroxidases. Sections were further blocked with Background Sniper blocking reagent (BioCare Medical, CA, USA) and incubated with rabbit anti-HA antibody (Cell Signaling C29F4; 1:1000 Liver, 1:2000 Tumor) and/or rabbit anti-HAdV-5 (Abcam ab6982; 1:2000) for 1 hr at room temperature. Antibody binding was visualized using the MAHC4™ + DAB detection system according to the manufacturer's instruction (BioCare Medical). Slides were then counterstained with Hematoxylin, and images obtained using the Zeiss Mirax Midi whole slide digital scanner. Images were analyzed using Pannoramic Viewer software (3DHISTECH, build 1.15.4.43061, Budapest, Hungary), and compiled using Inkscape software[164].

Statistical Analysis.

Statistical analyses were performed using GraphPad Prism 6 (Version 6.01, GraphPad Software Inc, San Diego, CA, USA). All bar graphs represent mean, while error bars represent standard deviation. MTS data presented in bar graphs were compared by two-way ANOVA, followed by Tukey's HSD post-hoc analysis. 4T1 tumor growth rates presented in bar graphs were modeled using tumor specific growth rate, as described by Mehrara et al.[165], and compared by one-way ANOVA. For the A549 xenograft tumor model, a linear growth model

was chosen to analyze growth, as the r^2 values overall were higher for linear regression (0.53 for PBS, 0.33 for CRAd and 0.42 for CRAdFAST) than for non-linear regression using an exponential model (0.38 for PBS, 0.27 for CRAd and 0.34 for CRAdFAST). There is significant evidence that tumors often take on a linear growth pattern [166].

Chapter 3: p14 FAST improves oncolytic efficacy of CRAd *in vitro* but not *in vivo* in a mouse cancer cell line

3.1 Introduction

In previous studies, our lab explored the efficacy of an early region 1 (E1)-deleted, replication-deficient HAdV expressing the p14 FAST protein (AdFAST) in both immune-deficient human A549 lung adenocarcinoma xenograft-bearing CD1/nude mice and immune-competent Balb/c 4T1 tumor models [80, 98]. Interestingly, during the course of our *in vitro* studies, we showed that under conditions in which the E1-deleted AdFAST virus can replicate (*i.e.* in the E1-complementing 293 cell line), p14 FAST protein caused significantly more efficient cell fusion and killing with greatly reduced quantity of vector. This observation suggests that p14 FAST protein may provide the greatest efficacy when expressed from a replicating, oncolytic HAdV. In this context, expression of the p14 FAST protein would amplify as the virus replicates, potentially providing greater cell fusion and enhanced induction of apoptosis, in addition to facilitating vector spread through the tumor.

This observation led to the generation of CRAdFAST, a conditionally replicating HAdV vector (CRAd) expressing the p14 FAST protein from the E3 region as a pseudo “late” gene. Using human and mouse cell lines, I first characterized the structure of CRAdFAST by PCR, western blot, qPCR and viral titer assay. I next tested whether the newly characterized CRAdFAST virus had oncolytic properties in 4T1 mouse cells. These cells were chosen for their ability to form tumors in immune-competent mice, an important model as the immunological aspects of oncolytic viruses have been increasingly found to be profoundly integral to efficacy [167]. This model allowed us to eventually examine the oncolytic effect of CRAdFAST *in vivo*. I show that the CRAdFAST vector replicates and expresses high levels

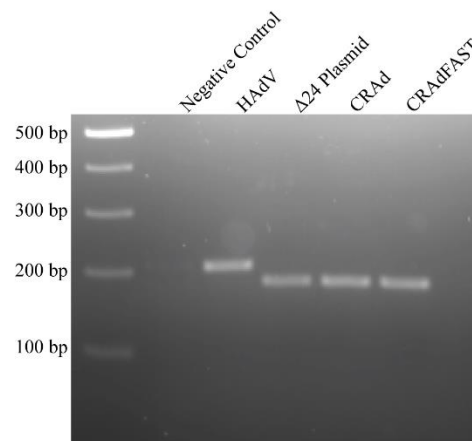
of p14 FAST and has improved oncolytic capacity *in vitro*, but does not provide benefit after intratumoral injection of 4T1 tumor bearing mice.

3.2 Results

CRAd and CRAdFAST retain the $\Delta 24$ deletion in E1A following viral amplification

Both CRAd and CRAdFAST were initially rescued in 293 cells. These cells, developed by transformation with HAdV DNA, contain roughly 4000bp of the HAdV genome and express the entire E1 region and the pIX gene [143, 168]. A major function of CRAd, i.e. conditional replication, is conferred by a 24bp deletion in the E1A region. Since this region is highly homologous with the HAdV wild-type DNA found in 293 cells, double crossover events leading to recombination and reversion to wild-type E1A are possible, particularly in CRAdFAST, which underwent serial expansion in 293 cells due to initially low yield [169]. To assess whether recombination had occurred, PCR and sequencing were performed on rescued CRAd and CRAdFAST vectors. DNA was extracted from purified HAdV, CRAd and CRAdFAST virions for PCR. Primers were designed flanking a 200bp region containing the $\Delta 24$ deletion. As is evident in gel electrophoresis of the PCR products obtained using wild-type HAdV, the CRAd plasmid (positive control) and extracted CRAd and CRAdFAST viral DNA, there appears to be a significant size reduction in $\Delta 24$ containing sequences, consistent with the deletion of 24bp (Figure 4A). To confirm that the deletion in this virus was in the correct location, the CRAdFAST PCR product was sequenced and aligned to the HAdV E1A sequence obtained from NCBI GenBank using MEGA7 software [170, 171]. When aligned, there is a clear 24 bp deletion in the CRAdFAST genome in the region originally intended

A.



B.

```

HAdV E1A  CCGGCAGCCCGAGCAGCCGGAGCAGAGAGCCTTGGGTCCGGTTTCTATGCCAAACCTTGT
          |||||||
CRAAdFAST CCGGCAGCCCGAGCAGCCGGAGCAGAGAGCCTTGGGTCCGGTTTCTATGCCAAACCTTGT

          ACCGGAGGTGATCGATCTTACCTGCCACGAGGCTGGCTTTCCACCCAGTGACGACGAGGA
          |||||||
          ACCGGAGGTGATCGA-----TCCACCCAGTGACGACGAGGA

          TGAAGAGGGTGAGGAGTTTGTGTTAGATTATGTGGAGCACCCGGGCACGGTTGCAGGTC
          |||||||
          TGAAGAGGGTGAGGAGTTTGTGTTAGATTATGTGGAGCACCCGGGCACGGTTGCAGGTC

          TTGTCATTATCACCGAGGAATACGGGGGACCCAGATATTATGTGTTGCTTTGCTATAT
          |||||||
          TTGTCATTATCACCGAGGAATACGGGGGACCCAGATATTATGTGTTGCTTTGCTATAT

          GAGGACCTGTGGCATGTTTGTCTACAGT
          |||||||
          GAGGACCTGTGGCATGTTTGTCTACAGT

```

Figure 4. CRAd and CRAdFAST retain 24bp deletion following expansion in 293 cells.

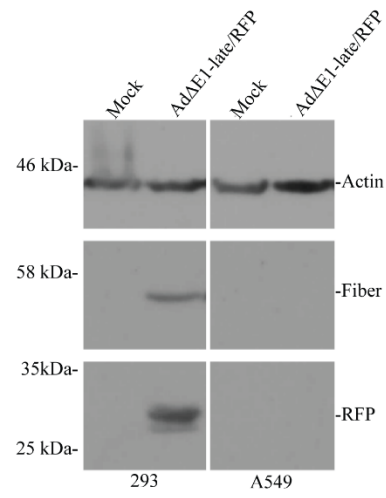
A) PCR was performed on DNA extracted from Ad Wildtype, CRAd and CRAdFAST using primers flanking the 24bp deletion in E1 and the product was separated by agarose electrophoresis. The $\Delta 24$ plasmid was used as a positive control. B) CRAdFAST was sequenced at this region using identical primers. CRAdFAST sequence was aligned to the HAdV E1A sequence using BLAST software to visualize the 24bp deletion [160].

(Figure 4B)[147]. Thus, the majority of purified CRAd and CRAdFAST virions do indeed contain the 24bp deletion necessary to confer cancer specific conditional replication.

Characterization of CRAdFAST p14 FAST expression patterns

CRAdFAST contains the p14 FAST cDNA within the E3 region, which is preceded by a splice acceptor. Theoretically, this allows the expression of the transgene of interest to be under the control of the HAdV major late promoter, and effectively ties transgene expression to viral replication. This mechanism has previously been exploited successfully by our lab [35]. To confirm that the inclusion of a splice acceptor immediately preceding a gene placed in the E3 region confers “late” gene expression, we constructed AdGFP-late/RFP, which contains a CMV-GFP expression cassette in the E1 region, and the RFP cDNA in the E3 region, preceded by a splice acceptor. We predict that this E1-deleted virus will only express RFP in cells in which it can replicate (i.e. E1-complementing 293 cells) but not in non-permissive cells (i.e. A549 cells). 293 and A549 cells were infected at MOI 1 with AdGFP-late/RFP, and crude protein extracts were harvested for western blot at 24 hpi (Figure 5A). As expected, viral replication (as noted by HAdV fiber expression) was limited to 293 cells which complement the E1 deficiency in this vector. Ad-late/RFP was only able to express the reporter RFP construct in the 293 cells in which it was able to replicate. Next, to show the importance of the splice acceptor for late gene expression, we created a CRAdFAST construct that contained the FAST insertion in E3, but lacked the splice acceptor (designated CRAdFAST/-SA). 293 cells were either mock infected or infected at MOI of 1 with CRAdFAST/-SA or CRAdFAST, and crude cell lysates were harvested for western blot at 24 hpi. As expected, both CRAdFAST and CRAdFAST/-SA replicate and express the HAdV late fiber protein, however, CRAdFAST/-SA failed to express the p14 FAST transgene at high levels (Figure 5B), re-affirming the importance of the splice acceptor to the late-gene control of the inserted

A.



B.

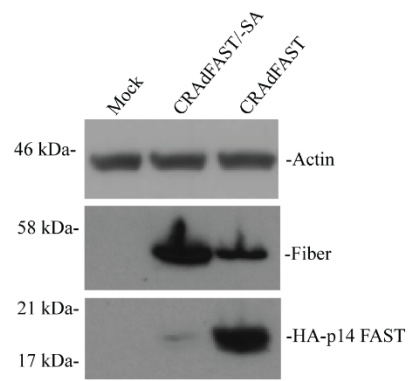


Figure 5. Transgenes placed in the HAdV E3 region require HAdV replication and must be preceded by a splice acceptor for detectable protein expression. A) 293 and A549 cells were either mock infected or infected at MOI 1 with AdGFP-late/RFP and cells were harvested at 24 hpi. Immunoblots were probed for Fiber and RFP, actin was used as a loading control. B) 293 cells were either mock infected or infected at MOI 1 with CRAdFAST/-SA or CRAdFAST, and cells were harvested at 24 hpi. Immunoblots were probed for Fiber and HA-FAST, actin was used as a loading control.

transgene. Taken together, these results show that the approach used to express the p14 FAST protein from CRAdFAST restricts expression of the transgene to cells in which the virus can replicate, and the splice acceptor is crucial for this activity.

I also examined whether expression of p14 FAST protein altered CRAd replication kinetics by examining virus yield at varying times post infection. As shown in Figure 6, after infection at an MOI of 10, A549 cells infected with both CRAd and CRAdFAST showed a ~300-fold increase in viral titer between 4 and 24 hpi, which further increased by 72 hpi. Importantly, there was no difference in virus recovery between CRAd containing or lacking the p14 FAST gene, suggesting expression of p14 FAST protein did not adversely affect virus growth and recovery. This result was surprising, as obtaining high titer large-scale preparations of purified CRAdFAST virions was difficult. It is possible that the replication kinetics of relatively high MOI infection in tissue culture dishes are more favorable for CRAdFAST burst than in large-scale suspension cultures, for reasons which are unclear.

CRAdFAST can replicate and express viral genes but does not produce progeny virions in mouse 4T1 cells

Previously, we showed that vectors based on HAdV-5 infected 4T1 mouse mammary carcinoma cells at an efficiency approximately one-sixth that of A549 cells, a cell line that infects well with HAdV and is used commonly in many HAdV studies [98]. HAdV can replicate its DNA and express both early and late genes in many mouse cell lines, but typically infection is not productive (*i.e.* progeny virions are not produced). I therefore examined whether HAdV infection was productive in 4T1 cells. 4T1 cells were infected at an MOI of 3 with AdFAST (E1-deleted, replication defective) or CRAdFAST (E1A Δ 24, replication competent) and total DNA was

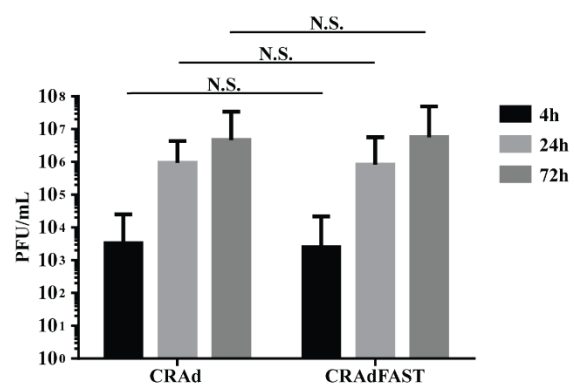
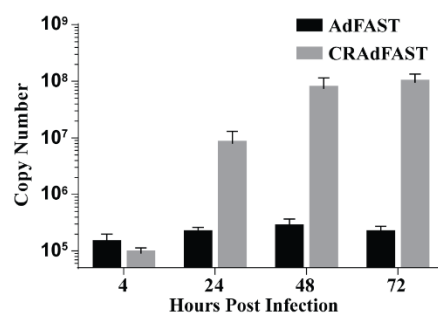


Figure 6. Expression of p14 FAST does not inhibit CRAd growth in A549 cells. A549 cells were infected at MOI 10 with either CRAd or CRAdFAST. Cells and media were harvested at 4, 24 and 72 hpi. Viral titers were determined by plaque assay on 293 cells.

harvested for qPCR analysis of viral genome content 4 to 72 hr later. As shown Figure 7A, as expected, infection of 4T1 cells with the replication-defective AdFAST vector did not result in an increase in viral genome copy number over the course of the experiment. In contrast, I observed an approximate 1000-fold increase in viral genome copy number in CRAdFAST-treated 4T1 cells between 4 and 72 hpi, clearly showing that the virus can replicate its DNA in this cell line. To determine if infection of 4T1 cells was productive, cells were infected at an MOI of 10 with CRAd or CRAdFAST, the cells were harvested into the medium at various time post-infection and titered for virus. As shown in Figure 7B, no increase in the amount of recovered virus between 4 and 72 hpi was observed, suggesting that CRAd and CRAdFAST did not give rise to progeny virions during this time period. However, the increase in genome copy number as the virus replicates its DNA should effectively amplify viral gene expression, leading to accumulation of high-levels of virus-encoded proteins, including the p14 FAST protein within the infected cells.

I next evaluated expression of virus-encoded proteins, specifically the native viral fiber and p14 FAST. CRAdFAST contains the p14 FAST cDNA preceded by a splice acceptor site replacing the E3 deletion, which should allow for high-level expression of p14 FAST only when the virus replicates, a strategy that we and others have employed previously (Figure 5A) [35, 149, 172]. 4T1 cells were infected with AdFAST (E1-deleted, replication defective) or CRAdFAST and protein expression was examined at 24, 48 and 72 hpi. As expected, treatment of cells with AdFAST did not result in detectable expression of fiber protein (Figure 8A). At this low MOI of 3, p14 FAST protein expression was also not detectable in AdFAST-treated cells, consistent with our previous observation that high MOI (>100) is required to visualize FAST expression in this cell line [98]. In contrast, likely due to the increase in template copy

A.



B.

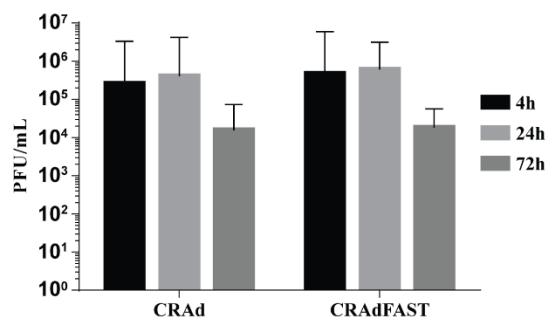
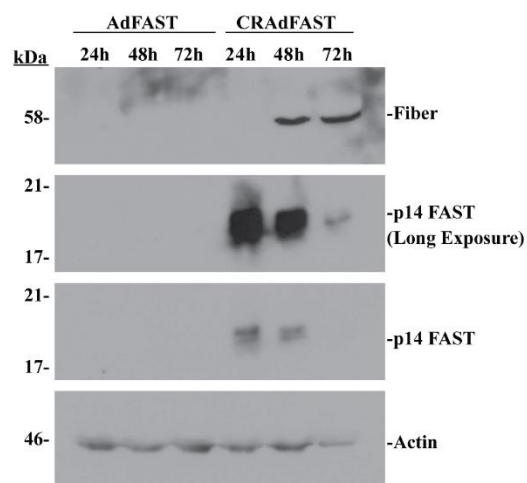


Figure 7. CRAdFAST replicates but does not produce progeny virions in 4T1 cells. A) 4T1 cells were infected with either AdFAST or CRAdFAST at MOI 10. At 4, 24, 48 and 72 hpi, media was removed and the cells were harvested in SDS/Proteinase K buffer for DNA extraction. qPCR was performed on 200ng of total isolated DNA for the Ad hexon genome region. B) 4T1 cells were infected with either CRAd or CRAdFAST at MOI 10. Cells and medium were collected at 4, 24 and 72 hpi. Viral titers were determined by plaque assay on 293 cells.

A.



B.

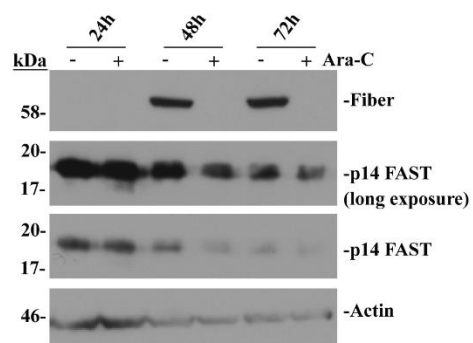


Figure 8. Expression of p14 FAST is not tied to virus replication in CRAdFAST infected 4T1 cells. A) 4T1 cells were infected with either AdFAST or CRAdFAST at MOI 3 and harvested at 24, 48 and 72 hpi. Immunoblot analysis was performed to examine HAdV fiber and HA (p14 FAST) protein levels, and actin was used as a loading control. B) 4T1 cells were infected at MOI 50 with CRAdFAST for 1 hr, and overlaid with medium lacking (-) or containing (+) 20µg/mL of cytosine arabinoside (Ara-C) to inhibit DNA replication. Crude protein samples were harvested at 24, 48 and 72 hpi, and assayed by immunoblot for expression of fiber protein, and p14 FAST protein, actin was used as a loading control.

number within the cells infected with CRAdFAST (Figure 8A), expression of both p14 FAST protein and the late fiber protein was easily detectable for this virus (Figure 8A). Interestingly, p14 FAST protein was detected at a much earlier time point compared to fiber protein. If onset of fiber protein expression correlates with active virus DNA replication, this suggests that p14 FAST protein expression may precede replication (i.e. is under “early” regulation) in 4T1 cells. To assess this possibility, I examined expression of fiber and p14 FAST protein in the presence or absence of Ara-C, a compound that blocks viral DNA replication. Since the major late promoter is not fully activated until viral genome replication begins [173], treatment with Ara-C should prevent expression of fiber and p14 FAST protein if they are indeed under “late” regulation. 4T1 cells were infected with CRAdFAST at an MOI 50 in the absence or presence of 20 µg/mL of Ara-C, and harvested at 24, 48 and 72 hpi. As shown in figure 8B, while fiber expression is indeed inhibited by treatment with Ara-C, p14 FAST expression is unaffected by this treatment. Thus, although CRAdFAST can provide very high-level expression of p14 FAST protein in the 4T1 mouse cell line, unlike endogenous viral genes (i.e. fiber), its expression does not appear to be conditionally tied to active virus replication.

Expression of p14 FAST protein from CRAd enhances 4T1 cell killing in vitro

I next examined whether the elevated expression of p14 FAST protein from CRAdFAST enhanced the ability of the oncolytic vector to mediate cell fusion and cause cancer cell death in 4T1 cells in culture. 4T1 cells were infected with AdFAST, CRAd or CRAdFAST at an MOI of 100, or mock infected with PBS, and the cells analyzed visually for cell fusion by phase-contrast microscopy at varying times post infection. I did not observe evidence of fusion in mock or AdFAST-treated cells at any of the time points (Figure 9),

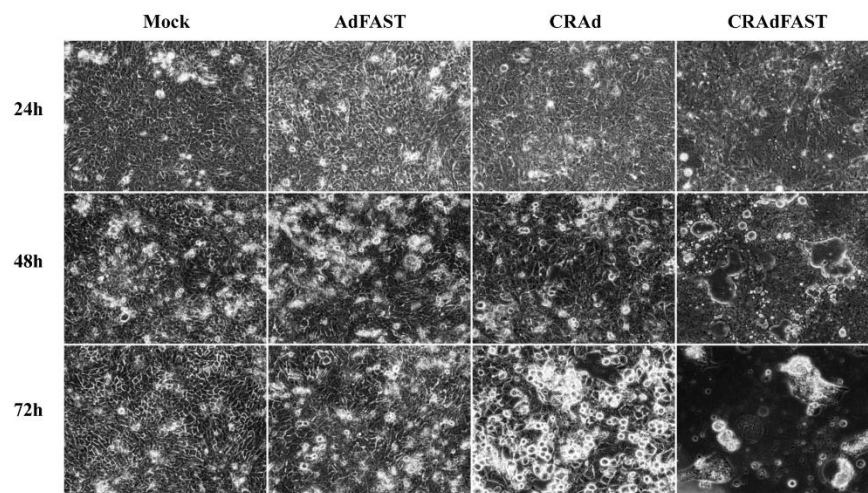
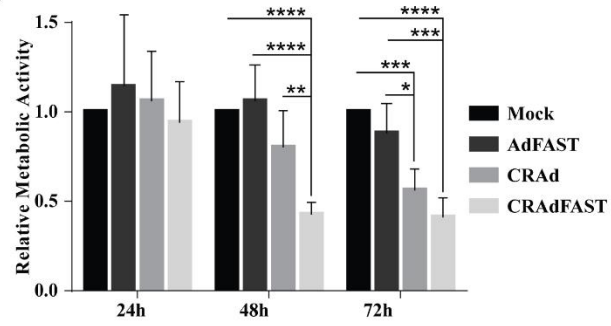


Figure 9. CRAdFAST infection leads to monolayer fusion and destruction in 4T1 cells. 4T1 cells were mock infected, or infected with AdFAST, CRAd or CRAdFAST at an MOI 100. Phase-contrast images were obtained at 24, 48, and 72 hpi.

consistent with our previous work showing that very high MOI of AdFAST was required to achieve visible fusion in 4T1 cells [98]. Cells treated with CRAd showed evidence of cytopathic effect (CPE), but only at the 72 hr time point, and no cell fusion occurred. However, CRAdFAST-treated 4T1 cells showed clear evidence of cell fusion as early as 24 hpi, which increased over the 72 hr time course. By 72 hpi, the entire monolayer of CRAdFAST-treated 4T1 cells had fused and exhibited extensive CPE. Thus, treatment of 4T1 cells with the replication-competent CRAdFAST results in greatly enhanced fusion relative to the replication-defective AdFAST.

To determine whether enhanced cell fusion mediated by CRAdFAST was associated with 4T1 cell death, metabolic activity of the treated cells over time was assessed. 4T1 cells were infected at an MOI of 1000 with AdFAST, CRAd, and CRAdFAST, or mock-infected with PBS, and metabolic activity was examined at 24 hr intervals by MTS assay. Treatment of cells with AdFAST did not result in a decrease in metabolic activity at any of the time points examined (Figure 10A). In contrast, CRAdFAST significantly reduced metabolic activity of the cells as early as 48 hpi. By 72 hpi, treatment with CRAd also caused a decrease in metabolic activity compared to AdFAST and mock treated cells. I also examined the cells for evidence of apoptotic cell death by immunoblot analysis for cleaved caspase 3 (Figure 10B). Treatment of 4T1 cells with CRAd did not result in enhanced levels of cleaved caspase 3 compared to untreated cells. In contrast, treatment of cells with CRAdFAST did result in enhanced cleavage of caspase 3, but only at high MOI. Taken together, these results indicate that treatment of 4T1 cells with CRAdFAST results in high-level expression of p14 FAST protein and significant cell fusion, resulting in a reduction in cellular metabolic activity and the onset of apoptosis-like cell death. Importantly, all of these effects are enhanced relative to 4T1 cells treated with

A.



B.

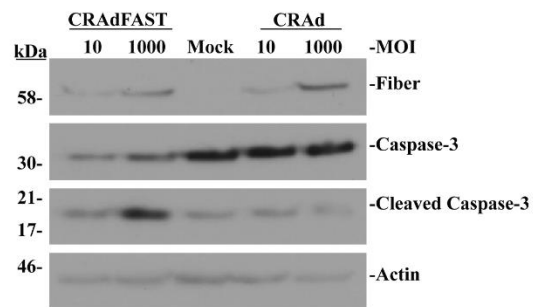


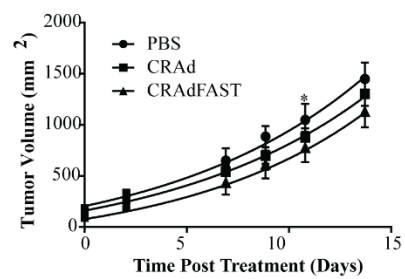
Figure 10. CRAdFAST reduces metabolic activity and leads to apoptosis of 4T1 cells. A) 4T1 cells were mock infected or infected with AdFAST, CRAd, or CRAdFAST at an MOI 1000 in triplicate wells (n=6 technical replicates, n=2 independent experimental replicates). At 48 hpi, metabolic activity was assessed by MTS assay, and the values are reported relative to metabolic activity in mock infected cells. Mean values reported $\pm$ SD (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). B) 4T1 cells were either mock infected or infected with CRAd or CRAdFAST at an MOI 10 and MOI 1000 and protein samples were harvested at 48 hpi. Protein lysates from infected cells were subjected to immunoblot for HAdV fiber, full length caspase-3 and cleaved caspase-3, actin was used as a loading control.

AdFAST or CRAd, suggesting that arming an oncolytic HAdV vector with p14 FAST protein enhances the anti-neoplastic potential of the vector.

Expression of p14 FAST protein from CRAd does not improve therapeutic efficacy in the 4T1 murine model of cancer in immune competent mice

I next examined whether p14 FAST protein expression could improve the efficacy of CRAd in the immune competent 4T1 mouse model of cancer. Balb/c mice bearing 4T1 subcutaneous tumors were injected intratumorally with either PBS, or 7×10^9 PFU of CRAd or CRAdFAST, and tumor size was measured by caliper three times weekly. As shown in Figure 11A and B, treatment of tumors with either CRAd or CRAdFAST resulted in a trend towards a reduced growth rate, but did not reach statistical significance. Tumor specific growth rates of PBS treated tumors (0.159 %/day) were not significantly different than those of CRAd (0.143 %/day, $p=0.74$) or of CRAdFAST-treated tumors (0.110 %/day, $p=0.097$) (Figure 11B). Tumor regression or stasis was not observed, and all animals reached endpoint and were euthanized at day 14. Tumor tissues removed at day 5 post-treatment were stained for HA to detect p14 FAST protein and examined visually for evidence of syncytia. As expected, CRAd treated tumors showed no evidence of p14 FAST protein or syncytial formation. p14 FAST protein was detected in tumors from CRAdFAST-treated mice, and syncytia were observed in isolated regions within the treated tumors (Figure 12). Livers from mice injected with the CRAd or CRAdFAST showed no obvious pathology. Thus, CRAdFAST can infect 4T1 tumors, giving rise to expression of p14 FAST protein, resulting in syncytium formation but, ultimately, does not reduce the tumor growth rate or enhance animal survival.

A.



B.

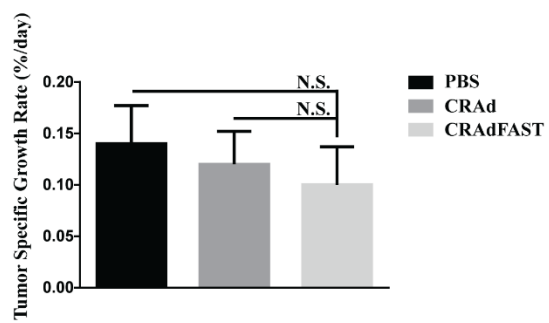


Figure 11. Expression of p14 FAST protein does not improve oncolytic efficacy of CRAd in the murine 4T1 model of cancer. Tumor-bearing mice were injected intratumorally with PBS, or 7×10^9 PFU of CRAd or CRAdFAST, and tumor volumes were measured for eight mice per group three times weekly. A) Symbols represent mean calculated tumor volumes over time. Lines represent non-linear regression model of tumor growth rates. At day 11, PBS treated tumors were significantly larger than CRAdFAST treated tumors. (* $p=0.03$). Error bars represent standard error. B) Tumor specific growth rates were calculated for each tumor and averaged, error bars represent standard deviation.

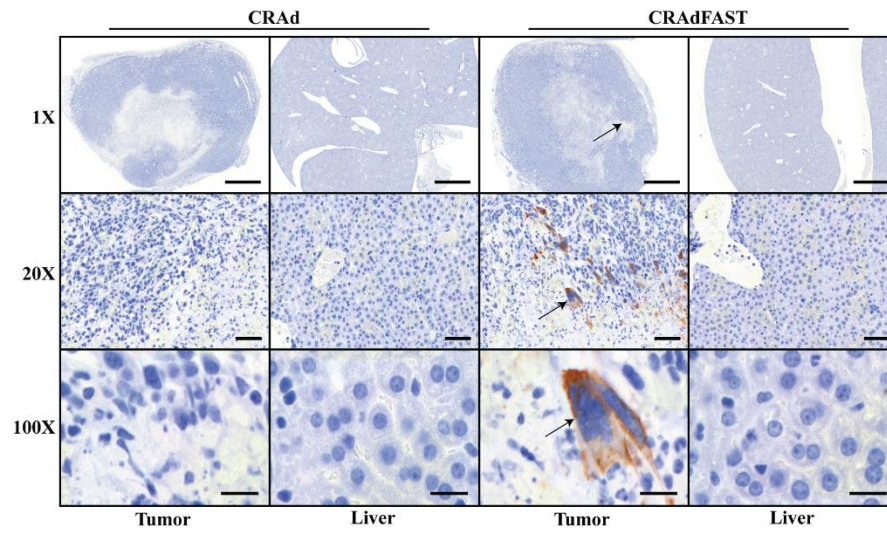


Figure 12. CRAdFAST induces syncytium formation in subcutaneous 4T1 tumors in vivo. Sections of tumor and liver from mice sacrificed at day 5 post-injection were probed with anti-HA antibody (p14 FAST) and counter-stained with hematoxylin to assess p14 FAST expression. At 1x, 20x and 100x magnifications, scale bars represent 2000 μ m, 100 μ m and 20 μ m, respectively.

3.3 Discussion

In preliminary experiments, I showed that placing a transgene in the E3 region preceded by a splice acceptor allowed for replication-specific expression of the transgene in human cells, and that the splice acceptor was necessary for this high level of expression. Due to the uniquely immense knowledge base on HAdV cloning and transcriptional control, there are many options for transgene insertion in CRAds. There are a variety of mechanisms available to control transgene transcription, including internal ribosome entry sites (IRES), P2A ribosome skipping, and splice acceptor insertion [174]. Importantly, splice acceptor-based expression requires the HAdV transcription to be initiated naturally, tying transgene expression intimately with genome replication. Indeed, splice-acceptor based transgene expression has been shown to confer high specificity to the replicating genome compared to IRES insertion [175]. Quirin et al. showed that, in the presence of Ara-C, CRAds expressing transgenes preceded by a splice acceptor were more significantly impacted than those preceded by either an IRES or a P2A sequence, indicating a tighter control of transgene expression [175].

Interestingly, in 4T1 cells in culture, we observed different temporal expression patterns between p14 FAST and fiber protein (Figure 8A). The p14 FAST protein was detected at an earlier time-point (24 hpi) and declined at later time-points (72 hpi), whereas HAdV fiber was first detected at 48 hpi and remained constant at 72 hpi. This led us to perform experiments with Ara-C, a DNA replication inhibitor which allows the differentiation between early and late gene expression. In the 4T1 cell line, p14 FAST expression from CRAdFAST appears to be under early, rather than late, transcriptional control (Figure 8). Although the majority of the E3 region is deleted in this viral construct, the early E3 promoter is retained, and may be

influencing the expression of p14 FAST. In human cells, analogous constructs were highly dependent on HAdV DNA replication and on the presence of the splice acceptor, indicating that late gene splicing was crucial for p14 FAST protein expression (Figure 5). Furthermore, I show that HAdV fiber protein is strongly regulated by the presence of Ara-C in 4T1 mouse cells (Figure 8B), suggesting that this drug is indeed functioning as expected in this cell line. The splice site used in this vector was isolated from the HAd40 long fiber gene, and thus, is not identical to the HAdV fiber splice site [148]. It is possible that incorrect splice site recognition is leading to a lack of expression of p14 FAST off of the major late transcript in these mouse cells. Despite a high degree of conservation of splicing machinery and recognition patterns among mammals, some studies have shown important species dependence of splice acceptor recognition between human and mouse cells [176-178]. It is indeed possible that these differences contribute to species-specific replication of HAdV.

Despite this interesting result, high level expression and robust monolayer fusion compared to AdFAST was shown, and I next examined the oncolytic capacity of CRAdFAST in this cell line. The efficacy of oncolytic vectors is attributed in part to their ability to directly replicate in and kill cancer cells, and also through their ability to act as an adjuvant to induce antitumor immunity [167, 179]. Thus, we initially tested CRAdFAST in the 4T1 immunocompetent mouse model of cancer.

In general, HAdV-5 has a relatively poor ability to infect mouse cells, including 4T1 cells [98], and infection is typically not productive (Figure 7). Although treatment of 4T1 cells in culture with CRAdFAST led to cell fusion, a reduction in cellular metabolic activity and induction of apoptosis (Figures 9 and 10), no beneficial effect was achieved *in vivo* (Figure 11). However, we did observe regions of syncytia formation in CRAdFAST-treated 4T1

tumors, which was not observed in CRAd-treated animals, suggesting that p14 FAST protein could mediate cell fusion *in vivo* (Figure 12). While inclusion of retargeting ligands can increase infection efficiency [35, 36, 180-183], the inability of HAdV to productively replicate in mouse cells is a distinct limitation to testing HAdV in mouse models of cancer.

The replication characteristics and oncolytic capacity of CRAdFAST have been characterized in the mouse 4T1 cell line. Despite genome replication, high level p14 FAST expression, and efficient monolayer destruction *in vitro*, CRAdFAST was ineffective after intratumoral administration *in vivo* in this cell line. To assess the direct antitumor oncolytic capacity of CRAdFAST, a cell line must be used that supports robust HAdV replication and progeny generation in order to amplify the oncolytic effect and spread throughout a treated tumor mass.

Chapter 4: CRAdFAST infection results in replication-dependent p14 FAST mediated fusion and cellular destruction in human cell culture and xenograft models of cancer

4.1 Introduction

Oncolytic viruses based on HAdV have a unique challenge in that most aspects of their viral life cycle are highly perturbed in mouse cell lines[184, 185]. This means that, in order to assess their oncolytic capacity *in vivo* in a pre-clinical setting, human tumors must be established in mice that cannot mount a non-self anti-tumor adaptive immune response. These mice, typically athymic “nude” mice, have drastically reduced T-cell numbers and cannot reject the human tumor[186]. Unfortunately, this also means that an anti-tumor adaptive immune response elicited by viral oncolysis is also not possible. Nevertheless, it is incredibly important to assess the direct anti-tumor impact and destruction induced by a CRAd, and it is straightforward to do so in the context of an eliminated adaptive immune response.

In the previous chapter, I showed that neither CRAd nor CRAdFAST provided any benefit *in vivo* after intratumoral injection into 4T1-bearing Balb/C mice. I hypothesized that this failure resulted from a combination of barriers that exist for HAdV infection, replication and progeny production in mouse cells. In this chapter, I have assessed the replication characteristics and oncolytic efficacy of CRAdFAST in human A549 lung adenocarcinoma cells. I show that, in the context of expression from a CRAd, p14 FAST expression mediates enhanced spread of the oncolytic effect *in vitro*, which leads to higher oncolytic efficiency per infectious unit. This increase in efficiency conferred an enhanced capacity to eliminate human xenograft tumor cells *in vivo* through improved intratumoral spread and enhanced cellular lysis resulting in reduced tumor growth rate.

4.2 Results

CRAdFAST shows improved replication characteristics in a human cancer cell line in vitro

In general, HAdV has an enhanced ability to infect human cell lines compared to mouse cell lines. To examine this in our system, A549 and 4T1 cells were infected at an MOI of 10 with CRAdFAST, crude protein samples were collected 48 hpi, and subjected to immunoblot for the relative levels of HAdV fiber and p14 FAST protein (Figure 13). Undiluted 4T1-infected cell sample was compared to a dilution series of the A549-infected cell sample, to get a more accurate estimation of the relative viral protein expression. As shown in Figure 13, the quantity of HAdV fiber protein in the 4T1 sample was between 1/100 and 1/400 that present in A549 cells, which would reflect the cumulative effect of differences in infection efficiency, genome replication and gene expression between the two cell lines. Interestingly, the ratio of p14 FAST protein present in the CRAdFAST-infected 4T1 cells compared to A549 cells appeared proportionately higher than that of fiber. Nevertheless, CRAdFAST can achieve higher levels of infection and gene expression in A549 cells compared to 4T1 cells.

To confirm that p14 FAST protein expression was tied to virus replication in human A549 cells, I again examined gene expression in the presence or absence of the DNA replication inhibitor Ara-C. A549 cells were infected at an MOI of 10 with CRAd, Ad-late/RFP or CRAdFAST, in the presence or absence of 20 $\mu\text{g/mL}$ Ara-C, and protein expression was examined 24 hr later. Ad-late/RFP is similar in structure to CRAdFAST, but contains a wildtype E1 region and a splice acceptor (SA)-RFP expression cassette instead of SA-p14 FAST within the E3 region [149]. As shown in Figure 14, treatment of cells with Ara-C abrogated expression of the late fiber protein for all of the viruses, and also inhibited

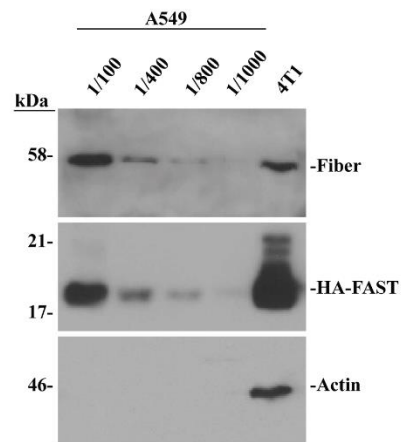


Figure 13. CRAdFAST expresses fiber and p14 FAST more efficiently in A549 cells than 4T1 cells. A549 and 4T1 cells were infected at an MOI of 10 and harvested at 48 hpi. A549 samples were diluted to 1/100, 1/400, 1/800 and 1/1000 to compare protein levels to that obtained in 4T1 cells. Immunoblots were probed with fiber and HA (p14 FAST), actin was used as a loading control.

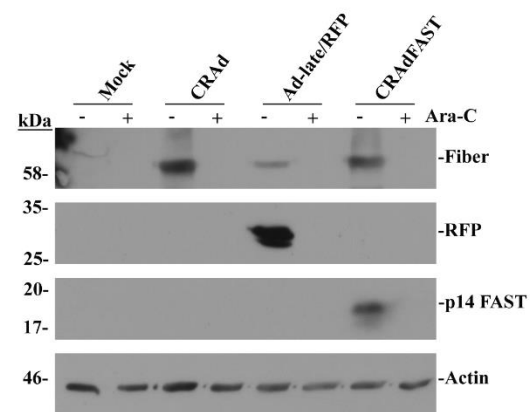


Figure 14. p14 FAST expression is replication dependent in A549 cells infected with CRAdFAST. A549 cells were infected at MOI 3 with CRAd, AdRFP or CRAdFAST, or mock infected, and overlayed with medium lacking (-) or containing (+) 20µg/mL of cytosine arabinoside (Ara-C) to inhibit viral DNA replication. Cells were harvested at 24 hpi and immunoblot analysis was performed to examine HAdV fiber, RFP and HA (p14 FAST) protein levels, actin was used as a loading control.

expression of RFP and p14 FAST from Ad-late/RFP and CRAdFAST, respectively. Thus, inclusion of an expression cassette consisting of a splice acceptor and transgene replacing the E3 region of a HAdV-5 vector allows for replication-dependent expression of the transgene in human A549 cells, but not the murine 4T1 cell line (Figure 8B).

CRAdFAST results in enhanced cancer cell killing in vitro in human A549 cells.

I next examined whether expression of p14 FAST protein from CRAdFAST led to fusion and cell death of A549 cells in culture. A549 cells were infected at MOI 1, 0.1 and 0.01 with AdFAST, CRAd and CRAdFAST, and phase contrast images were obtained at 24 and 48 hpi. No fusion was observed in AdFAST-infected cells (Figure 15), which is consistent with our previous studies showing that an MOI of ~50 of this replication-defective virus is required to achieve fusion in this cell line [80]. Only minor evidence of CPE was observed in CRAd-infected cells at the highest MOI (MOI=1) and at the latest time point (48 hpi) examined. In contrast, syncytia were observed in cells treated with CRAdFAST at an MOI as low as 0.1 and as early as 24 hpi. By 48 hpi, even an MOI as low as 0.01 yielded complete monolayer fusion, suggesting that CRAdFAST is very efficient at inducing fusion in A549 cells.

To determine if CRAdFAST-mediated cell fusion impacted A549 cell viability, A549 cells were infected with AdFAST, CRAd, or CRAdFAST, or mock infected with PBS, and metabolic activity was assayed at varying time post infection. Treatment of A549 cells with CRAdFAST caused a dramatic decrease in metabolic activity relative to cells treated with AdFAST or CRAd (Figure 16A). At 48 hpi, cells treated with CRAdFAST showed a metabolic activity of only 4% of untreated cells, which was also significantly lower than that observed for CRAd and AdFAST (117% and 118%, respectively, compared to 4% for CRAdFAST, $p < 0.0001$). By 72 hpi, metabolic activity of CRAdFAST-infected cells remained significantly

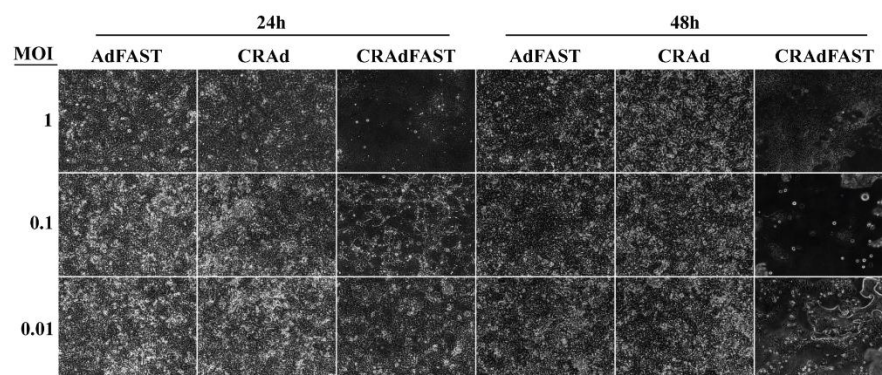
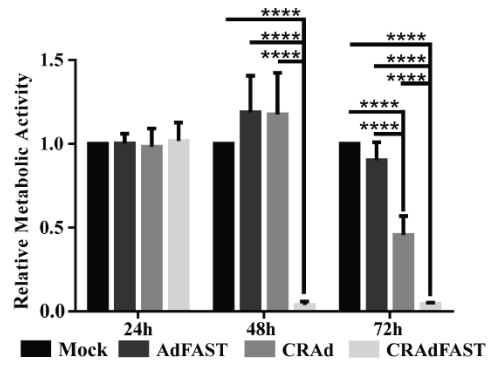


Figure 15. CRAdFAST infection leads to efficient monolayer destruction in A549 cells. Phase-contrast images taken at 24 and 48 hpi of A549 cells infected at an MOI of 0.01, 0.1 and 1 with AdFAST, CRAd or CRAdFAST.

A.



B.

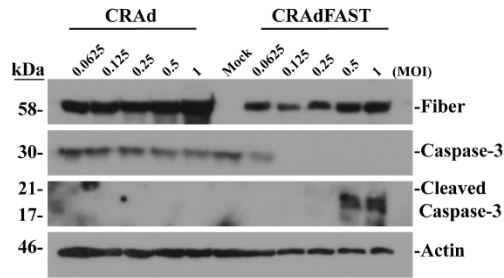


Figure 16. CRAdFAST reduces metabolic activity and leads to apoptosis of A549 cells. A) A549 cells were either mock-infected or infected with AdFAST, CRAd, or CRAdFAST at MOI 1 in triplicate (n=6 technical replicates, n=2 independent experimental replicates). At 24, 48 and 72 hpi, metabolic activity was assessed by MTS assay, values reported are relative to mock-infected cells, and represent mean $\pm$ SD (**** p<0.0001). B) A549 cells were either mock-infected or infected at an MOI of 0.0625, 0.125, 0.25, 0.5 and 1 with CRAd or CRAdFAST and harvested for immunoblot analysis at 48 hpi. Blots were probed for HAdV fiber, full length caspase-3 or cleaved caspase-3, and actin was used as a loading control.

lower than cells treated with the control viruses. To determine whether this decrease in metabolic activity was associated with cell death, treated cells were examined for the presence of cleaved caspase-3 by immunoblot (Figure 16B). A549 cells were either mock infected or infected with varying amounts of virus (MOI = 0.0625 to 1), and crude protein extracts were prepared 48 hr later. Infection of cells with CRAd did not lead to a decline in the cellular quantity of full length caspase 3, nor formation of the active cleavage product. In contrast, in cells treated with CRAdFAST, full-length caspase-3 is only detectable at the lowest MOI tested, suggesting processing of the protein to its active form, and cleaved caspase-3 is readily detectable at an MOI of 0.5 and higher. Taken together, these data indicate that expression of p14 FAST protein from a CRAd enhances the oncolytic potential of the virus, resulting in significant cell fusion, and a decline in cellular metabolic activity due to the induction of apoptosis-like cell death in A549 cells.

p14 FAST induces marked upregulation of extracellular vesicle production in CRAdFAST-infected cells

Previous work has shown that during syncytium formation in cancer cells mediated by GALV and measles fusion proteins, there is an enhanced release of extracellular vesicles (EV) (termed “syncytiosomes”) relative to normal cells [79, 187]. These vesicles are remarkably efficient at transferring their contents to dendritic cells, which then can functionally cross-present antigen to CD8⁺ T-cells for anti-tumor recognition. Thus, I explored whether CRAdFAST infection led to similar increases in syncytiosomes, which could potentially play a pro-oncolytic role by potentiating an anti-cancer adaptive immune response.

To determine whether fusion mediated by CRAdFAST causes enhanced release of syncytiosomes, A549 cells were infected with CRAd or CRAdFAST at an MOI of 3, or mock

infected with PBS, and supernatant was harvested 72 hours later. Extracellular particle concentration was determined using Zeta-View nanoparticle tracking. Infection of cells with CRAdFAST resulted in a roughly 10-fold increase in the quantity of extracellular vesicles present in the medium (Figure 17), but with similar size profiles to uninfected or CRAd infected vesicles (Figure 18). Exosome-sized particles were then further isolated by differential centrifugation and their protein contents were assayed by immunoblot (equal volumes of resuspended exosomes were applied to the gel). Figure 19 shows a clear and robust increase in typical exosome marker proteins Alix and Flotillin, as well as the presence of p14 FAST protein within these exosome-like particles. Thus, cells expressing p14 FAST in the context of a conditionally replicating HAdV release significantly more extracellular vesicles, which likely contain tumor antigen and may facilitate the development of an anti-tumor adaptive immune response.

CRAdFAST shows enhanced cancer cell killing in vivo in human A549 cells

As CRAdFAST showed significant efficacy as an oncolytic virus in A549 cells in culture, we tested its potency *in vivo* using an A549 xenograft model of cancer. CD1 nude mice bearing A549 tumors were injected with a single dose of either vehicle (PBS), or 1×10^9 PFU of CRAd or CRAdFAST, and treated tumors were measured by caliper three times weekly. A statistically significant decrease in tumor size was achieved with both CRAd (beginning at Day 27) and CRAdFAST (beginning at Day 24), relative to animals treated with vehicle alone (Figure 20A). Although CRAdFAST treated tumors trended toward a smaller size than those treated with CRAd, there was no statistically significant difference between these treatments by day 50 when mice were euthanized. However, tumors treated with

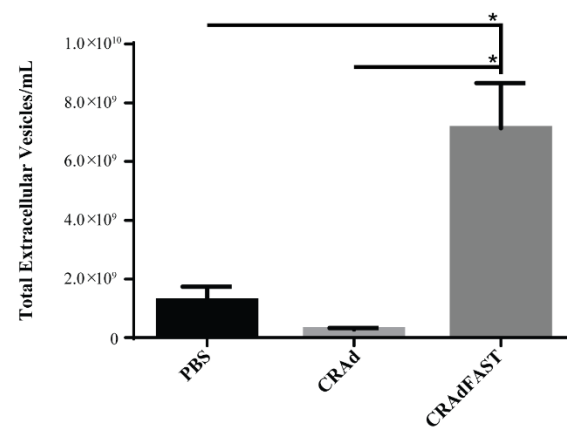


Figure 17. Expression of p14 FAST enhances extracellular vesicle release in CRAd infected A549 cells. A549 cells were either mock infected, infected with CRAd or CRAdFAST at MOI 3. At 72 hpi, extracellular vesicles were isolated from media by differential centrifugation. Extracellular vesicle concentrations were measured by Zeta-View nanoparticle tracking. Error bars represent standard deviation (**** $p < 0.0001$).

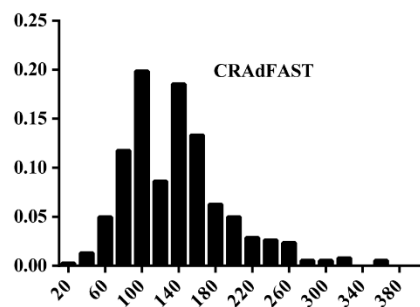
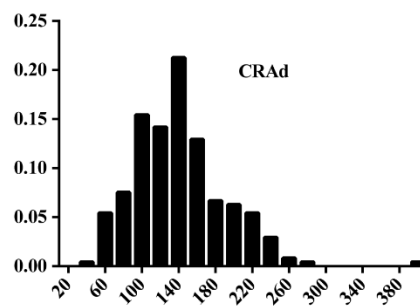
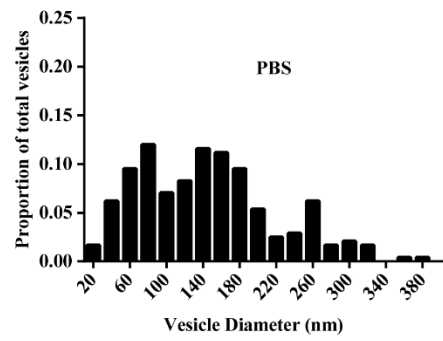


Figure 18. Vesicles from CRAdFAST infected cells have similar size distributions to mock and CRAd treated cells. A549 cells were either mock infected, infected with CRAd or CRAdFAST at MOI 3. At 72 hpi, extracellular vesicles were isolated from media by differential centrifugation. Extracellular vesicle size distributions were measured by Zeta-View nanoparticle tracking.

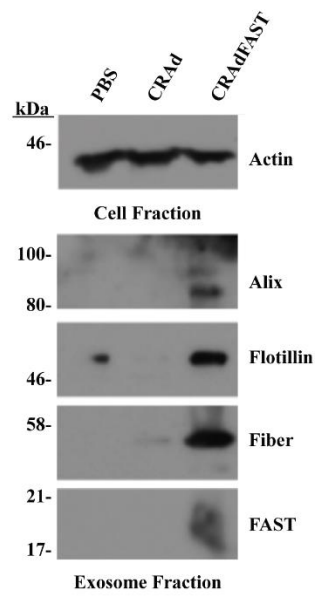
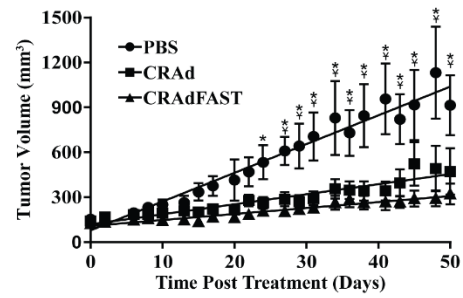


Figure 19. Vesicles from CRAdFAST infected cells contain exosome marker proteins. A549 cells were either mock infected, infected with CRAd or CRAdFAST at MOI 3. At 72 hpi, exosomes were isolated from media by differential centrifugation. Exosome pellets were harvested and probed by immunoblot for alix, flotillin, Ad fiber, and HA (p14 FAST). Cell lysates were probed for actin as a loading control.

A.



B.

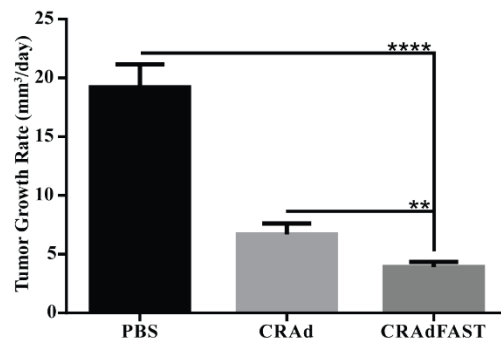


Figure 20. Expression of p14 FAST protein significantly improves oncolytic efficacy of CRAd following intratumoral injection in a human A549 xenograft model of cancer. Mice bearing A549 tumors were injected intratumorally with PBS, CRAd, or CRAdFAST. Tumor volumes were measured for five mice per group, three times weekly. A) Symbols represent calculated tumor volumes over time. Lines represent linear regression model of tumor growth rates. Beyond day 29, PBS treated tumors are significantly larger than both CRAd (¥) and CRAdFAST (*) treated tumors. Error bars represent standard deviation. B) Tumor growth rates were calculated for each tumor and averaged, error bars represents standard deviation (** $p=0.0013$, **** $p<0.0001$).

CRAdFAST showed a statistically significant reduction in growth rate (CRAdFAST = $3.9 \pm 0.45 \text{ mm}^3/\text{day}$) compared to tumors treated with either PBS ($19.2 \pm 1.95 \text{ mm}^3/\text{day}$, $p < 0.0001$) or CRAd ($6.7 \pm 0.92 \text{ mm}^3/\text{day}$, $p = 0.0013$) (Figure 20B). Thus, arming an oncolytic HAdV with p14 FAST enhanced the oncolytic efficacy of the vector *in vivo* in A549 tumors.

We next examined tumor sections histologically. Since vectors based on HAdV tend to accumulate in the liver, even following intratumoral injection [188], we also examined the livers from treated animals for signs of syncytia or other pathology. Tumor and liver tissue were harvested at 5 and 50 days post-treatment, processed, and stained with anti-HAdV antibody to detect viral gene expression and anti-HA antibody to detect expression of p14 FAST protein. Anti-HAdV antibody primarily detects structural (late) proteins which are only expressed after DNA replication has initiated, and is thus a surrogate marker for active virus replication [189]. At 5 days post-injection, both CRAd and CRAdFAST appear to have replicated efficiently in A549 tumors, as evidenced by the presence of viral late proteins, however the two viruses showed strikingly different patterns of spatial spread (Figure 21A). While CRAd-treated tumors appear to have intense HAdV staining localized to pockets in the interior of the tumor, sections from CRAdFAST-treated tumors show more diffuse staining spread throughout the tumor mass. As expected, immunohistochemistry for the HA-tagged p14 FAST protein was negative in CRAd treated tumors, and there was no evidence of multinucleated syncytia at either Day 5 or Day 50 (Figure 22). In contrast, in CRAdFAST-treated animals, expression of the HA-tagged p14 FAST protein was readily detected in tumors at Day 5 and, to a much lesser extent, also at Day 50 (Figure 22). Importantly, HA-positive areas of the tumor showed clear evidence of syncytium formation. Interestingly, although livers from mice injected with CRAdFAST stained positive with anti-HAdV at day 5 post vector injection, no anti-HAdV staining was observed for CRAd (Figure 23A). Staining of

Tumor - α Ad - Day 5

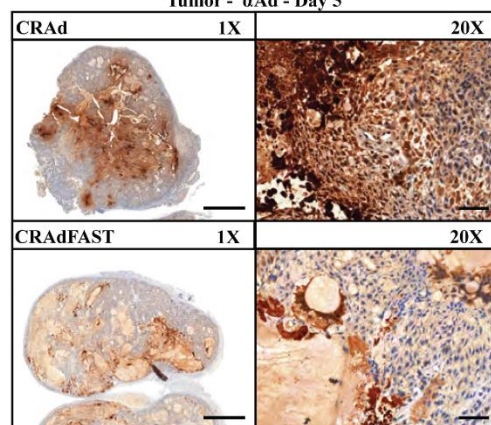


Figure 21. Intratumoral injection of CRAdFAST leads to improved spread relative to CRAd. Sections of A549 xenograft tumors from mice treated with CRAd or CRAdFAST and sacrificed at day 5 post-injection were probed with anti-HAdV-5 antibody and counter-stained with hematoxylin to assess HAdV replication. Images taken at 1x and 20x magnification.

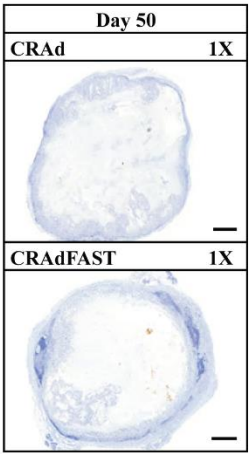
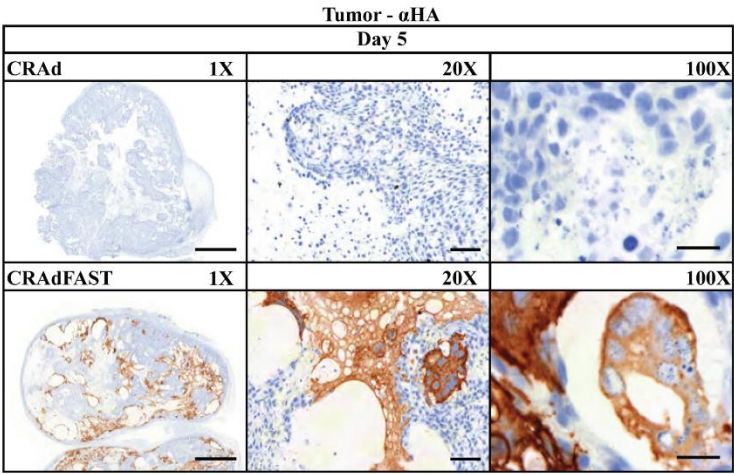
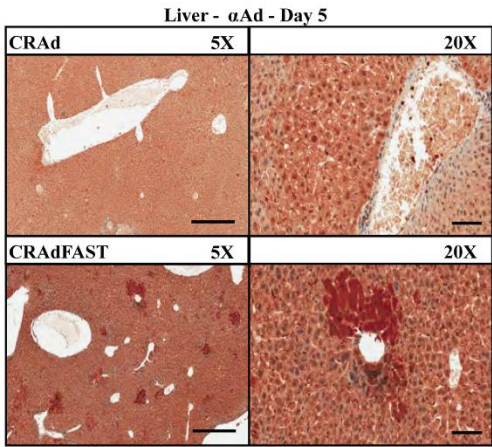


Figure 22. Treatment with CRAdFAST induces syncytium formation in A549 tumors in vivo. Tumor sections from mice treated with CRAd or CRAdFAST and sacrificed at day 5 and day 50 post-injection were probed with anti-HA (p14 FAST) antibody and counter-stained with hematoxylin to assess p14 FAST expression. Images taken at 1x, 20x and 100x magnification.

A.



B.

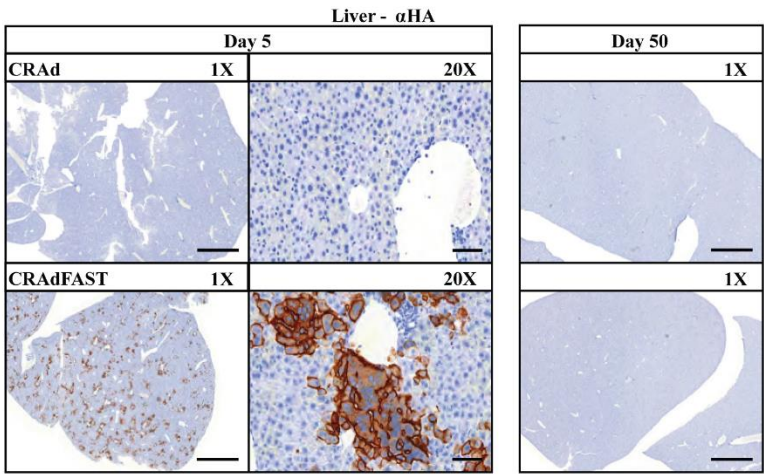


Figure 23. Intratumoral injection of CRAdFAST induces p14 FAST associated fusion in mouse livers. A) Liver sections from mice treated with CRAd or CRAdFAST and sacrificed at day 5 post-injection were probed with anti-HAdV-5 and counter-stained with hematoxylin to assess adenoviral replication. Images taken at 5x and 20x magnification. At 1x, 5x, 20x and 100x, scale bars represent 2000 μ m, 500 μ m, 100 μ m and 20 μ m, respectively. B) Liver sections from mice treated with CRAd or CRAdFAST and sacrificed at day 5 and day 50 post-injection were probed with anti-HA (p14 FAST) and counter-stained with hematoxylin to assess p14 FAST expression. Images taken at 1x and 20x magnification.

liver sections for the HA-tagged p14 FAST protein showed small regions of positive staining around blood vessels at day 5 (Figure 23B), accompanied by apparent syncytia formation, which was resolved by Day 50, suggesting this transient effect resolved without intervention and produced no obvious long-term effect on liver health.

Taken together, our results indicate that treatment of A549 cells with a CRAd vector expressing p14 FAST protein induces dramatic cell fusion, with a concomitant decline in cellular metabolic activity and induction of apoptosis, resulting in a statistically significant reduction in tumor growth rate *in vivo*, relative to CRAd alone.

4.3 Discussion

I have evaluated CRAdFAST in a human A549 xenograft model of cancer in immunodeficient mice. In this model, CRAd and CRAdFAST would be expected to replicate and spread to adjacent cells, but the effect of these vectors on antitumor immunity cannot be evaluated. In tissue culture, a very low quantity of CRAdFAST was required to achieve complete fusion of the cell monolayer, which was accompanied by a dramatic decline in cellular metabolic activity and induction of apoptotic-like cell death, as indicated by cleavage of caspase 3 (Figures 15 and 16). *In vivo*, treatment with CRAdFAST caused a statistically significant decline in tumor growth rate (Figure 20). ICOVIR16, a conditionally replicating HAdV expressing the GALV fusion protein induced a similar reduction in SkMel-28 tumor growth rates following a single intratumoral injection [88]. Indeed, inclusion of the GALV fusion protein in ICOVIR16 appeared to have a greater therapeutic effect than we observed for p14 FAST. It is possible that the efficiency with which HAdV can replicate and spread within A549 cells may mask some of the benefits induced by intratumoral syncytia formation in our experiments. Immunohistological examination of tumors treated with CRAd showed

intense staining primarily in the center regions of the tumor, whereas CRAdFAST showed less intense but more diffuse staining (Figure 21). In addition, CRAdFAST treated tumors had large acellular pockets bordered by regions of significant anti-HA (p14 FAST) staining (Figure 22). Multinucleated syncytia were also noted in CRAdFAST-treated A549 tumors. Nuclear fragmentation, which is a hallmark of apoptotic cell death, was evident in both CRAd and CRAdFAST treated tumors (Figure 22, 100X). Interestingly, not all fusogenic proteins induce apoptosis [78, 82], suggesting that different fusogenic proteins may induce alternate forms of cell death.

Since a large proportion of the initial injectate can escape the tumor and infect the liver [23, 188], we also examined pathology of the liver in treated animals. Interestingly, although the livers of all animals did not show any gross abnormal pathology at 5 days post treatment, we did observe significant anti-Ad staining primarily adjacent to blood vessels in the liver of CRAdFAST-treated animals, which was not present in animals treated with CRAd (Figure 23A). Similarly, anti-HA (p14 FAST) staining revealed formation of small syncytia in regions adjacent to blood vessels in the liver of CRAdFAST-treated animals (Figure 23B). The lack of anti-Ad staining in the liver of mice treated with CRAd suggests that p14 FAST conferred some property which allowed for accumulation of viral proteins in the liver of CRAdFAST-treated animals. p14 FAST protein may allow for enhanced replication of CRAd in mouse hepatocytes, although the mechanism by which this would occur is unclear. Cell fusion or syncytium formation within the CRAdFAST-infected tumor may allow for enhanced release of progeny virus into circulation, resulting in enhanced uptake of virus in the liver, which would not occur in CRAd-treated animals. In this scenario, the presence of HAdV proteins in the liver would be due to *de novo* gene expression in the liver, which may be possible without DNA replication given the aberrant p14 FAST expression seen in infected 4T1 cells in chapter

3 in the presence of Ara-C. However, the lack of anti-HA staining in the livers of mice bearing 4T1 tumors in the previous chapter points to an alternative mechanism. We and others have shown that expression of fusion proteins, including p14 FAST protein [98], can lead to enhanced release of exosome-like particles termed syncytiosomes [112, 187]. Syncytiosomes are hypothesized to have the capacity to transport tumor associated antigen outside of the tumor micro-environment. Thus, the HAdV and p14 FAST proteins present in the liver in CRAdFAST-treated animals may have been released from infected tumor cells in syncytiosomes which were subsequently taken up by the liver. Consistent with this scenario, we have observed enhanced release of exosome-like particles from CRAdFAST-infected cells (Figures 17, 18 and 19). Syncytiosomes are also extremely efficient at delivering their contents to dendritic cells, thereby enhancing tumor associated antigen cross-presentation and activation of T-cells [112, 187]. This latter point would obviously be extremely beneficial for the formation of anti-tumor immunity to help mediate tumor regression, thus improving CRAd efficacy in permissive and immunocompetent animal models of cancer and, ultimately, human patients.

Chapter 5: Adenovirus death protein (ADP) and p14 FAST synergize to improve oncolytic capacity of CRAd expressing p14 FAST *in vitro* but not *in vivo* in an HAdV semi-permissive mouse cell line.

5.1 Introduction

In the two previous chapters, I explored the oncolytic capacity of CRAdFAST, a CRAd expressing the p14 FAST protein that replicates and induces significant cell fusion in mouse 4T1 and human A549 cells. To maximize the potential of this virus, we developed a new vector which includes an additional oncolytic transgene, which is possible due to the small size of p14 FAST. Previous work has shown that a significant improvement in oncolytic efficacy can be achieved *in vivo* when ADP is over-expressed from CRAd, by improving nuclear lysis and progeny virion release [30, 138, 139, 190]. CRAdFAST, an E3-deleted vector, lacks the native copy of ADP. Since fusogenic HAdV do not spread within nuclei of a syncytium [141], we hypothesize that cellular fusion (mediated by p14 FAST protein) followed by nuclear lysis (mediated by ADP) may elicit a synergetic anti-cancer effect. An oncolytic CRAd expressing both FAST and ADP was constructed, designated CRAdFAST-ADP.

In previous experiments, mouse 4T1 cells proved to be fairly resistant to CRAd infection, while A549 cells could not be used in an immune-competent mouse, and thus did not accurately recapitulate clinical reality. CMT-64 cells appear significantly more susceptible to HAdV infection than other mouse cell lines [191, 192], and such cell lines are described as “semi-permissive” for HAdV replication. This cell line, a spontaneously occurring lung adenocarcinoma from C57BL/6CRF mice, forms solid tumors when injected subcutaneously, and shows metastatic potential [145]. I tested the efficacy of CRAdFAST and CRAdFAST-

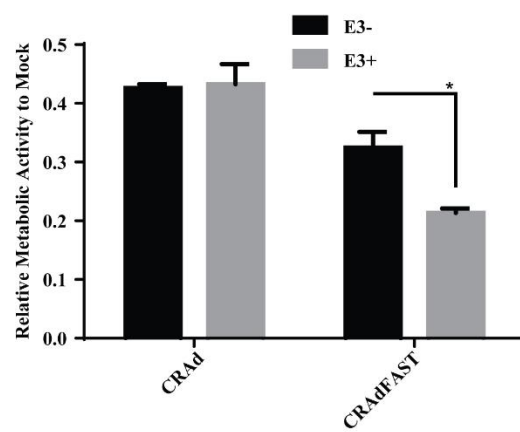
ADP in cell culture and *in vivo* using CMT-64 cells. I show that p14 FAST and ADP proteins function synergistically *in vitro* in CMT-64 cells, but do not provide an increased benefit *in vivo* compared to CRAdFAST following intratumoral injection.

5.2 Results

E3 and p14 FAST synergize to promote cell death in CRAd infected A549 cells

We first assessed whether p14 FAST and ADP (E3) could synergize in a simple co-infection experiment. A549 cells were simultaneously infected with an MOI of 5 of either AdRP3200 or AdRP3201 combined with an MOI of 0.1 of either CRAd or CRAdFAST, and metabolic activity of co-infected cells was assessed at 72 hours post-infection. AdRP3200 is an E1+ virus but is deleted of the E3 region, while AdRP3201 is identical to AdRP3200 but contains an intact E3 region. A low MOI was chosen for CRAdFAST as a higher MOI of this virus alone significantly reduces metabolic activity, which may mask any enhancing effect mediated by E3 proteins. We chose 72 hpi, since this is consistent with the known functional timeline of ADP activity [27]. When cells were infected with CRAd, the presence (AdRP3201) or absence (AdRP3200) of the E3 region did not significantly change metabolic activity of the infected cells (Figure 24A). However, for CRAdFAST, co-infection with the E3-containing AdRP3201 significantly reduced cellular metabolic activity compared to AdRP3200 (Figure 24A, $p=0.015$). To visually assess E3 and p14 FAST synergy, we examined the cells by phase-contrast microscopy at 72 hpi. As shown in figure 24B, there is a noticeable difference in fused monolayer integrity when CRAdFAST infected cells were co-infected with AdRP3201. Co-infection of CRAdFAST and AdRP3200 led to typical

A.



B.

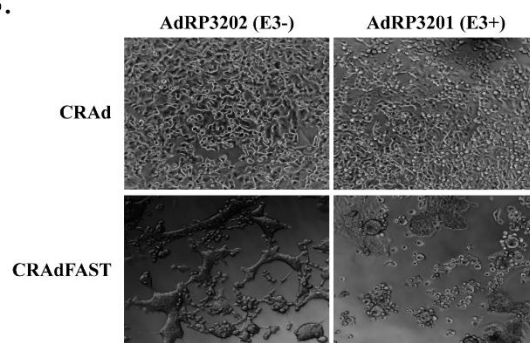


Figure 24. E3 and p14 FAST synergize to promote cell death in CRAd infected A549 cells. A549 cells were simultaneously infected with an MOI of 5 of either AdRP3200 or AdRP3201 combined with an MOI of 0.1 of either CRAd or CRAdFAST. A) Metabolic activity of co-infected cells was assessed at 72 hours post-infection by MTS assay. B) Cells were examined by phase-contrast microscopy at 72 hours post-infection.

syncytium formation seen in CRAdFAST infection. Interestingly, when co-infected with AdRP3201, and thus a functional E3 region, individual syncytia are smaller and lifted from the plate, suggesting that multiple mechanisms may be contributing to monolayer destruction. Taken together, these preliminary results suggest that the E3 region and p14 FAST protein may synergize to promote oncolysis. Since the immunomodulatory properties of the other E3 proteins are unlikely to have a function *in vitro*, it is likely that ADP is responsible for E3 and p14 FAST synergy.

To take advantage of this synergy, a CRAdFAST-like virus co-expressing p14 FAST and ADP was constructed [151]. This vector makes use of the P2A ribosomal skipping motif, which allows equi-molar expression of the gene proceeding and following a defined recognition amino acid sequence (GSGATNFSLLKQAGDVEENPGP) [152, 153]. To assess whether the newly designed CRAdFAST-ADP indeed supported co-expression of p14 FAST and ADP proteins, A549 cells were infected with an MOI of 1 of CRAdFAST and CRAdFAST-ADP, and harvested for western blot at 24 and 48 hpi. As shown in figure 25, CRAdFAST-ADP successfully expresses ADP. Interestingly, despite high structural similarity, CRAdFAST-ADP expresses significantly less p14 FAST than CRAdFAST. Also of note, p14 FAST from CRAdFAST-ADP appears as a larger band in western blot analyses. Since P2A is “cleaved” after the c-terminal glycine in the recognition motif, 21 additional amino acids of the motif are retained on the c-terminus of p14 FAST, giving rise to the observed size-shift of the p14 FAST protein produced from CRAdFAST-ADP [151, 152].

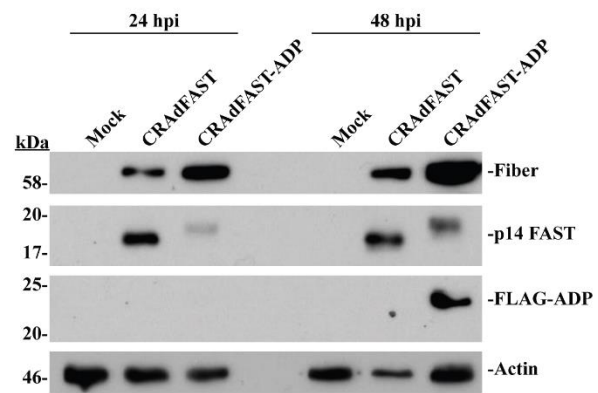


Figure 25. CRAdFAST-ADP co-expresses p14 FAST and HAdV ADP. A549 cells were either mock infected or infected with CRAdFAST or CRAdFAST-ADP at MOI 1. Immunoblot analysis was conducted on whole cell lysates harvested at 24 and 48 hpi. Samples were probed for fiber, HA (p14 FAST), and FLAG (ADP). Actin was used as a loading control. This figure was provided courtesy of Ryan Clarkin.

CMT-64.6 cells show enhanced susceptibility to CRAd and CRAdFAST infection versus the resistant 4T1 cell line

In chapter 3, it was evident that 4T1 cells lack the permissivity to HAdV infection of human cancer cells, which was a major barrier to investigation of CRAdFAST. In chapter 4, A549 cells highlighted the direct antitumor capacity of CRAdFAST and, although the virus replicated and induced significant intratumoral syncytial formation, we were unable to evaluate the contribution of an adaptive immune response in this model. Since the evidence presented by Bateman et al. suggests that syncytiosomes generated during CRAd induced fusion (Figure 17-19) have robust anti-tumor potential, I searched for a model that would allow the investigation of CRAdFAST and CRAdFAST-ADP antitumor activity in the presence of a functional mouse immune system [193]. Other groups have shown that CMT-64 cells are easily infected by HAdV and permit the production of progeny virions [191, 192]. There have been conflicting data presented regarding the capacity of this cell line to produce progeny virions [191, 194]. Halldén et al. showed that CMT-64 cells were fully permissive to HAdV replication, although the calculated burst size is unclear [191]. On the contrary, others showed a viral burst size of roughly 1 transducing unit per cell, indicating that this cell line is only semi-permissive to HAdV virion production [194]. Rincón et al. isolated a CMT-64 sub-clone (CMT-64.6) that allowed roughly five times greater ability of HAdV to internalize and give rise to GFP expression than the parental cell line [194]. For simplicity, I will refer to the process of HAdV attachment, internalization and initial gene expression as “infection”, since I measure the end result of this process and I did not perform experiments to identify details of any of these steps individually. After receiving the CMT64 and CMT64.6 cell lines as a kind gift from Dr. Ramon Alemany (IDIBELL, Barcelona, Spain), I first investigated whether I could recapitulate the above mentioned characteristics. To establish the relative susceptibility

of 4T1, CMT-64 and CMT-64.6 cells to HAdV infection, cells were infected with AdCA35, a non-replicating virus that expresses the β -Galactosidase (β Gal) gene from the mCMV promoter/enhancer at an MOI of 0.01 [155]. At 24 hpi, cells were fixed and stained with X-Gal in order to visualize instances where AdCA35 infection was successful. As shown in figure 26, AdCA35 successfully infected cells roughly 30 times more frequently in CMT-64 cells and 70 times more frequently in CMT-64.6 cells than in 4T1 cells, indicating that this cell line is more permissive to HAdV infection than either 4T1 or CMT-64 cells. CMT-64.6 cells were used in place of CMT-64 cells in all future experiments.

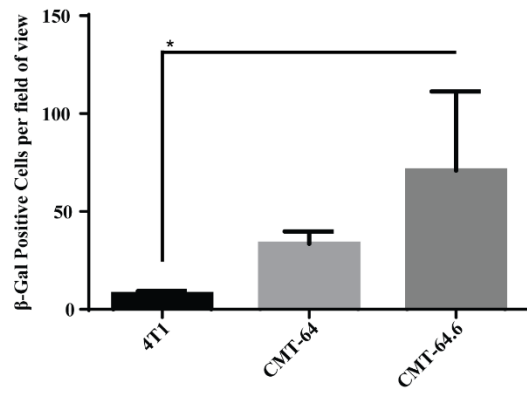


Figure 26. HAdV infectivity is significantly higher in CMT-64.6 cells than in 4T1 cells. 4T1, CMT-64 and CMT-64.6 cells were infected with AdCA35 at MOI 0.01. At 24hpi, cells were fixed and stained with X-Gal in order to visualize instances where AdCA35 successfully internalized and expressed the β Gal transgene.

To assess the capacity of CMT-64.6 cells to support late-gene and p14 FAST expression, CMT-64.6 cells were infected at an MOI of 10 with either AdFAST or CRAdFAST and harvested at 24, 48 and 72 hpi. As expected, no fiber protein signal was detectable in cells infected with the non-replicating AdFAST virus (Figure 27). Despite the improved infectivity of CMT-64.6 cells, the non-replicating AdFAST was also unable to generate detectable quantities of p14 FAST protein. In CRAdFAST-infected cells, fiber expression is detectable at 48 hpi and continues to increase by 72 hpi. Interestingly, p14 FAST protein expression is evident earlier, at 24 hpi, and continues to increase thereafter. Unlike in 4T1 cells (Figure 8A), p14 FAST expression remains high at late times in infection. Taken together, these data indicate that CMT-64.6 cells sustain expression of p14 FAST and CRAd fiber proteins.

To determine whether CMT-64.6 cells could support a productive CRAd infection, A549, 4T1 and CMT-64.6 cells were infected with CRAd at MOI 10, and virus yield was assayed at varying time post infection by plaque assay. We chose CRAd rather than CRAdFAST for this experiment to avoid confounding factors that may occur as a result of p14 FAST-induced syncytium formation. At 4, 24 and 72 hpi, cells and medium were collected, and infectious viral units were determined by plaque assay on 293 cells. As shown in figure 28, highly permissive A549 cells allowed an increase of viral infectious units of roughly 4 logs between 4 and 24 hours post infection. In 4T1 cells, no increase in infectious units was detected at 24 hpi, and a decrease in infectious units followed by 72 hpi. Interestingly, in CMT-64.6-infected cells, there was no drop in infectious viral units from 24 hpi to 72 hpi as was seen in 4T1 cells. It is possible that this

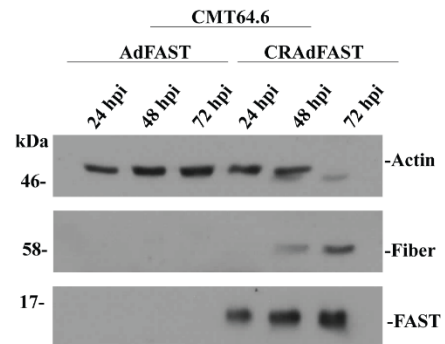


Figure 27. CMT-64.6 cells support CRAdFAST fiber and p14 FAST expression. CMT-64.6 cells were infected at MOI 10 with either AdFAST or CRAdFAST and harvested at 24, 48 and 72hpi. Cell lysates were subjected to immunoblot and probed for fiber and HA (p14 FAST). Actin was used as a loading control.

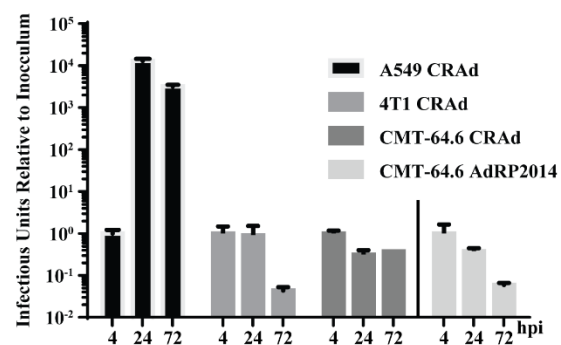
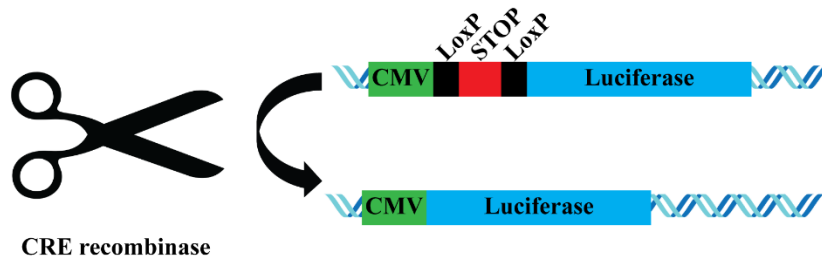


Figure 28. CRAd infected CMT-64.6 cells do not support a significant viral burst. A549, 4T1 and CMT-64.6 cells were infected with CRAd (CMT-64.6 cells were infected with RP2014 in a subsequent experiment) at MOI 10. Cells and medium were collected at 4, 24 and 72 hpi. Viral titers were determined by plaque assay on 293 cells.

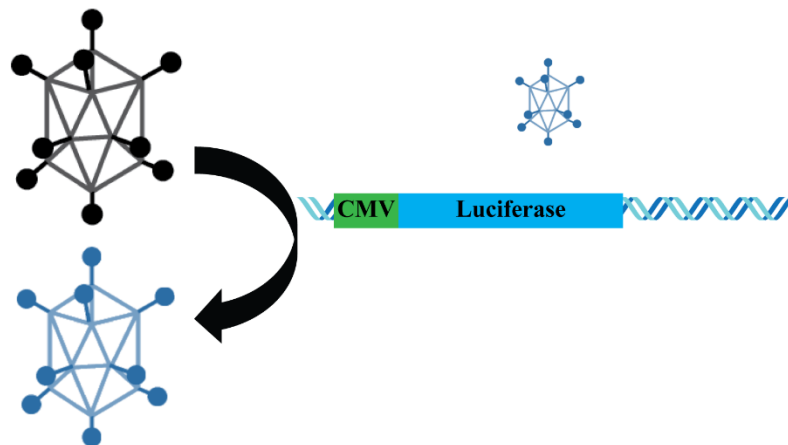
sustained number of infectious viral units is the result of the production of a small number of new infectious particles. To assess this possibility, the experiment was repeated and CMT-64.6 cells were now infected with AdRP2014, an E1-E3 deleted non-replicating HAdV vector. This virus is not expected to have the capacity to produce progeny virions, and thus functions as a control for virus that persists from the initial inoculum. CRAd infection in CMT-64.6 cells results in significantly higher infectious particle numbers at 72hpi than AdRP2014 infection, suggesting there may be new progeny CRAd virions produced in CRAd infected CMT64.4 cells, albeit at a greatly reduced frequency than from the A549 permissive cell line.

To conclusively determine if progeny HAdV virions were produced in the CMT64.6 cell line, we performed the following experiment. CMT64.6 cells were co-infected at an MOI of 10 with a combination of 3 viruses: (1) an E1-deleted virus expressing Cre (AdCre), (2) an E1-deleted virus expressing a Cre-activatable luciferase expression cassette (AdFloxLuc), and (3) CRAd. Infection with CRAd will allow for co-replication and packaging of the E1-deleted vectors. At 24hpi, the infected cells are collected into the medium, freeze/thawed to release any virions, and then used to infect naive, recipient A549 cells. If we detect significant luciferase activity in the recipient cells, it suggests that AdFloxLuc had been exposed to Cre in the triple infected cells (i.e. the DNA had been ‘unpackaged’), thus activating the luciferase expression cassette, and that the Cre-recombined DNA had been repackaged into infectious virions that allowed for luciferase expression in the recipient cells (Figure 29). If we do not observe luciferase activity in the recipient cells, it suggests the virus we observe at 24hpi was never “unpackaged” and may simply be residual virus from the initial infecting inoculum. Appropriate controls were also included to evaluate background luciferase expression from AdFlox-Luc in the

1. The protein product of AdCRE interacts with the AdFloxLuc genome to produce an AdLuc genome



2. Virion produced by CRAd is packaged with AdLuc genome



3. Progeny virions induce luciferase expression in infected A549 cells

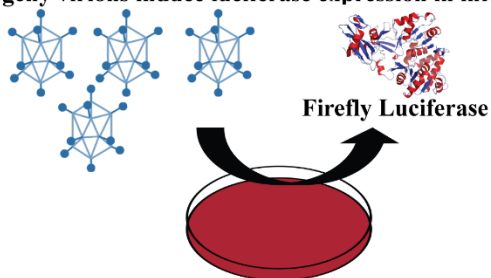


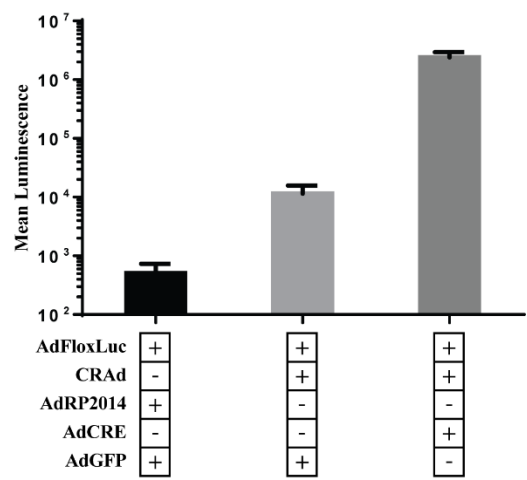
Figure 29. Experimental schematic. 1. The protein product of AdCRE interacts with the AdFloxLuc genome to produce an AdLuc genome. 2. Virion produced by CRAd is packaged with AdLuc genome. 3. Progeny virions induce luciferase expression in infected 549 cells.

presence and absence of CRAd (i.e. with and without DNA replication) and in the presence and absence of AdCre.

As seen in figure 30A, lysates from cells infected with a combination of AdFloxLuc, CRAd, and AdCRE expressed over 2 log greater luciferase than those from cells infected with AdFloxLuc, CRAd and AdGFP (no Cre expression), and over 4 log greater than cells infected with AdFloxLuc, AdRP2014 and AdGFP (no Cre expression, and no de novo virus DNA replication). Luciferase detected in the latter case is likely leaky expression from the floxed luciferase transcript which has been transferred into the secondary infection. An increase in this leaky expression is detected in the AdFloxLuc, CRAd and AdGFP infection which indicates CRAd replication is likely to be leading to repackaging of AdFloxLuc into novel virions, which undergo further leaky expression in the secondary, A549 cell infection. Finally, the 2 log increase seen following the replacement of AdCRE for AdGFP indicates that the gene product of AdCRE is acting upon the unpackaged AdFloxLuc genome to generate AdLuc, which is then re-packaged and freely infects the A549 cells in the secondary infection.

It is possible that, if sufficient quantity of the virus from the initial infection in CMT-64.6 was present and remained intact following the 24 hour infection, co-infection of AdCRE and AdFloxLuc could occur in the A549 recipient cell line, leading to activation of luciferase expression. To assess this possibility, cells and media collected from CMT-64.6 cells infected with AdFloxLuc, AdCRE and CRAd were serially diluted for infection on A549 cells. If the luciferase (luminescence) detected in the secondary A549 infection were the result of a single virus particle (as opposed to co-infection of two virus particles, AdCre and AdFloxLuc), a linear relationship between inoculum and luciferase expression (luminescence) should be detected. Conversely, if co-infection were taking place in this

A.



B.

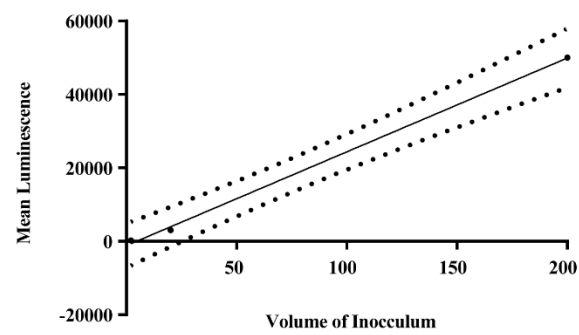


Figure 30. CRAd produces progeny virions upon infection of CMT-64.6 cells. CMT-64.6 cells were infected with AdFloxLuc, AdCRE (or AdGFP), and CRAd (or RP2014). Cells and media were harvested at 24hpi. A) 200 μ L of lysate was transferred to A549 cells for infection. 12 hours later, A549 cells were harvested, lysed, and assessed by luciferase assay for viral luciferase expression. B) A549 cells were infected with a dilution series of 2 μ L 20 μ L and 200 μ L of lysate from AdFloxLuc-AdCRE-CRAd infected cells. 12 hours later, A549 cells were harvested, lysed, and assessed by luciferase assay for viral luciferase expression. Dotted lines represent 90% confidence band of the best fit line. See text for more information.

follow-up infection (i.e. AdCRE and AdFloxLuc leftover from the initial infection were co-infecting A549 cells), a non-linear relationship between inoculum and luciferase expression (luminescence) should be observed, as the efficiency of co-infection would decline dramatically on serial dilution of the inoculum. As seen in figure 30B, the luminescence measured in A549 cells infected with 2, 20 and 200 μ L of inoculum takes on a linear relationship and does not deviate significantly from linearity by runs test ($p=1.000$), strongly suggesting that luciferase expression is dependent on single virus particles and not co-infection of two viruses in the A549 recipient cell line. Taken together, these results indicate that CMT-64.6 cells have increased susceptibility to CRAd attachment/internalization, protein expression and may support a low quantity of viral progeny production (burst size $\sim$ 1).

ADP expression improves oncolytic efficacy of CRAdFAST in vitro in mouse CMT-64.6 cells

To visually assess the effect of CRAdFAST-ADP *in vitro*, CMT-64.6 cells were either mock infected or infected at MOI 10 with CRAd, CRAdFAST or CRAdFAST-ADP, and phase-contrast images were taken at 72hpi. As shown in figure 31, although CRAdFAST induces profound monolayer fusion at this MOI, CRAdFAST-ADP results in significantly greater monolayer destruction, with fused cells having evidently lifted from the plate and only small patches of the monolayer remaining attached.

To assess whether CRAdFAST-ADP shows enhanced oncolytic capacity relative to CRAd or CRAdFAST in CMT-64.6 cells, CMT-64.6 cells were either mock infected or infected at MOI 10 with CRAd, CRAdFAST and CRAdFAST-ADP, and assessed for metabolic activity by MTS assay at 24, 48 and 72hpi. As shown in figure 32, treatment with

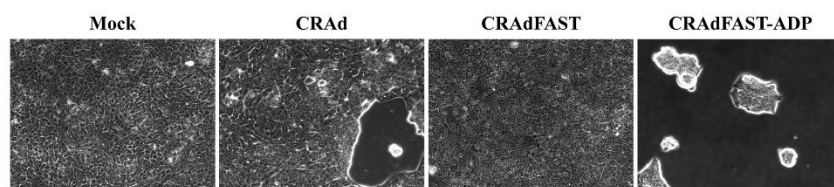


Figure 31. CRAdFAST-ADP induces monolayer destruction at low MOI. CMT-64.6 cells were either mock infected or infected at MOI 10 with CRAd, CRAdFAST or CRAdFAST-ADP, and phase-contrast images were taken at 72hpi.

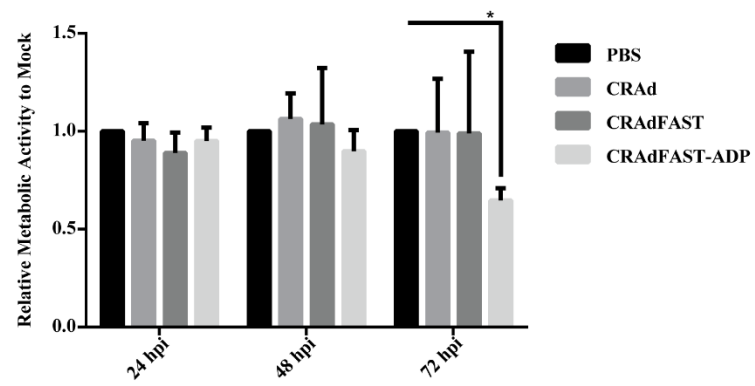


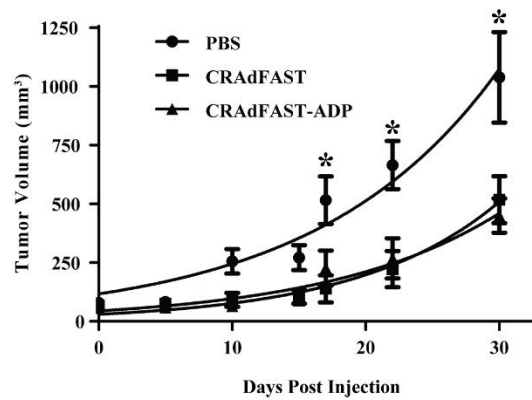
Figure 32. CRAdFAST-ADP reduces metabolic activity of infected CMT-64.6 cells. CMT-64.6 cells were either mock infected or infected at MOI 10 with CRAd, CRAdFAST and CRAdFAST-ADP, and assessed by MTS metabolic activity assay at 24, 48 and 72hpi

CRAAd or CRAAdFAST did not lead to a detectable reduction in metabolic activity. Importantly, at this low MOI in a mouse cell line, only CRAAdFAST-ADP infection led to a significant reduction in metabolic activity of CMT-64.6 cells by 72 hpi (Figure 32), consistent with our co-infection synergy experiment results in A549 cells (Figure 24). These results indicate that significant synergy exists *in vitro* between p14 FAST and ADP proteins in the context of expression from a CRAAd.

CRAAdFAST and CRAAdFAST-ADP delay tumor growth to a similar degree in an immune competent, semi-permissive mouse model of cancer

To assess whether ADP expression improves the oncolytic capacity of CRAAdFAST *in vivo*, CMT-64.6 tumors were established subcutaneously in C57BL/6J mice and injected intratumorally with PBS, 1×10^9 PFU of CRAAdFAST or CRAAdFAST-ADP in a total volume of 50 μ L. Tumors were measured by caliper three times weekly until day 30, when all mice were culled for humane reasons, as significant ulceration was apparent in all three groups. Calculated tumor volumes indicated a significantly smaller average tumor volume for both CRAAdFAST and CRAAdFAST-ADP from day 17 onward (Figure 33A). As shown in figure 33B, tumors excised at day 30 from both CRAAdFAST and CRAAdFAST-ADP treated mice appeared smaller in general than those from PBS treated mice. Despite lowered p14 FAST expression (Figure 25), CRAAdFAST-ADP was able to reduce tumor growth rate to a similar extent as CRAAdFAST in this immune-competent mouse model of cancer, suggesting that the presence of ADP may counteract the evident reduction in p14 FAST expression from this vector.

A.



B.

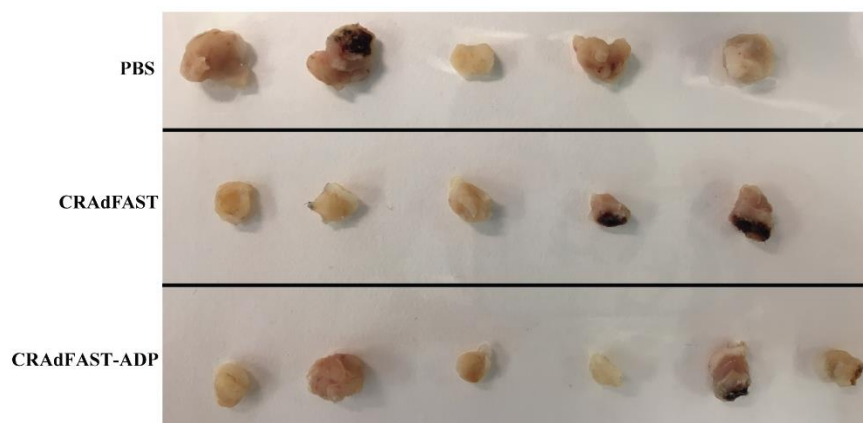


Figure 33. CRAdFAST delays tumor growth in an immune competent, semi-permissive mouse model of cancer, but ADP does not offer further benefit. CMT-64.6 tumors were established subcutaneously in C57BL/6J mice and injected intratumorally with PBS, 1×10^9 PFU of CRAdFAST or CRAdFAST-ADP in a total volume of 50 μ L. Tumors were measured by caliper three times weekly until day 30, when all mice were culled for humane purposes as significant ulceration was apparent in all three groups. A) Symbols represent calculated tumor volumes over time. Lines represent non-linear regression model of tumor growth rates. B) Tumors were removed from mice at day 30 for images of gross-morphology and size.

5.3 Discussion

Much is known about the HAdV E3 region. Many of the proteins encoded by this region protect the cell from the host immune system, such as inducing degradation of surface receptors and inhibition of MHC class I insertion into the cell membrane (to hide infected cells from the adaptive immune response) [130]. Of the proteins encoded by the E3 region, ADP stands out as being pro-lysis, but only at late times in infection. ADP inserts into the nuclear envelope, and induces nuclear lysis by an unknown mechanism [27, 195, 196]. Since HAdV virions are assembled in the nucleus, it is likely this aspect of ADP function that aids virus release. Historically, CRAds have been deleted of E3 to both make room for therapeutic/oncolytic transgenes and to increase the immunogenicity of infection. A number of groups have investigated whether re-insertion of ADP into a CRAd genome would provide an oncolytic benefit, as efficient lysis may improve spread of virus through an infected tumor [30, 138, 139, 190]. Indeed, these studies showed that ADP can improve the therapeutic effect of the vector. We hypothesized that syncytia formed by CRAdFAST may benefit from an improved virus-mediated lysis at late times post-infection. As was shown in figures 24 and 31, in our hands, ADP and p14 FAST appear to have synergistic roles in inducing cancer cell death. To my knowledge, this is the first time that ADP has been expressed from a syncytium-inducing virus. In his MSc thesis work, Ryan Clarkin showed that ADP did not enhance cell-cell spread of a CRAd encoding the p14 FAST protein, however, this vector expressed only low levels of p14 FAST protein, and was thus inefficient at inducing cell fusion [151]. The morphology visible in figure 24B and figure 31 suggests that p14 FAST-mediated cell fusion remains efficient in the presence of ADP, with a novel fusion phenotype which is potentially due to the lytic capacity of ADP.

Infection of CMT-64.6 cells at an MOI of 10 with CRAdFAST caused near-total monolayer fusion, while infection with CRAdFAST-ADP lead to profound CPE of the fused monolayer. Interestingly, at the same MOI, metabolic activity of CRAdFAST-infected cells was apparently not affected, while CRAdFAST-ADP induced a modest but significant reduction in metabolic activity at 72 hpi. It has been shown that syncytia remain metabolically active until late times post-infection, leading to apparent differences in visible CPE and loss of metabolic activity of the monolayer [76, 81]. Indeed, it is possible that CRAdFAST infected CMT-64.6 cells would need to be examined at later time-points to detect metabolic changes. In CRAdFAST-ADP infected cells, ADP may have begun to initiate nuclear lysis in the infected monolayer, and thus may lead to earlier metabolic perturbations.

In vivo, CMT-64.6 tumors in C57BL/6J mice grew very rapidly and induced significant ulceration, which led to an early end to the trial. Despite this early termination, there was ample time for viral replication, tumor cell destruction and the development of any potential antitumor adaptive immune response which could occur in this immunocompetent cancer model [96, 197]. As seen in figure 33, failure to stabilize tumor growth indicates that, if a successful adaptive immune response was formed, tumor growth outcompeted CD8⁺ T-cell-mediated tumor cell destruction. CMT-64 cells are known to have a down-regulation of the transporter of antigen processing-1 (TAP-1), which is common among many other cancer cell types [198-200]. Tap-1 protein is essential for transporting cytosolic antigen fragments into the ER, which are then bound by MHC I for presentation to and recognition by dendritic cells and cytotoxic lymphocytes [201]. For this reason, it is possible that the CMT-64.6 cells evaded an anti-tumor immune response regardless of CRAdFAST or CRAdFAST-ADP induced cellular lysis and syncytiosome release. In the future, developing a CRAdFAST-like virus

which can restore TAP-1 expression may provide an improvement in the oncolytic capacity of this virus *in vivo*.

Despite being more permissive to CRAd infection than 4T1 cells, CMT-64.6 cells remain a highly resistant cell line compared to human A549 adenocarcinoma cells. Currently, the evaluation of the oncolytic capacity of an HAdV-based virus in an immune competent mouse model represents a major barrier to the field. Interestingly, it is not known exactly why mouse cells are robustly resistant to HAdV infection. Although the early stages of infection are perturbed, (attachment, internalization), they do not appear to be the main barrier to progeny production [202]. Indeed, there appear to be complex perturbations to early gene expression levels and timing as well [185]. To overcome this barrier, exploration of alternative *in vivo* models of CRAd oncolysis is necessary. Syrian hamster models of cancer, which are more susceptible to CRAd infection than mouse cells, may allow for important insight into the immunological ramifications of oncolysis [203, 204]. Interestingly, HAdV replication in swine tissue appears to be highly efficient compared to other animals, indicating this large animal model may be useful for evaluating CRAd safety in late-stage preclinical analyses [202, 205]. CRAd-infected cell vaccines, a technique where cancer cells infected *ex vivo* are re-introduced to provide protection or to treat established tumors [206], may present a logistically simpler route of exploration of the immunological capacity of CRAds in mouse models, since we have the ability to achieve high MOI infection and significant virus-mediated destruction of mouse cells *in vitro*. Regardless, the continued exploration of fusogenic proteins to augment the direct antitumor activity and immunogenicity of oncolytic vectors remains promising.

Chapter 6: General Discussion

Reaching more tumor tissue: implications of improvements in viral spread

Over 50 clinical trials have been performed worldwide exploring the use of conditionally replicating HAdV in cancer treatment, 40 of which have an “open” status in the Gene Therapy Clinical Trials Worldwide database [19]. As a result of these studies, it is likely that a large quantity of clinical data regarding the safety and efficacy of these viruses will be made available in the coming years. 23 of these clinical trials involve the use of an arming transgene, most frequently GM-CSF, HSV-TK and hyaluronidase [19]. Interestingly, the latter, is an enzyme that catalyses the hydrolysis of hyaluronic acid, a major constituent of the extracellular matrix [207]. By increasing the permeability of tumor tissues, CRAds expressing hyaluronidase have an increased capacity to spread within the tumor and reach a greater proportion of the tumor mass. The problem with insufficient HAdV spread was first identified in non-replicating viruses designed for delivery of cytotoxic or immunogenic transgenes. As seen in a phase I study performed by Lang et al., a non-replicating virus expressing the p53 tumor suppressor transgene did not perfuse well within the injected tumor, and affected an area of roughly 5mm surrounding the perfusion catheter [62]. Later, studies done on conditionally replicating vectors showed similar restrictions, namely, minor areas of effect upon intratumoral delivery. In a phase 1 trial of DNX-2401, a $\Delta 24$ based CRAd, patient tumors were resected post-perfusion and IHC was performed [208]. Despite viral replication as noted by intratumoral expression of E1A and HAdV hexon proteins, the area of effect within these tumors was minimal. Despite this, and importantly, DNX-2401 induced intratumoral infiltration of CD8⁺ T-cells and lead to an overall improvement in 3 year survival. These data

suggest a vector with improved capacity to reach a greater proportion of an injected tumor could have substantial antitumor effect.

A variety of methods have been tested in pre-clinical studies to improve CRAd spread throughout an injected tumor. As discussed above, the use of hyaluronidase, or other extracellular matrix degrading proteins has been explored in some studies [29, 197]. One interesting technique involved the use of HAdV-3 penton dodecahedral particles (PtDd) which were shown in the native HAdV-3 virus to be released in high quantity from infected cells and induce epithelial junction opening [31]. An HAdV based CRAd expressing a secreted form of the PtDd junction opening domains following the HAdV fiber transcript and inserted via IRES was studied [31]. This CRAd controlled tumor growth to a greater extent than CRAd without a transgene, however, no evidence was provided for greater intratumoral spread of this vector. Other groups have over-expressed ADP to improve viral cell lysis and CRAd spread [30, 138, 139, 190]. A large-plaque phenotype and a boost in efficiency with which a CRAd can destroy a monolayer was observed in all studies. In one interesting experiment, replication competent HAdVs expressing the human sodium iodide symporter (hNIS) as a reporter gene were used to calculate the volume of infected tissue following serial stacking of 2D images [190]. Nude mice bearing human tumor xenografts were given contralateral injections of one HAdV lacking ADP and one expressing ADP. The presence of ADP lead to an increase of 2.5 fold in reporter gene expression volume in mouse tumor xenografts, suggesting that improved nuclear lysis can allow CRAds to spread to a greater extent within an injected tumor. Finally, as described above, hyaluronidase was used to arm a CRAd based on the E1A Δ 24 motif [29]. This virus was shown to have improved intratumoral spread based on IHC staining for HAdV proteins in tumor sections.

An improvement in vector spread due to the inclusion of fusogenic proteins has now been directly shown in two studies. First, an E1A Δ 24-based CRAd expressing the GaLV fusion protein showed statistically greater regions of CRAd spread within tumors, and the formation of small syncytia within tumors following systemic administration [88]. And next, in my work, high intratumoral p14 FAST expression led to robust cellular fusion and increased spread of the viral effect within injected tumors at day 5 (Figures 20 and 21). Indeed, these are likely the most striking images of intratumoral syncytia generated to date. But why is it so important to increase the spread of a CRAd within an infected tumor?

Without considering the immunological benefits of CRAd infection, one might ask why, if a tumor is not completely resectable, are incremental improvements in CRAd spread important? Indeed, if cells are left unaffected, their eventual regrowth is a significant concern. Similarities can be drawn between this concept and a surgical concept otherwise known as tumor debulking. Tumor debulking, typically done surgically, involves the removal of as much tumor material as possible, despite knowing that the cancer is advanced and not completely resectable [209]. Debulking is a first line treatment in many advanced cancers, and if optimal debulking is possible (a large proportion of the tumor is resected), there can be significantly greater outcomes for patients [210-212]. Aside from the obvious physiological benefit of having a reduced tumor burden in the body, a number of mechanisms by which tumor debulking offers benefit have been proposed, including improved access to tumor tissues for systemically administered chemotherapy, or the elimination of chemotherapy resistant clones from the cellular pool within the tumor[209, 211]. Indeed, it appears that for these reasons, a

CRAAd that can reach and destroy large sections of an infected tumor may offer distinct advantages in clinical use, especially when combined with chemotherapy.

Another distinct advantage of improving the volume of effect of an injected CRAAd comes from the now widely known concept of cellular heterogeneity within tumors [144, 213, 214]. Individual tumors frequently have distinct sub-populations of cells, with the possibility of distinct neoantigen pools and resistances to chemotherapeutic treatment [215]. For this reason, mounting an anti-tumor immune response against one particular tumor antigen may only allow for the development of a CTL attack against a minor proportion of the tumor population. By facilitating the spread of CRAAd-induced cell death through a tumor mass, and therefore theoretically through multiple sub-populations of tumor cells, it is possible that a more varied anti-tumor immune response is generated, leading to more efficient tumor clearance.

Finally, another identified barrier to cancer treatment are cancer stem cells. These somewhat controversial cells have been postulated to provide continuous self-renewal properties to tumors while evading current chemotherapeutic interventions through a senescence-like phenotype [216, 217]. Chemotherapeutic drugs target cells which are rapidly dividing, and thus these cells may evade destruction [217]. Furthermore, it may be that these cells are also resistant to CRAAd infection, as the typical methods for attenuating replication of the virus involve the inability to replicate in a non-dividing cell. Interestingly, there are no obvious mechanisms by which cancer stem cells would be resistant to fusion with

neighbouring cells. In this proposed model, the physical fusion and destruction of cancer stem cells would be possible regardless of their cell cycle state, and if combined with chemotherapy, longer-lasting tumor eradication may be possible.

Implications of poor mouse model quality in pre-clinical evaluation of oncolytic HAdV

In a large number of studies describing the use of novel engineered CRAds in an *in vivo* setting, a statement can be found which describes the fact that, since oncolytic HAdV does not replicate well in mouse cells, (1) a human tumor xenograft model was used, or (2) a slightly more permissive mouse cell line was used to generate tumors. Importantly, even in the case of mouse cell lines that permit some level of viral replication and transgene expression, the non-tumor tissues of the mouse are also highly resistant to CRAd infection, and thus, safety in the pre-clinical setting is almost impossible to ascertain [191]. Some studies have shown CRAd efficacy in otherwise highly aggressive tumor cell lines. For instance, Guo et al. showed 4T1 tumors treated intratumorally with a CRAd based on E1A Δ 24 and a non-replicating HAdV expressing TRAIL were significantly smaller than untreated counterparts [218]. In these instances, very little intratumoral CRAd replication was evident, and the CRAd induced effect may be mediated mostly by immunostimulatory transgenes. Indeed, a CRAd co-expressing IL-12 and decorin (a TGF- β inhibitor) at very high levels from two CMV promoter/enhancers was able to completely inhibit 4T1 growth following three intratumoral injections [219]. Significant immune cell infiltration was shown in treated tumors with some diffuse E1A staining but no late-protein was visible in the tissue sections, as assessed by IHC. It is possible that the immunological and direct anti-tumor effects of a CRAd must be assessed in different mouse models. Unfortunately, for a CRAd that requires replication and cellular

destruction for immunological activity (such as, theoretically, CRAdFAST and CRAdFAST-ADP), a suitable mouse model is unavailable.

In our hands, 4T1 cells were highly resistant to both HAdV infection and generation of progeny virions. Wong et al. showed that AdFAST, a non-replicating vector expressing p14 FAST protein from a CMV promoter/enhancer, was able to elicit fusion at very high MOI *in vitro*, but p14 FAST expression was undetectable in treated tumors *in vivo* [98]. CRAdFAST showed improved efficacy, and induced 4T1 cell fusion at 10x lower MOI compared to AdFAST (100 vs 1000), and a small amount of p14 FAST protein expression was detectable in tumors *in vivo* (Figures 9 and 12, respectively). However, relative to human A549 cells, in which the virus induced complete CPE in the monolayer at an MOI of 0.01 by 48hpi (an MOI 10,000x lower than required in 4T1, Figure 15) and robust formation of syncytia in tumors (Figure 22), the effect elicited in 4T1 mouse cells was insignificant. I next tested CMT-64.6 cells, as they have been reported to have higher infectivity and support productive CRAd replication. These cells were roughly 9x more susceptible to infection than 4T1 cells (Figure 26) and permitted CRAdFAST-induced fusion and CRAdFAST-ADP-induced monolayer destruction at 10x lower MOI (Figure 31). Despite these improvements, neither CRAdFAST nor CRAdFAST-ADP were able to halt CMT-64.6 tumor growth *in vivo*. Given that, regardless of their improved infectivity over 4T1 cells, CMT-64.6 cells require 1000x higher MOI to induce monolayer fusion than A549 cells, a reduced *in vivo* efficacy compared to A549 cells was likely.

Despite the fact that pre-clinical CRAd experimentation could potentially benefit immensely from an improved understanding of HAdV infection and progeny production

impairment in mouse cells, there is a lack of robust research in this topic. Seminal work on this subject was published in the 1980s [185, 220]. Blair et al. performed an investigation of HAdV viral DNA and RNA expression conducted in mouse cells compared to human cells [185]. E1A mRNA levels were shown to be relatively unperturbed, but E1B mRNA was almost undetectable in HAdV infected mouse cells compared to human cells. Hexon protein levels were detectable but low in two mouse cell lines tested, but hexon mRNA was not determined, and so whether late gene expression or translation was effected was not established. Evidence for a block of late gene expression and translation, without a block of DNA replication, has been generated for HAdV and HAdV-II infection of mouse cell lines [220, 221]. Our results coincide with the results of these early works. I have shown that the mouse cell lines tested are less infectable by HAdV (Figure 26) and have lower late protein expression following CRAd infection (Figure 13) with successful genome replication (Figure 7) but a failure to produce progeny virions in significant quantities (Figure 28). The unexpected disruption of replication-dependent expression of p14 FAST following CRAdFAST infection of 4T1 cells, and possibly CMT64.6 cells, may point to a mechanism by which these late gene expression defects are founded (Figure 8). It is possible that promoter utilization and/or failure of splicing recognition leads to the significant reduction in late structural protein accumulation, and finally in the failure of progeny virion assembly.

Ultimately, alternative models for the pre-clinical examination of CRAd efficacy and safety are likely necessary, of which, Syrian hamsters, are likely the cheapest and most widely available. Syrian hamsters are naturally susceptible to infection by HAdV, and there are a number of cancer cell lines available for these animals [222]. Indeed, CRAd vector efficacy and safety have both been tested in Syrian hamster models, which, importantly, resulted in

clinical translation of the experimental CRAd on at least one occasion [88, 223]. These animals cost roughly twice as much as most immune competent mice, and less than half of athymic mice (Charles River Laboratories), and thus are a suitable replacement for most CRAd vector early preclinical analyses. For more in-depth preclinical mechanistic assessments of oncolytic CRAd, however, the Syrian hamster model lacks a diverse array of readily available transgenic animals. For an improved, patient-centric model that may more closely resemble the clinical situation, mice with humanized immune systems have been developed which can host human tumors [224]. Importantly, patient derived xenografts from human tumor explants can be used to generate tumors in these mice [225]. Unfortunately, due in part to the extreme complexity involved in recreating a suitable humanized immune system in mice, there are significant financial barriers for their use in oncolytic virus research as they cost roughly 10x more than an equivalent immune-competent mouse (Charles River Laboratories). Finally, in recent years, porcine models of cancer have received more attention. Currently, there are a number of engineered pig models of spontaneously arising cancers under development [226]. Further, there is at least one transplantable cancer model developed for pigs, however, these remain to be explored in depth [205]. Since HAdV replicates well in pig tissues, these models may have an excellent capacity to study CRAd safety in late, pre-clinical investigations [202, 227].

Conclusions

I have shown that, in the context of expression from a conditionally replicating HAdV, p14 FAST improves oncolytic capacity in human and mouse tumor cells through improved efficiency and intratumoral spread. I have also shown that p14 FAST has the capacity to synergize with ADP to further improve tumor cell killing. Despite this, there still exist major barriers to the translation of pre-clinical investigations of CRAds. We were unable to

thoroughly assess the immunological consequences of robust tumor cell fusion or the true safety profiles of either CRAdFAST or CRAdFAST-ADP due to the non-permissive nature of mouse cells to HAdV infection. Future studies should be done in novel models such as Syrian hamsters and humanized mice in order to accurately assess the immunological contribution to and safety of CRAd antitumor effect. By identifying and utilizing novel, improved pre-clinical models of cancer, significantly greater correlations between pre-clinical and clinical outcomes will be achieved, resulting in a more efficient transition from bench to bedside, and ultimately, improved patient outcomes.

References

1. Torre, L.A., et al., *Global cancer statistics, 2012*. CA: a cancer journal for clinicians, 2015. **65**(2): p. 87-108.
2. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries*. CA: a cancer journal for clinicians, 2018. **68**(6): p. 394-424.
3. Schwab, M., *Encyclopedia of cancer*. 2008: Springer Science & Business Media.
4. Green, D.M., et al., *Relevance of historical therapeutic approaches to the contemporary treatment of pediatric solid tumors*. Pediatric blood & cancer, 2013. **60**(7): p. 1083-1094.
5. García-Cao, I., et al., 'Super p53' mice exhibit enhanced DNA damage response, are tumor resistant and age normally. The EMBO journal, 2002. **21**(22): p. 6225-6235.
6. Gottesman, M.M., *Mechanisms of cancer drug resistance*. Annual review of medicine, 2002. **53**(1): p. 615-627.
7. Bauerschmitz, G.J., S.D. Barker, and A. Hemminki, *Adenoviral gene therapy for cancer: from vectors to targeted and replication competent agents (review)*. International journal of oncology, 2002. **21**(6): p. 1161-1174.
8. Heise, C. and D.H. Kim, *Replication-selective adenoviruses as oncolytic agents*. The Journal of clinical investigation, 2000. **105**(7): p. 847-851.
9. Kim, D.H., et al., *Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus*. PLoS Med, 2007. **4**(12): p. e353.
10. Senzer, N.N., et al., *Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor–encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma*. Journal of Clinical Oncology, 2009. **27**(34): p. 5763-5771.
11. Lichty, B.D., et al., *Vesicular stomatitis virus: re-inventing the bullet*. Trends in molecular medicine, 2004. **10**(5): p. 210-216.
12. Khuri, F.R., et al., *A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer*. Nature medicine, 2000. **6**(8): p. 879-885.
13. Greig, S.L., *Talimogene laherparepvec: first global approval*. Drugs, 2016. **76**(1): p. 147-154.
14. Peng, Z., *Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers*. Human gene therapy, 2005. **16**(9): p. 1016-1027.
15. Xia, Z., et al., *Phase III randomized clinical trial of intratumoral injection of E1B gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus*. Chinese journal of cancer, 2004. **23**(12): p. 1666-70.
16. Lu, W., et al., *Intra-tumor injection of H101, a recombinant adenovirus, in combination with chemotherapy in patients with advanced cancers: a pilot phase II clinical trial*. World J Gastroenterol, 2004. **10**(24): p. 3634-3638.
17. Zhang, W.-W., et al., *The first approved gene therapy product for cancer Ad-p53 (Gendicine): 12 years in the clinic*. Human gene therapy, 2018. **29**(2): p. 160-179.
18. Liang, M., *Oncorine, the world first oncolytic virus medicine and its update in China*. Current cancer drug targets, 2018. **18**(2): p. 171-176.

19. Edelstein, M. *Gene Therapy Clinical Trials Worldwide*. 2018 [cited 2019 June]; Available from: <http://www.abedia.com/wiley/>.
20. DeWeese, T.L., et al., *A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy*. *Cancer research*, 2001. **61**(20): p. 7464-7472.
21. Vecil, G.G. and F.F. Lang, *Clinical trials of adenoviruses in brain tumors: a review of Ad-p53 and oncolytic adenoviruses*. *Journal of neuro-oncology*, 2003. **65**(3): p. 237-246.
22. Sauthoff, H., et al., *Intratumoral spread of wild-type adenovirus is limited after local injection of human xenograft tumors: virus persists and spreads systemically at late time points*. *Hum Gene Ther*, 2003. **14**(5): p. 425-33.
23. Bernt, K.M., et al., *The effect of sequestration by nontarget tissues on anti-tumor efficacy of systemically applied, conditionally replicating adenovirus vectors*. *Molecular Therapy*, 2003. **8**(5): p. 746-755.
24. Ross, P.J. and R.J. Parks, *Oncolytic adenovirus: getting there is half the battle*. *Molecular Therapy*, 2003. **8**(5): p. 705-706.
25. Heise, C.C., et al., *Efficacy of a replication-competent adenovirus (ONYX-015) following intratumoral injection: intratumoral spread and distribution effects*. *Cancer gene therapy*, 1999. **6**(6): p. 499-05.
26. Wang, Y., et al., *Systemic dissemination of viral vectors during intratumoral injection*. *Molecular Cancer Therapeutics*, 2003. **2**(11): p. 1233-1242.
27. Tollefson, A.E., et al., *The adenovirus death protein (E3-11.6 K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells*. *Journal of virology*, 1996. **70**(4): p. 2296-2306.
28. Harrison, D., et al., *Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved-deletion of the viral E1b-19-kD gene increases the viral oncolytic effect*. *Human gene therapy*, 2001. **12**(10): p. 1323-1332.
29. Guedan, S., et al., *Hyaluronidase expression by an oncolytic adenovirus enhances its intratumoral spread and suppresses tumor growth*. *Mol Ther*, 2010. **18**(7): p. 1275-83.
30. Toth, K., et al., *An oncolytic adenovirus vector combining enhanced cell-to-cell spreading, mediated by the ADP cytolytic protein, with selective replication in cancer cells with deregulated wnt signaling*. *Cancer Res*, 2004. **64**(10): p. 3638-44.
31. Yumul, R., et al., *Epithelial Junction Opener Improves Oncolytic Adenovirus Therapy in Mouse Tumor Models*. *Hum Gene Ther*, 2016. **27**(4): p. 325-37.
32. Dmitriev, I., et al., *An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism*. *Journal of virology*, 1998. **72**(12): p. 9706-9713.
33. Li, Y., et al., *Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy*. *Cancer research*, 1999. **59**(2): p. 325-330.
34. Vigne, E., et al., *RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection*. *Journal of virology*, 1999. **73**(6): p. 5156-5161.
35. Poulin, K.L., et al., *Retargeting of adenovirus vectors through genetic fusion of a single-chain or single-domain antibody to capsid protein IX*. *Journal of virology*, 2010. **84**(19): p. 10074-10086.

36. Poulin, K.L., et al., *Use of Cre/loxP recombination to swap cell binding motifs on the adenoviral capsid protein IX*. Virology, 2011. **420**(2): p. 146-155.
37. Nwanegbo, E., et al., *Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States*. Clinical and diagnostic laboratory immunology, 2004. **11**(2): p. 351-357.
38. Mast, T.C., et al., *International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials*. Vaccine, 2010. **28**(4): p. 950-957.
39. Tsai, V., et al., *Impact of human neutralizing antibodies on antitumor efficacy of an oncolytic adenovirus in a murine model*. Clinical cancer research, 2004. **10**(21): p. 7199-7206.
40. Joyce, J.A. and D.T. Fearon, *T cell exclusion, immune privilege, and the tumor microenvironment*. Science, 2015. **348**(6230): p. 74-80.
41. Binder, A.M., et al., *Human adenovirus surveillance—United States, 2003–2016*. MMWR. Morbidity and mortality weekly report, 2017. **66**(39): p. 1039.
42. Kojaoghlanian, T., P. Flomenberg, and M.S. Horwitz, *The impact of adenovirus infection on the immunocompromised host*. Reviews in medical virology, 2003. **13**(3): p. 155-171.
43. Martin, M., et al., *Adenoviridae: the viruses and their replication*. Fields' Virology, 2007. **2**: p. 2355-2395.
44. Giberson, A.N., A.R. Davidson, and R.J. Parks, *Chromatin structure of adenovirus DNA throughout infection*. Nucleic acids research, 2012. **40**(6): p. 2369-2376.
45. Davison, A.J., M. Benkő, and B. Harrach, *Genetic content and evolution of adenoviruses*. Journal of general virology, 2003. **84**(11): p. 2895-2908.
46. Tollefson, A.E., et al., *The E3-11.6-kDa Adenovirus Death Protein (ADP) Is Required for Efficient Cell Death: Characterization of Cells Infected with ADP Mutants*. Virology, 1996. **220**(1): p. 152-162.
47. Akusjärvi, G. and H. Persson, *Controls of RNA splicing and termination in the major late adenovirus transcription unit*. Nature, 1981. **292**(5822): p. 420.
48. Smith, A.C., K.L. Poulin, and R.J. Parks, *DNA genome size affects the stability of the adenovirus virion*. Journal of virology, 2009. **83**(4): p. 2025-2028.
49. Christensen, J.B., et al., *Presence of the adenovirus IVa2 protein at a single vertex of the mature virion*. Journal of virology, 2008. **82**(18): p. 9086-9093.
50. O'Malley, R.P., et al., *A mechanism for the control of protein synthesis by adenovirus VA RNA I*. Cell, 1986. **44**(3): p. 391-400.
51. Aparicio, O., et al., *Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production*. Journal of virology, 2006. **80**(3): p. 1376-1384.
52. Saha, B., C.M. Wong, and R.J. Parks, *The adenovirus genome contributes to the structural stability of the virion*. viruses, 2014. **6**(9): p. 3563-3583.
53. Nelson, J.E. and M.A. Kay, *Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication*. Journal of virology, 1997. **71**(11): p. 8902-8907.
54. Wolf, J.K., et al., *A phase I study of Adp53 (INGN 201; ADVEXIN) for patients with platinum-and paclitaxel-resistant epithelial ovarian cancer*. Gynecologic oncology, 2004. **94**(2): p. 442-448.

55. Miller, G., S. Lahrs, and R.P. DeMatteo, *Overexpression of interleukin-12 enables dendritic cells to activate NK cells and confer systemic antitumor immunity*. The FASEB Journal, 2003. **17**(6): p. 728-730.
56. Rodriguez, R., et al., *Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells*. Cancer Research, 1997. **57**(13): p. 2559-2563.
57. Ding, M., et al., *Prostate cancer-specific and potent antitumor effect of a DD3-controlled oncolytic virus harboring the PTEN gene*. PLoS One, 2012. **7**(4): p. e35153.
58. Oh, E., et al., *A hypoxia-and telomerase-responsive oncolytic adenovirus expressing secretable trimeric TRAIL triggers tumour-specific apoptosis and promotes viral dispersion in TRAIL-resistant glioblastoma*. Scientific reports, 2018. **8**(1): p. 1420.
59. Bandara, L.R. and N.B. La Thangue, *Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor*. Nature 1991. **351**: p. 494-497.
60. Jiang, H., et al., *Downmodulation of El A Protein Expression as a Novel Strategy to Design Cancer-Selective Adenoviruses*. Neoplasia, 2005. **7**(8): p. 723-729.
61. Nevins, J.R., *The Rb/E2F pathway and cancer*. Human molecular genetics, 2001. **10**(7): p. 699-703.
62. Lang, F.F., et al., *Phase I trial of adenovirus-mediated p53 gene therapy for recurrent glioma: biological and clinical results*. Journal of Clinical Oncology, 2003. **21**(13): p. 2508-2518.
63. Smith, E., J. Breznik, and B.D. Lichty, *Strategies to enhance viral penetration of solid tumors*. Human gene therapy, 2011. **22**(9): p. 1053-1060.
64. Vedrenne, C. and H.P. Hauri, *Morphogenesis of the endoplasmic reticulum: beyond active membrane expansion*. Traffic, 2006. **7**(6): p. 639-646.
65. Chernomordik, L.V. and M.M. Kozlov, *Protein-lipid interplay in fusion and fission of biological membranes*. Annual review of biochemistry, 2003. **72**(1): p. 175-207.
66. Jahn, R., T. Lang, and T.C. Südhof, *Membrane fusion*. Cell, 2003. **112**(4): p. 519-533.
67. Cohen, F. and G. Melikyan, *The energetics of membrane fusion from binding, through hemifusion, pore formation, and pore enlargement*. The Journal of membrane biology, 2004. **199**(1): p. 1-14.
68. Jahn, R. and R.H. Scheller, *SNAREs—engines for membrane fusion*. Nature reviews Molecular cell biology, 2006. **7**(9): p. 631-643.
69. Eitzen, G., *Actin remodeling to facilitate membrane fusion*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2003. **1641**(2): p. 175-181.
70. Weissenhorn, W., et al., *Structural basis for membrane fusion by enveloped viruses*. Molecular membrane biology, 1999. **16**(1): p. 3-9.
71. Eckert, D.M. and P.S. Kim, *Mechanisms of viral membrane fusion and its inhibition*. Annual review of biochemistry, 2001. **70**(1): p. 777-810.
72. Epand, R.M., *Fusion peptides and the mechanism of viral fusion*. Biochimica et Biophysica Acta (BBA)-Biomembranes, 2003. **1614**(1): p. 116-121.
73. Durell, S.R., et al., *What studies of fusion peptides tell us about viral envelope glycoprotein-mediated membrane fusion (review)*. Molecular membrane biology, 1997. **14**(3): p. 97-112.
74. White, J.M., *Viral and cellular membrane fusion proteins*. Annual review of physiology, 1990. **52**(1): p. 675-697.

75. Laliberte, J.P., A.S. Weisberg, and B. Moss, *The membrane fusion step of vaccinia virus entry is cooperatively mediated by multiple viral proteins and host cell components*. PLoS Pathog, 2011. **7**(12): p. e1002446.
76. Higuchi, H., et al., *Viral fusogenic membrane glycoprotein expression causes syncytia formation with bioenergetic cell death: implications for gene therapy*. Cancer research, 2000. **60**(22): p. 6396-6402.
77. Chernomordik, L.V. and M.M. Kozlov, *Mechanics of membrane fusion*. Nature structural & molecular biology, 2008. **15**(7): p. 675-683.
78. Bateman, A., et al., *Fusogenic membrane glycoproteins as a novel class of genes for the local and immune-mediated control of tumor growth*. Cancer Res, 2000. **60**(6): p. 1492-7.
79. Bateman, A., et al., *Viral fusogenic membrane glycoproteins kill solid tumor cells by nonapoptotic mechanisms that promote cross presentation of tumor antigens by dendritic cells*. Cancer Res, 2002. **62**(22): p. 6566-78.
80. Wong, C.M., et al., *Adenovirus-Mediated Expression of the p14 Fusion-Associated Small Transmembrane Protein Promotes Cancer Cell Fusion and Apoptosis In Vitro but Does Not Provide Therapeutic Efficacy in a Xenograft Mouse Model of Cancer*. PLoS One, 2016. **11**(3): p. e0151516.
81. Salsman, J., et al., *Extensive syncytium formation mediated by the reovirus FAST proteins triggers apoptosis-induced membrane instability*. J Virol, 2005. **79**(13): p. 8090-100.
82. Tan, L., et al., *Inhibition of NF- κ B in fusogenic membrane glycoprotein causing HL-60 cell death: Implications for acute myeloid leukemia*. Cancer letters, 2009. **273**(1): p. 114-121.
83. Allen, C., et al., *Adenoviral vectors expressing fusogenic membrane glycoproteins activated via matrix metalloproteinase cleavable linkers have significant antitumor potential in the gene therapy of gliomas*. J Gene Med, 2004. **6**(11): p. 1216-27.
84. Brade, A.M., et al., *Heat-directed tumor cell fusion*. Hum Gene Ther, 2003. **14**(5): p. 447-61.
85. Chen, H.H., et al., *Active adenoviral vascular penetration by targeted formation of heterocellular endothelial-epithelial syncytia*. Mol Ther, 2011. **19**(1): p. 67-75.
86. Ahmed, A., et al., *Intratumoral expression of a fusogenic membrane glycoprotein enhances the efficacy of replicating adenovirus therapy*. Gene Ther, 2003. **10**(19): p. 1663-71.
87. Guedan, S., et al., *Syncytia formation affects the yield and cytotoxicity of an adenovirus expressing a fusogenic glycoprotein at a late stage of replication*. Gene Ther, 2008. **15**(17): p. 1240-5.
88. Guedan, S., et al., *GALV expression enhances the therapeutic efficacy of an oncolytic adenovirus by inducing cell fusion and enhancing virus distribution*. Gene Ther, 2012. **19**(11): p. 1048-57.
89. Alkhatib, G., C. Richardson, and S.H. Shen, *Intracellular processing, glycosylation, and cell-surface expression of the measles virus fusion protein (F) encoded by a recombinant adenovirus*. Virology, 1990. **175**(1): p. 262-70.
90. Galanis, E., et al., *Use of viral fusogenic membrane glycoproteins as novel therapeutic transgenes in gliomas*. Hum Gene Ther, 2001. **12**(7): p. 811-21.

91. Hoffmann, D., W. Bayer, and O. Wildner, *In situ tumor vaccination with adenovirus vectors encoding measles virus fusogenic membrane proteins and cytokines*. World J Gastroenterol, 2007. **13**(22): p. 3063-70.
92. Hoffmann, D. and O. Wildner, *Enhanced killing of pancreatic cancer cells by expression of fusogenic membrane glycoproteins in combination with chemotherapy*. Mol Cancer Ther, 2006. **5**(8): p. 2013-22.
93. Hoffmann, D., et al., *Synergy between expression of fusogenic membrane proteins, chemotherapy and facultative virotherapy in colorectal cancer*. Gene Ther, 2006. **13**(21): p. 1534-44.
94. Hoffmann, D., et al., *Evaluation of twenty-one human adenovirus types and one infectivity-enhanced adenovirus for the treatment of malignant melanoma*. J Invest Dermatol, 2008. **128**(4): p. 988-98.
95. Hoffmann, D., et al., *Intratumoral expression of respiratory syncytial virus fusion protein in combination with cytokines encoded by adenoviral vectors as in situ tumor vaccine for colorectal cancer*. Mol Cancer Ther, 2007. **6**(7): p. 1942-50.
96. Hoffmann, D., W. Bayer, and O. Wildner, *Therapeutic immune response induced by intratumoral expression of the fusogenic membrane protein of vesicular stomatitis virus and cytokines encoded by adenoviral vectors*. Int J Mol Med, 2007. **20**(5): p. 673-81.
97. Gomez-Trevino, A., et al., *Effects of adenovirus-mediated SV5 fusogenic glycoprotein expression on tumor cells*. J Gene Med, 2003. **5**(6): p. 483-92.
98. Wong, C.M., et al., *Expression of the fusogenic p14 FAST protein from a replication-defective adenovirus vector does not provide a therapeutic benefit in an immunocompetent mouse model of cancer*. Cancer Gene Therapy, 2016: p. In Press.
99. Dewar, R.L., et al., *Biosynthesis and processing of human immunodeficiency virus type 1 envelope glycoproteins: effects of monensin on glycosylation and transport*. Journal of virology, 1989. **63**(6): p. 2452-2456.
100. Li, H., et al., *Human immunodeficiency virus type 1-mediated syncytium formation is compatible with adenovirus replication and facilitates efficient dispersion of viral gene products and de novo-synthesized virus particles*. Hum Gene Ther, 2001. **12**(18): p. 2155-65.
101. Van Zeijl, M., et al., *A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family*. Proceedings of the national academy of sciences, 1994. **91**(3): p. 1168-1172.
102. Kavanaugh, M.P. and D. Kabat, *Identification and characterization of a widely expressed phosphate transporter/retrovirus receptor family*. Kidney international, 1996. **49**(4): p. 959-963.
103. Forestell, S.P., et al., *Novel retroviral packaging cell lines: complementary tropisms and improved vector production for efficient gene transfer*. Gene Ther, 1997. **4**(6): p. 600-10.
104. Fielding, A.K., et al., *A hyperfusogenic gibbon ape leukemia envelope glycoprotein: targeting of a cytotoxic gene by ligand display*. Hum Gene Ther, 2000. **11**(6): p. 817-26.
105. Johnson, K.J., et al., *Targeting the cytotoxicity of fusogenic membrane glycoproteins in gliomas through protease-substrate interaction*. Gene Ther, 2003. **10**(9): p. 725-32.
106. Wust, P., et al., *Hyperthermia in combined treatment of cancer*. The Lancet Oncology, 2002. **3**(8): p. 487-497.

107. Griffin, D.E. and M.B. Oldstone, *Measles: history and basic biology*. Vol. 329. 2008: Springer Science & Business Media.
108. Plattet, P., et al., *Measles Virus Fusion Protein: Structure, Function and Inhibition*. Viruses, 2016. **8**(4).
109. Nakamura, T., et al., *Antibody-targeted cell fusion*. Nat Biotechnol, 2004. **22**(3): p. 331-6.
110. Hammond, A.L., et al., *Single-chain antibody displayed on a recombinant measles virus confers entry through the tumor-associated carcinoembryonic antigen*. J Virol, 2001. **75**(5): p. 2087-96.
111. Peng, K.W., et al., *Oncolytic measles viruses displaying a single-chain antibody against CD38, a myeloma cell marker*. Blood, 2003. **101**(7): p. 2557-62.
112. Linardakis, E., et al., *Enhancing the efficacy of a weak allogeneic melanoma vaccine by viral fusogenic membrane glycoprotein-mediated tumor cell-tumor cell fusion*. Cancer Res, 2002. **62**(19): p. 5495-504.
113. Tietge, U.J., et al., *A tetracycline-regulated adenoviral expression system for in vivo delivery of transgenes to lung and liver*. The journal of gene medicine, 2003. **5**(7): p. 567-575.
114. Shmulevitz, M. and R. Duncan, *A new class of fusion-associated small transmembrane (FAST) proteins encoded by the non-enveloped fusogenic reoviruses*. EMBO Journal, 2000. **19**(5): p. 902-12.
115. Joklik, W.K., *The reoviridae*. 2013: Springer Science & Business Media.
116. Ciechonska, M. and R. Duncan, *Reovirus FAST proteins: virus-encoded cellular fusogens*. Trends in microbiology, 2014. **22**(12): p. 715-724.
117. Nibert, M.L. and R. Duncan, *Bioinformatics of recent aqua-and orthoreovirus isolates from fish: evolutionary gain or loss of FAST and fiber proteins and taxonomic implications*. PLoS One, 2013. **8**(7): p. e68607.
118. Clancy, E.K. and R. Duncan, *Reovirus FAST protein transmembrane domains function in a modular, primary sequence-independent manner to mediate cell-cell membrane fusion*. Journal of virology, 2009. **83**(7): p. 2941-2950.
119. Parmar, H.B., et al., *Golgi complex-plasma membrane trafficking directed by an autonomous, tribasic Golgi export signal*. Molecular biology of the cell, 2014. **25**(6): p. 866-878.
120. Corcoran, J.A. and R. Duncan, *Reptilian reovirus utilizes a small type III protein with an external myristylated amino terminus to mediate cell-cell fusion*. Journal of virology, 2004. **78**(8): p. 4342-4351.
121. Top, D., et al., *Enhanced fusion pore expansion mediated by the trans-acting Endodomain of the reovirus FAST proteins*. PLoS Pathog, 2009. **5**(3): p. e1000331.
122. Barry, C. and R. Duncan, *Multifaceted sequence-dependent and-independent roles for reovirus FAST protein cytoplasmic tails in fusion pore formation and syncytiogenesis*. Journal of virology, 2009. **83**(23): p. 12185-12195.
123. Corcoran, J.A., E.K. Clancy, and R. Duncan, *Homomultimerization of the reovirus p14 fusion-associated small transmembrane protein during transit through the ER-Golgi complex secretory pathway*. Journal of General Virology, 2011. **92**(1): p. 162-166.
124. Corcoran, J.A., et al., *The p14 fusion-associated small transmembrane (FAST) protein effects membrane fusion from a subset of membrane microdomains*. Journal of Biological Chemistry, 2006. **281**(42): p. 31778-31789.

125. Brown, C.W., et al., *The p14 FAST protein of reptilian reovirus increases vesicular stomatitis virus neuropathogenesis*. Journal of virology, 2009. **83**(2): p. 552-561.
126. Stojdl, D.F., et al., *VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents*. Cancer cell, 2003. **4**(4): p. 263-275.
127. Le Boeuf, F., et al., *Synergistic interaction between oncolytic viruses augments tumor killing*. Molecular Therapy, 2010. **18**(5): p. 888-895.
128. Le Boeuf, F., et al., *Reovirus FAST Protein Enhances Vesicular Stomatitis Virus Oncolytic Virotherapy in Primary and Metastatic Tumor Models*. Molecular Therapy - Oncolytics, 2017. **6**: p. 80-89.
129. Symons, J.A., A. Alcamí, and G.L. Smith, *Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species soecificity*. Cell, 1995. **81**(4): p. 551-560.
130. Lichtenstein, D.L., et al., *Functions and mechanisms of action of the adenovirus E3 proteins*. International reviews of immunology, 2004. **23**(1-2): p. 75-111.
131. Scaria, A., et al., *The E3-11.6 K protein of adenovirus is an Asn-glycosylated integral membrane protein that localizes to the nuclear membrane*. Virology, 1992. **191**(2): p. 743-753.
132. Tollefson, A.E., et al., *The 11,600-MW protein encoded by region E3 of adenovirus is expressed early but is greatly amplified at late stages of infection*. J Virol, 1992. **66**(6): p. 3633-42.
133. Amalfitano, A., et al., *Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted*. Journal of virology, 1998. **72**(2): p. 926-933.
134. Suzuki, K., et al., *The presence of the adenovirus E3 region improves the oncolytic potency of conditionally replicative adenoviruses*. Clinical cancer research, 2002. **8**(11): p. 3348-3359.
135. Wang, Y., et al., *E3 gene manipulations affect oncolytic adenovirus activity in immunocompetent tumor models*. Nature biotechnology, 2003. **21**(11): p. 1328.
136. Doronin, K., et al., *Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein*. Journal of virology, 2000. **74**(13): p. 6147-6155.
137. Ramachandra, M., et al., *Re-engineering adenovirus regulatory pathways to enhance oncolytic specificity and efficacy*. Nature biotechnology, 2001. **19**(11): p. 1035.
138. Doronin, K., et al., *Overexpression of the ADP (E3-11.6 K) protein increases cell lysis and spread of adenovirus*. Virology, 2003. **305**(2): p. 378-387.
139. Yun, C.-O., et al., *ADP-overexpressing adenovirus elicits enhanced cytopathic effect by induction of apoptosis*. Cancer gene therapy, 2005. **12**(1): p. 61.
140. Greber, U.F., et al., *The role of the nuclear pore complex in adenovirus DNA entry*. The EMBO journal, 1997. **16**(19): p. 5998-6007.
141. Horn, G.P., et al., *Enhanced cytotoxicity without internuclear spread of adenovirus upon cell fusion by measles virus glycoproteins*. Journal of virology, 2005. **79**(3): p. 1911-1917.
142. Giard, D.J., et al., *In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors*. Journal of the National Cancer Institute, 1973. **51**(5): p. 1417-1423.
143. Graham, F.L., et al., *Characteristics of a human cell line transformed by DNA from human adenovirus type 5*. Journal of General Virology, 1977. **36**(1): p. 59-72.

144. Dexter, D.L., et al., *Heterogeneity of tumor cells from a single mouse mammary tumor*. Cancer research, 1978. **38**(10): p. 3174-3181.
145. Franks, L.M., et al., *Metastasizing tumors from serum-supplemented and serum-free cell lines from a C57BL mouse lung tumor*. Cancer Res, 1976. **36**(3): p. 1049-55.
146. Chartier, C., et al., *Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli*. Journal of virology, 1996. **70**(7): p. 4805-4810.
147. Fueyo, J., et al., *A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo*. Oncogene, 2000. **19**(1): p. 2-12.
148. Carette, J.E., et al., *Replication-dependent transgene expression from a conditionally replicating adenovirus via alternative splicing to a heterologous splice-acceptor site*. journal of gene medicine, 2005. **7**(8): p. 1053-1062.
149. Saha, B. and R.J. Parks, *Histone deacetylase inhibitor SAHA suppresses human adenovirus gene expression and replication*. Journal of virology, 2019: p. JVI. 00088-19.
150. Thomas, G.P. and M.B. Mathews, *DNA replication and the early to late transition in adenovirus infection*. Cell, 1980. **22**(2): p. 523-533.
151. Clarkin, R.G., *Enhancing Oncolytic Adenovirus Vector Efficacy through Co-expression of the p14 Fusion-associated Small Transmembrane Protein and Adenovirus Death Protein*, in *Biochemistry, Microbiology and Immunology*. 2018, Université d'Ottawa / University of Ottawa.
152. Funston, G.M., et al., *Expression of heterologous genes in oncolytic adenoviruses using picornaviral 2A sequences that trigger ribosome skipping*. Journal of General Virology, 2008. **89**(2): p. 389-396.
153. Donnelly, M.L., et al., *Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'*. Journal of General Virology, 2001. **82**(5): p. 1013-1025.
154. Bett, A.J., et al., *An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3*. Proceedings of the National Academy of Sciences, 1994. **91**(19): p. 8802-8806.
155. Addison, C.L., et al., *Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors*. J Gen Virol, 1997. **78** (Pt 7): p. 1653-61.
156. Anton, M. and F.L. Graham, *Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression*. Journal of virology, 1995. **69**(8): p. 4600-4606.
157. Graham, F., *Growth of 293 cells in suspension culture*. Journal of general virology, 1987. **68**(3): p. 937-940.
158. Ross, P.J. and R.J. Parks, *Construction and characterization of adenovirus vectors*. cold spring harbor protocols, 2009. **2009**(5): p. pdb. prot5011.
159. Steven Rozen, H.J.S., *Primer3 on the WWW for general users and for biologist programmers*, in *Bioinformatic Methods and Protocols: Methods in Molecular Biology*, M.S. Krawetz S, Editor. 2000, Humana Press: Totowa, NJ. p. 365-386.
160. Altschul, S.F., et al., *Basic local alignment search tool*. Journal of molecular biology, 1990. **215**(3): p. 403-410.

161. Whelan, J.A., N.B. Russell, and M.A. Whelan, *A method for the absolute quantification of cDNA using real-time PCR*. Journal of immunological methods, 2003. **278**(1-2): p. 261-269.
162. Kimball, S. and P. Mattis. *GNU Image Manipulation Program V2.8.22*. 1995.
163. Parks, R.J., et al., *A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal*. Proceedings of the National Academy of Sciences, 1996. **93**(24): p. 13565-13570.
164. Harrington, B.e.a. *Inkscape*. 2004-2005; Available from: <http://www.Inkscape.org>.
165. Mehrara, E., et al., *Specific growth rate versus doubling time for quantitative characterization of tumor growth rate*. Cancer research, 2007. **67**(8): p. 3970-3975.
166. Brú, A., et al., *The universal dynamics of tumor growth*. Biophysical journal, 2003. **85**(5): p. 2948-2961.
167. Lindenmann, J. and P.A. Klein, *Immunological aspects of viral oncolysis*. Vol. 9. 2012: Springer Science & Business Media.
168. Louis, N., C. Eveleigh, and F.L. Graham, *Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line*. Virology, 1997. **233**(2): p. 423-9.
169. Kovesdi, I. and S.J. Hedley, *Adenoviral producer cells*. Viruses, 2010. **2**(8): p. 1681-703.
170. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets*. Molecular biology and evolution, 2016: p. msw054.
171. Benson, D.A., et al., *GenBank*. Nucleic acids research, 2013. **41**(D1): p. D36-D42.
172. Carette, J.E., et al., *Replication-dependent transgene expression from a conditionally replicating adenovirus via alternative splicing to a heterologous splice-acceptor site*. Journal of Gene Medicine, 2005. **7**(8): p. 1053-1062.
173. Gaynor, R.B., et al., *Enhanced expression of adenovirus transforming proteins*. Journal of virology, 1982. **44**(1): p. 276-285.
174. Nettelbeck, D.M., *Cellular genetic tools to control oncolytic adenoviruses for virotherapy of cancer*. Journal of molecular medicine, 2008. **86**(4): p. 363-377.
175. Quirin, C., et al., *Selectivity and efficiency of late transgene expression by transcriptionally targeted oncolytic adenoviruses are dependent on the transgene insertion strategy*. Human gene therapy, 2010. **22**(4): p. 389-404.
176. Yeo, G., et al., *Variation in sequence and organization of splicing regulatory elements in vertebrate genes*. Proceedings of the National Academy of Sciences, 2004. **101**(44): p. 15700-15705.
177. Garanto, A., L. Duijkers, and R. Collin, *Species-dependent splice recognition of a cryptic exon resulting from a recurrent intronic CEP290 mutation that causes congenital blindness*. International journal of molecular sciences, 2015. **16**(3): p. 5285-5298.
178. Garanto, A., et al., *High transcriptional complexity of the retinitis pigmentosa CERKL gene in human and mouse*. Investigative ophthalmology & visual science, 2011. **52**(8): p. 5202-5214.
179. Parato, K.A., et al., *Recent progress in the battle between oncolytic viruses and tumours*. Nature Reviews: Cancer, 2005. **5**(12): p. 965.

180. Smith, T.A., et al., *Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates*. Human gene therapy, 2003. **14**(17): p. 1595-1604.
181. Tao, N., et al., *Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver*. Molecular Therapy, 2001. **3**(1): p. 28-35.
182. Zhang, Z., et al., *Systemic delivery of a novel liver-detargeted oncolytic adenovirus causes reduced liver toxicity but maintains the antitumor response in a breast cancer bone metastasis model*. Human gene therapy, 2011. **22**(9): p. 1137-1142.
183. Coughlan, L., et al., *Combined fiber modifications both to target $\alpha\beta 6$ and detarget the coxsackievirus-adenovirus receptor improve virus toxicity profiles in vivo but fail to improve antitumoral efficacy relative to adenovirus serotype 5*. Human gene therapy, 2012. **23**(9): p. 960-979.
184. Silverstein, G. and W. Strohl, *Restricted replication of adenovirus type 2 in mouse Balb/3T3 cells*. Archives of virology, 1986. **87**(3-4): p. 241-264.
185. Blair, G.E., et al., *Restricted replication of human adenovirus type 5 in mouse cell lines*. Virus research, 1989. **14**(4): p. 339-346.
186. Kelland, L., *"Of mice and men": values and liabilities of the athymic nude mouse model in anticancer drug development*. European Journal of Cancer, 2004. **40**(6): p. 827-836.
187. Errington, F., et al., *Fusogenic membrane glycoprotein-mediated tumour cell fusion activates human dendritic cells for enhanced IL-12 production and T-cell priming*. Gene Ther, 2006. **13**(2): p. 138-49.
188. Bramson, J., et al., *Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination*. Gene therapy, 1997. **4**(10): p. 1069-1076.
189. Saha, B. and R.J. Parks, *Human adenovirus type 5 vectors deleted of early region 1 (E1) undergo limited expression of early replicative E2 proteins and DNA replication in non-permissive cells*. PloS one, 2017. **12**(7): p. e0181012.
190. Barton, K.N., et al., *Second-generation replication-competent oncolytic adenovirus armed with improved suicide genes and ADP gene demonstrates greater efficacy without increased toxicity*. Molecular Therapy, 2006. **13**(2): p. 347-356.
191. Halldén, G., et al., *Novel immunocompetent murine tumor models for the assessment of replication-competent oncolytic adenovirus efficacy*. Molecular Therapy, 2003. **8**(3): p. 412-424.
192. Oberg, D., et al., *Improved potency and selectivity of an oncolytic E1ACR2 and E1B19K deleted adenoviral mutant in prostate and pancreatic cancers*. Clin Cancer Res, 2010. **16**(2): p. 541-53.
193. Bateman, A.R., et al., *Viral fusogenic membrane glycoproteins kill solid tumor cells by nonapoptotic mechanisms that promote cross presentation of tumor antigens by dendritic cells*. Cancer research, 2002. **62**(22): p. 6566-6578.
194. Rincón, E., et al., *Mesenchymal stem cell carriers enhance antitumor efficacy of oncolytic adenoviruses in an immunocompetent mouse model*. Oncotarget, 2017. **8**(28): p. 45415.
195. Lichtenstein, D.L., et al., *Functions and mechanisms of action of the adenovirus E3 proteins*. Int Rev Immunol, 2004. **23**(1-2): p. 75-111.
196. Murali, V.K., et al., *Adenovirus death protein (ADP) is required for lytic infection of human lymphocytes*. J Virol, 2014. **88**(2): p. 903-12.

197. Al-Zaher, A.A., et al., *Evidence of anti-tumoral efficacy in an immune competent setting with an iRGD modified hyaluronidase-armed oncolytic adenovirus*. Molecular Therapy - Oncolytics, 2018.
198. Ling, A., et al., *TAP1 down-regulation elicits immune escape and poor prognosis in colorectal cancer*. Oncoimmunology, 2017. **6**(11): p. e1356143.
199. Henle, A.M., et al., *Downregulation of TAP1 and TAP2 in early stage breast cancer*. PloS one, 2017. **12**(11): p. e0187323.
200. Johnsen, A., et al., *Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis*. The Journal of Immunology, 1999. **163**(8): p. 4224-4231.
201. Van Kaer, L., et al., *TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4⁺ 8⁺ T cells*. Cell, 1992. **71**(7): p. 1205-1214.
202. Jogler, C., et al., *Replication properties of human adenovirus in vivo and in cultures of primary cells from different animal species*. Journal of virology, 2006. **80**(7): p. 3549-3558.
203. Thomas, M.A., et al., *Immunosuppression enhances oncolytic adenovirus replication and antitumor efficacy in the Syrian hamster model*. Molecular therapy, 2008. **16**(10): p. 1665-1673.
204. Li, X., et al., *The efficacy of oncolytic adenovirus is mediated by T-cell responses against virus and tumor in Syrian hamster model*. Clinical Cancer Research, 2017. **23**(1): p. 239-249.
205. Schenk, M., et al., *Development of a Transplantable GFP⁺ B-Cell Lymphoma Tumor Cell Line From MHC-Defined Miniature Swine: Potential for a Large Animal Tumor Model*. Front Oncol, 2019. **9**: p. 209.
206. Lemay, C.G., et al., *Harnessing oncolytic virus-mediated antitumor immunity in an infected cell vaccine*. Molecular Therapy, 2012. **20**(9): p. 1791-1799.
207. Kreil, G., *Hyaluronidases—a group of neglected enzymes*. Protein Science, 1995. **4**(9): p. 1666-1669.
208. Lang, F.F., et al., *Phase I study of DNX-2401 (Delta-24-RGD) oncolytic adenovirus: replication and immunotherapeutic effects in recurrent malignant glioma*. Journal of Clinical Oncology, 2018. **36**(14): p. 1419.
209. Silberman, A., *Surgical debulking of tumors*. Surgery, gynecology & obstetrics, 1982. **155**(4): p. 577-585.
210. Curtin, J.P., et al., *Stage IV Ovarian Cancer: Impact of Surgical Debulking*. Gynecologic oncology, 1997. **64**(1): p. 9-12.
211. Le, T., et al., *Does debulking surgery improve survival in biologically aggressive ovarian carcinoma?* Gynecologic oncology, 1997. **67**(2): p. 208-214.
212. Feldman, A.L., et al., *Analysis of factors associated with outcome in patients with malignant peritoneal mesothelioma undergoing surgical debulking and intraperitoneal chemotherapy*. Journal of Clinical Oncology, 2003. **21**(24): p. 4560-4567.
213. Schnipper, L.E., *Clinical implications of tumor-cell heterogeneity*. New England Journal of Medicine, 1986. **314**(22): p. 1423-1431.
214. Calbo, J., et al., *A functional role for tumor cell heterogeneity in a mouse model of small cell lung cancer*. Cancer cell, 2011. **19**(2): p. 244-256.
215. McGranahan, N. and C. Swanton, *Clonal heterogeneity and tumor evolution: past, present, and the future*. Cell, 2017. **168**(4): p. 613-628.

216. Erenpreisa, J., K. Salmina, and M.S. Cragg, *Accelerated Senescence of Cancer Stem Cells: A Failure to Thrive or a Route to Survival?*, in *Senescence-Physiology or Pathology*. 2017, IntechOpen.
217. Milanovic, M., et al., *Senescence-associated reprogramming promotes cancer stemness*. *Nature*, 2018. **553**(7686): p. 96.
218. Guo, W., et al., *Combination effect of oncolytic adenovirotherapy and TRAIL gene therapy in syngeneic murine breast cancer models*. *Cancer gene therapy*, 2006. **13**(1): p. 82-90.
219. Oh, E., et al., *Oncolytic adenovirus coexpressing interleukin-12 and decorin overcomes Treg-mediated immunosuppression inducing potent antitumor effects in a weakly immunogenic tumor model*. *Oncotarget*, 2017. **8**(3): p. 4730.
220. Cheng, C. and J. Praszkie, *Regulation of type 5 adenovirus replication in murine teratocarcinoma cell lines*. *Virology*, 1982. **123**(1): p. 45-59.
221. Eggerding, F.A. and W.C. Pierce, *Molecular biology of adenovirus type 2 semipermissive infections I. Viral growth and expression of viral replicative functions during restricted adenovirus infection*. *Virology*, 1986. **148**(1): p. 97-113.
222. Wold, W.S. and K. Toth, *Chapter three--Syrian hamster as an animal model to study oncolytic adenoviruses and to evaluate the efficacy of antiviral compounds*. *Adv Cancer Res*, 2012. **115**: p. 69-92.
223. Cerullo, V., et al., *Oncolytic adenovirus coding for granulocyte macrophage colony-stimulating factor induces antitumoral immunity in cancer patients*. *Cancer research*, 2010. **70**(11): p. 4297-4309.
224. Walsh, N.C., et al., *Humanized mouse models of clinical disease*. *Annual Review of Pathology: Mechanisms of Disease*, 2017. **12**: p. 187-215.
225. Wang, M., et al., *Humanized mice in studying efficacy and mechanisms of PD-1-targeted cancer immunotherapy*. *The FASEB Journal*, 2017. **32**(3): p. 1537-1549.
226. Watson, A.L., et al., *Engineered swine models of cancer*. *Frontiers in genetics*, 2016. **7**: p. 78.
227. Koodie, L., et al., *Rodents Versus Pig Model for Assessing the Performance of Serotype Chimeric Ad5/3 Oncolytic Adenoviruses*. *Cancers*, 2019. **11**(2): p. 198.

Contributions of Collaborators

All of the data presented herein were generated by the author, with the exception of figure 25 which is comprised of data generated by Ryan Clarkin and used with his permission. Kathy Poulin provided technical assistance. Julia Petryk and Dr. John Bell contributed to the design and implementation of animal trials. Dr. Robin Parks, my graduate supervisor, provided technical assistance with all cloning involved in virus generation, and provided guidance and feedback throughout my training. Dr. Parks also provided editorial comments on this work. This work contains significant components of a published review article (DOI:10.3390/v9010013) and a published manuscript (DOI:<https://doi.org/10.1016/j.omto.2019.05.001>), both of which I am first author, and both of which are permitted for use in this non-commercial thesis. These can be found as Appendix 1 and Appendix 2, respectively.

Appendix 1: Published review article



Review

Adenoviral Vectors Armed with Cell Fusion-Inducing Proteins as Anti-Cancer Agents

Joshua Del Papa^{1,2,3} and Robin J. Parks^{1,2,3,4,*}

¹ Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON K1H 8L6, Canada;

jdelpapa@ohri.ca

² Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON K1N 6N5, Canada

³ Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON K1N 6N5, Canada

⁴ Department of Medicine, University of Ottawa, Ottawa, ON K1N 6N5, Canada

* Correspondence: rparks@ohri.ca; Tel.: +1-613-737-8123

Academic Editor: Alexander Ploss

Received: 9 November 2016; Accepted: 15 January 2017; Published: 19 January 2017

Abstract: Cancer is a devastating disease that affects millions of patients every year, and causes an enormous economic burden on the health care system and emotional burden on affected families. The first line of defense against solid tumors is usually extraction of the tumor, when possible, by surgical methods. In cases where solid tumors can not be safely removed, chemotherapy is often the first line of treatment. As metastatic cancers often become vigorously resistant to treatments, the development of novel, more potent and selective anti-cancer strategies is of great importance. Adenovirus (Ad) is the most commonly used virus in cancer clinical trials, however, regardless of the nature of the Ad-based therapeutic, complete responses to treatment remain rare. A number of pre-clinical studies have shown that, for all vector systems, viral spread throughout the tumor mass can be a major limiting factor for complete tumor elimination. By expressing exogenous cell-fusion proteins, many groups have shown improved spread of Ad-based vectors. This review summarizes the research done to examine the potency of Ad vectors expressing fusogenic proteins as anti-cancer therapeutics.

Keywords: adenovirus; fusion protein; cancer; therapy

1. Introduction

Cancer is the second leading cause of death in high income countries, and the third leading cause of death in low and middle income countries, behind heart disease and infection [1]. Globally, in 2012, there were 14.1 million new cancer diagnoses, and 8.2 million cancer-related deaths. In the USA alone in 2011, the direct medical cost of cancer was a staggering 88.7 billion USD [2]. These numbers are expected to rise with global population and lifespan increase. Of all cancer cases discovered, 80% are classified as solid tumors [3]. When these tumors form in or near critical tissues, have diffuse borders, or occur in patients unable to tolerate surgery, they can be deemed inoperable. In such cases, chemotherapy (or other drug-based treatment) and radiation are the main modes of treatment. However, although these treatments can often reduce disease burden and increase lifespan, prognoses for inoperable cancers are often grim [4].

There are over 100 known major types of cancer that affect humans, each of which can have a diverse array of founding mutations, which influences pathology and treatment effectiveness [3]. A significant proportion of traditional chemotherapeutics aim to damage cellular DNA, and the differential response to DNA damage between normal and cancer cells is what underlies the effectiveness of many chemotherapeutic drugs. Upon DNA damage, normal cells undergo cell

cycle arrest and engage the DNA damage repair (DDR) machinery prior to further cell replication and division. Cancerous cells often exhibit defects in the cell cycle checkpoints that would normally halt division or activate the DDR, and therefore these cancer cells will continue to replicate, most often dying in the process [5]. A major barrier to the success of these therapeutics is that the survival of a single cancerous cell can lead to re-emergence of the tumor and, similar to the evolution of antibiotic resistance, these cells often have or gain additional mutations that allow for resistance to future treatments [6]. This, and other mechanisms, can lead to the proliferation of cancer cells that are resistant to traditional chemotherapies, highlighting the need for new more potent and more specific cancer treatments for inoperable solid tumors [7].

One promising candidate therapy under development, viral gene therapy, has the capacity to offer cancer strain-specific treatments to counteract the exceptional diversity that exists within this disease group. Two basic strategies exist when using engineered viral vectors against cancer: use of the virus to deliver genes encoding therapeutic proteins to cancer cells, or engineering of the virus such that it selectively replicates in and kills cancer cells [8,9]. A wide array of viruses have been explored in preclinical and clinical studies, including vaccinia virus, herpes virus, vesicular stomatitis virus, and adenovirus [10–13]. Indeed, an oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor (GM-CSF) has been approved recently for clinical use in North America [14].

For one of the most commonly used gene therapy vectors, adenovirus (Ad), two Ad-based vectors have been approved for cancer treatment in China—a non-replicating Ad encoding p53, termed Gendicine, and an oncolytic Ad that selectively replicates in cancer cells, termed Oncorine [15,16]. In stage II/III clinical trials, intratumoral treatment with Gendicine with radiation treatment resulted in significant increases in both partial and complete responses, with a three-fold increase in complete responses versus radiation alone. Oncorine combination treatment with chemotherapy resulted in similar success rates with a 72.7% response rate versus 40.4% with chemotherapy alone. Ad vectors have been used extensively in molecular biology applications for many decades, to achieve high-level gene expression of a desired transgene in mammalian cells. As of July 2015, 22% ($n = 506$) of all human gene therapy clinical trials used Ad-based vectors to deliver a therapeutic gene of interest, with the vast majority of these trials directed towards treatment of cancer [17]. Adenovirus efficiently infects a wide variety of human cell types, regardless of cell cycle status, and has a relatively large cloning capacity [8]. These desirable qualities have led to a vast amount of research into their use as oncolytic vectors or as delivery vehicles for anti-cancer therapeutic genes. Unfortunately, many preclinical and clinical studies have shown that Ad-based therapeutics frequently mediate only a partial response [18,19], partly due to limitations of the vector system combined with complexities of the target tissue. A major barrier to anti-cancer efficacy for Ad, and many other therapeutic viruses, is efficient spread or distribution of virus throughout the tumor mass [20]. Tumors typically exhibit a relatively high internal pressure, which inhibits effective spread of virus when injected directly into the tumor mass [20]. Tumors also contain a large amount of connective tissue, further impeding virus spread [21]. Many approaches have been investigated to overcome this barrier, including the expression of junction opening peptides or enzymes capable of dissociating the extracellular matrix, or increasing the rate of viral lysis and spread by overexpression of native viral components such as the Ad death protein [22–24]. In addition to mechanical barriers to intratumoral spread, there are also several biochemical barriers. Tumors often express low levels of the main adenovirus receptor, the Coxsackievirus and Adenovirus Receptor (CAR), which can reduce the ability of Ad to bind to and enter the tumor cells [25,26]. Several methods of structural modification of Ad to increase CAR independent cellular uptake have been used, such as alterations to the fiber domain to increase efficiency of viral entry and fusion of single-domain antibodies to various capsid proteins [27–29]. Finally, pre-existing neutralizing Ad antibodies are detectable in 30% to 60% of the USA population, with higher rates in developing countries [30,31]. The existence of these neutralizing antibodies in combination with the efficiency of complement recognition of Ad can in theory cause rapid elimination

of released virus following tumor cell lysis, further impeding spread throughout the tumor mass [32]. Interestingly, some studies suggest that presence or absence of pre-existing neutralizing Ad antibodies does not appear to affect treatment efficacy when Ad is delivered intratumorally [13,18].

An encouraging approach under investigation to enhance viral spread through a tumor involves heterologous expression of fusion-inducing proteins from the therapeutic vector. This approach has shown promise in not only increasing viral spread throughout the tumor mass, but also in facilitating activation of the host anti-tumor immune response. In this review, we will discuss recent studies in the development of vectors expressing fusogenic proteins for cancer therapy, focusing mainly on their use and delivery from Ad vectors.

2. Adenovirus Biology

Although there are roughly 50 distinct serotypes of human *Adenoviridae*, Ad serotypes 2 and 5, both of subclass C, are the most well-studied [33]. The genomes of Ad2 and Ad5 are ~95% identical at the nucleotide level and, for the purpose of this review, will be considered experimentally identical. Adenoviruses are icosahedral, non-enveloped, double stranded DNA viruses of approximately 70–100 nm in diameter for the main “body” of the virus, with varying lengths of the viral attachment protein, fiber, protruding from the vertices of the icosahedron [33]. Adenoviruses display a naturally diverse tropism for tissue types, which varies between viral subtypes, however, in nature, Ad5 most often infects cells of the upper respiratory tract [34]. The Ad5 genome is roughly 36 kb and is transcribed in essentially two stages upon entry into the host cell (Figure 1) [35]. Soon after the Ad genome reaches the nucleus, the Ad early genes are expressed, including early region 1 (E1), E3 and E4, which function to activate other transcription units on the Ad genome as well as modulate the host cell cycle and immune responses [36]. The E2 region encodes proteins directly involved in Ad DNA replication, such as the virally-encoded DNA polymerase. Only after the onset of DNA replication is the major late promoter (MLP) activated, which results in the production of a large (roughly 26 kb) transcript which subsequently is processed through alternative splicing to produce the late transcripts late region 1 (L1) to L5 [37]. These late regions encode the structural components necessary for Ad assembly, including the Ad capsid proteins [38]. There are also four small products produced at intermediate/late times of infection, including the structural protein IX (pIX), and the IVa2 protein that helps package viral DNA into immature virions [39]. The other intermediate/late products, viral associated (VA) RNA I and II, inhibit activation of the interferon response, impede cellular micro-RNA processing, and may influence expression of host genes [40,41]. The viral DNA also contains the origins of replication, the inverted terminal repeats (ITR), which comprise ~100 bp at each end of the genome and the packaging sequence, which is ~150 bp in length and is located immediately adjacent to the left ITR [42].

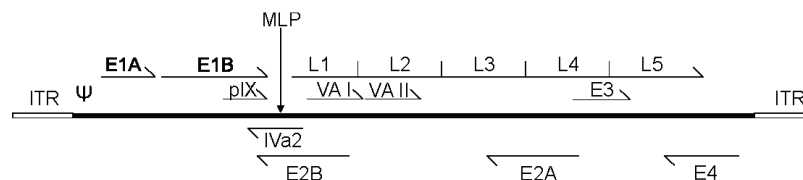


Figure 1. Schematic representation of the adenovirus genome. The inverted terminal repeat (ITR) sequences act as origins of viral replication, while Ψ represents the adenoviral packaging sequence. Adenovirus early transcripts (E1–E4) are transcribed upon viral entry, and expression of the intermediate transcripts pIX, IVa2, VA I and VA II follows. Activation of the major late promoter (MLP) occurs after the onset of adenovirus DNA replication, and generates a single transcript from which late transcription units, L1–L5, are spliced.

The most extensively utilized class of Ad vector is the replication-defective E1-deleted Ad. The E1A and E1B proteins encoded within the E1 region are absolutely required for virus replication, and thus E1-deleted vectors are incapable of replicating in most cells [43], but can efficiently deliver their DNA to the nucleus of cells and express an encoded transgene placed under the control of a heterologous promoter. Early region 1-deleted Ad have been explored extensively as delivery vehicles for genes encoding therapeutic proteins with cancer-mitigating effects such as, for example, p53 or interleukin (IL)-12 [44,45].

For oncolytic, or conditionally replicating Ad, there are two major approaches to achieve cancer-specific replication. When viral genes encoding proteins essential for viral replication, (e.g., E1) are placed under the control of a tissue (cancer)-specific promoter (e.g., the prostate specific antigen promoter), some selectivity can be achieved in viral replication (e.g., only in prostate cancer cells) [46,47]. Alternatively, the virus can be attenuated or mutated such that it can only replicate in cancer cells with mutations in cellular pathways required to promote virus replication. For example, one of the functions of the Ad E1A protein is to interact with the retinoblastoma (Rb) family of pocket proteins, to promote cell cycle progression [48]. Deletion of a 24 amino acid segment of conserved region 2 of the E1A protein creates a molecule that can no longer interact with the pocket proteins, and virus with this mutation can no longer replicate efficiently in normal cells [49]. However, since the Rb pathway is mutated in many cancers, Ad vectors containing the E1A Δ 24 mutation can selectively replicate in these cells but not normal tissue [50]. As stated above, two Ad-based cancer treatments are approved for use in China, one from each class of the above vector types—Gendicine and Oncorine (H101) [15,51]. Gendicine is a replication-deficient Ad expressing wild-type p53, which would reestablish expression of p53 in p53-deficient cancer cells, and Oncorine is a conditionally replicating Ad deleted of the E1B-55kD, which may also show specificity for cancers deficient in p53 [15,51].

As mentioned, a common problem with Ad is their restricted ability to disperse throughout a tumor, particularly following direct intratumoral injection [52–54]. Typically, Ad infection is limited to a 5 mm area surrounding the injection tract, which prevents efficient transgene product delivery to all cells of the tumor [19]. While this may not be a significant issue when Ad is used to deliver a gene for secreted proteins, such as cytokines, it may limit the efficacy of other types of therapeutic genes and proteins which require expression directly within the cell in order to exert their efficacy, such as, for example, the tumor suppressor p53. For oncolytic Ad vectors, preclinical studies have shown that complete tumor regression can be achieved with the oncolytic Ad ONYX-015 with initial infection of as little as 5% of the tumor cells, but these cells must be equally distributed throughout the tumor [52]. Consequently, distribution and efficacy can be improved through use of multiple, sequential injections of vector [52]. However, better, more effective approaches are required to enhance virus dispersion and spread within the tumor in order to achieve better efficacy.

3. Fusogenic Proteins

In an attempt to improve Ad vector distribution within the tumor, several research groups have explored whether inclusion of genes encoding fusogenic proteins can enhance therapeutic efficacy. Fusogenic proteins have the ability to promote cell–cell fusion, and thus their expression can promote lateral transfer of the virus or a co-expressed therapeutic gene product (Figure 2). The fusogenic proteins most often investigated tend to originate from enveloped viruses. These viruses, such as gibbon-ape leukemia virus (GALV), measles virus (MV) and respiratory syncytial virus (RSV), express membrane-bound fusogenic proteins within the viral envelope to facilitate viral entry into host cells [55–57]. There also exists a class of non-structural fusogenic proteins, the fusion associated small transmembrane (FAST) proteins, which are expressed from several species of non-enveloped *Orthoreovirus* [58]. Fusion associated small transmembrane proteins are not involved in virus entry but, instead, are believed to promote cell–cell fusion within the host as a means to increase viral lysis and progeny virus release.

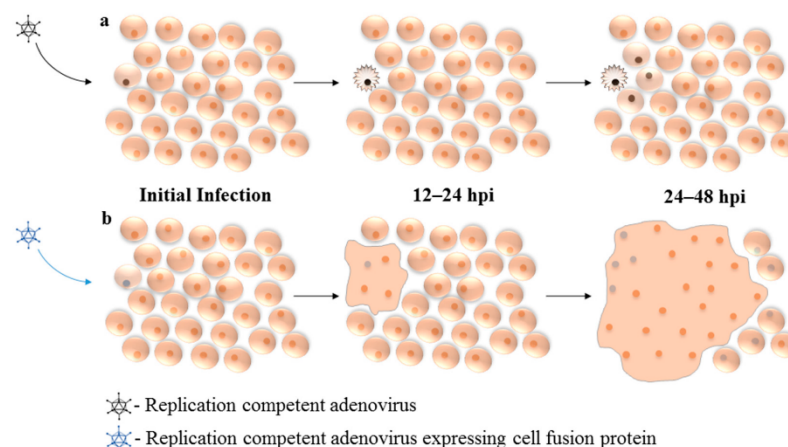


Figure 2. Mechanism of enhanced viral spread through expression of fusogenic protein: (a) Upon infection, replication-competent oncolytic adenovirus (Ad) must undergo productive replication and significantly damage the host cell in order to affect neighboring cells. (b) Cell-fusion proteins allow Ad to spread through densely packed cells with much greater efficiency, before replication-associated cell death or progeny release. Initial Infection: Virus enters host cell and initiates gene expression and early phase of replication. 12–24 hpi: Viral protein expression and progeny production. 24–48 hpi: Viral progeny released from initially infected cell, enter nearby cells. hpi: hours post-infection.

Regardless of the virus of origin, the basic mechanism by which all fusogenic proteins function involves reducing the mechanical energy required for lipid bilayer fusion to occur [59]. However, the biochemical mechanism by which cell fusion occurs differs drastically between viral fusogenic proteins. Nevertheless, a few characteristics are shared by all fusogenic proteins. Syncytia, multinucleated cells caused by the fusion of multiple cellular membranes, are initially viable, and remain active both metabolically and transcriptionally [60]. In tissue culture studies *in vitro*, initially viable syncytia eventually lift from the dish and undergo a type of cell death that has not been definitively characterized [61]. Multiple conflicting studies have provided evidence in support of either apoptotic or non-apoptotic cell death [60,62,63]. Some studies point to a necrotic cell death mechanism, with expression of heat shock protein 70, heat shock protein 90kDa beta member 1 (gp96) and retention of inhibitor of kappa B (I κ B α) in the cytoplasm, while others show elevation of cleaved caspase-3 and clear evidence of apoptotic DNA fragmentation [63,64]. However, the actual mechanism of cell death may be unique to fused cells, or even vary between fusogenic proteins.

During syncytium formation and associated cell death, blebbing at the cell surface results in the enhanced release of exosome-like particles referred to as syncytiosomes. These particles were shown to contain high levels of immune-stimulatory molecules, and lower levels of major histocompatibility complex I (MHC I) than exosomes naturally released from tumor cells [61]. Presence of these syncytiosomes promoted dendritic cell activation and resulted in activation of an anti-tumor T-cell response and tumor cell killing. Thus, fusogenic protein-induced formation of syncytia, and subsequent release of syncytiosomes, may enhance formation of anti-tumor immune responses, ultimately enhancing immune-mediated tumor rejection.

4. Expression of Fusogenic Proteins from Replication-Defective Adenovirus Vectors

The ability of viral fusogenic proteins to efficiently reduce cell viability while inciting a host anti-tumor response makes them excellent candidates as potential sole therapeutic molecules in

non-replicating Ad vectors, or to enhance spread of Ad throughout the tumor mass in the context of oncolytic Ad vectors. A number of viral fusogenic proteins have been investigated for their anti-cancer effects in the context of both non-replicating and oncolytic Ad vector (Table 1), as discussed below.

Table 1. Adenovirus expressing fusogenic proteins.

Origin of Fusion Protein	Replication-Defective Ad Vector	Replication-Competent Ad Vector	Reference
Gibbon-Ape Leukemia Virus	✓ -	- ✓	[65,66] [67] ¹ , [68] ² , [69,70]
Measles Virus	✓ -	- ✓	[71–73] [74–76] ³
Respiratory Syncytial Virus	✓	-	[77]
Vesicular Stomatitis Virus	✓	-	[78]
Simian Virus 5	✓	-	[79]
Reptilian Reovirus	✓	-	[62,80]
Human Immunodeficiency Virus	-	✓	[81,82]

¹ Early region 1 (E1)-deleted viruses used in E1-expressing 293 tumors; ² Plasmid expressing fusion protein combined with E1-competent Ad; ³ E1-deleted Ad trans-complemented with E1-competent Ad.

4.1. *Gibbon-Ape Leukemia Virus Fusogenic Membrane Glycoprotein*

Gibbon ape leukemia virus (GaLV) is an enveloped C-type retrovirus, whose cellular target for attachment, PiT-1, is ubiquitously expressed in human tissues [83,84]. Through the examination of retroviral packaging cell lines, the GaLV fusion protein was shown to cause efficient human cell–cell fusion after truncation of the C-terminal R-peptide (a negative regulatory element) of the protein [85]. Bateman et al. showed that the GaLV fusogenic membrane glycoprotein (FMG) was able to significantly reduce viability of cancer cells in vitro and in vivo upon in situ transfection with a FMG expression plasmid [60]. Interestingly, mice vaccinated with tumor cells that had been pre-transfected with the FMG expression plasmid showed significant immune protection, compared to mice treated with cells which had been transfected with a plasmid encoding a non-fusogenic form of the protein, when subsequently challenged with subcutaneous tumors. As mice and hamsters do not express the PiT-1 receptor necessary for GaLV binding and fusion, these models could not adequately examine the safety profile of this therapeutic approach.

One concern with the use of fusion proteins is that fusion can occur in any cell type, including cancer and normal cells. To counteract this safety concern with the GaLV fusion protein, several groups attempted to restrict its fusogenic capacity specifically to cancer cells. One such proof-of-concept effort involved fusing Epidermal growth factor (EGF) to the N-terminal targeting domain of the GaLV FMG via a factor Xa cleavage/linker domain, which was designated EXGaLV (EGF-X-GaLV) [86]. Incorporation of EXGaLV FMG into a retroviral vector (murine leukemia virus) limited infection to cells expressing the EGF receptor, and protease-mediated cleavage of the factor Xa domain resulted in rescue of viral infectivity [86]. Following this success, Johnson et al. sought to use a similar construct to target gliomas, which often overexpress the matrix metalloproteinase (MMP) enzymes MMP-2, MMP-9, MT-1 and MT-2, in stark contrast to the complete absence of MMPs in normal brain tissues [87]. The group used broad spectrum MMP-cleavable linkers to fuse the C-terminal domain of the cluster of differentiation 40 (CD40) ligand, to the N-terminus of the GaLV FMG. Addition of the fragment of the CD40 ligand prevents attachment through steric restriction, but would be cleaved from GaLV FMG in tumors expressing MMPs, thus permitting fusion. Transfection of a plasmid expressing this fusion protein into glioma cell lines in culture resulted in rampant cell fusion in cells with the highest expression of MMP, while no fusion was observed for normal human astrocyte (NHA) cells. When U87 glioblastoma cells were pre-transfected with a plasmid expressing the modified fusion protein and subsequently introduced into mice, MMP-mediated cleavage of the modified fusion protein was observed, along with fusion of the cancer cells and a concomitant significant reduction in tumor burden

compared to animals receiving unmodified U87 cells. Unfortunately, the reduction of tumor volume was greatest in wild-type GaLV-transfected cells, suggesting an overall reduction in fusogenic capacity of the engineered protein. All of the above studies involved plasmid-mediated expression of the fusion protein; however, in general, plasmid delivery in vivo is relatively inefficient, resulting in reduced overall efficacy.

To improve the delivery efficiency of the targeted GaLV genes in vivo, multiple groups have utilized non-replicating Ad vectors. An Ad vector expressing the MMP-dependant GaLV construct (AdM40) was compared to a vector expressing a non-cleavable version of the protein (AdN40) in in vitro and in vivo models of glioma [65]. Studies in tissue culture showed that AdN40 was able to efficiently infect cells, but was unable to induce fusion. However, AdM40 induced significant fusion which was dependent on the expression of MMP, as co-administration of MMP inhibitors eliminated fusion. Upon injection of the viruses into subcutaneous U87 glioma xenograft tumors in BALB/c nude mice, a significant reduction in tumor volume was observed in AdM40-treated mice relative to untreated or AdN40-treated mice. Treatment with AdM40 also significantly improved survival, with 30% of treated mice showing complete eradication of tumors and survival at the end of the study period (50 days), compared to AdN40 which showed no improvement relative to untreated mice.

Another study used a non-replicating Ad expressing GaLV FMG under the control of a modified human heat-shock protein HSP70b promoter (HSE.70b), for thermal-mediated regulation of expression [66]. Hyperthermia can be easily induced in localized tissues, such as tumors, and has shown significant potential when used in conjunction with chemotherapy or radiotherapy [88]. The ability of this HSE.70b promoter to increase transcript levels 950-fold in response to heating enables this construct to have impressive potential to restrict transgene expression both temporally and spatially [66]. A stably transfected HT-1080 fibrosarcoma cell line expressing the transgene grew unimpeded until challenged with hyperthermia, which induced syncytium formation and a 70% reduction in cell viability. The median survival of mice bearing tumors composed of this cell line increased from 4.3 to 52 days when tumors were treated with mild hyperthermia (44 °C for 30 min), compared to an increase from 7.9 to 12.5 days for mice bearing tumors established using the parental HT-1080 cell line which were also treated with the same hyperthermia regime. Treatment of a variety of cell lines with a replication-deficient Ad containing the same expression cassette (Ad.HSE70b.GALV) showed no evidence of fusion until mild hyperthermia (43 °C for 30 min) was applied. Incorporation of the expression cassette into conditionally replicating Ad was suggested as the next step for follow-up on these encouraging results but, this has not yet been reported.

4.2. Measles Virus Fusion Proteins

Measles virus (MeV) is an enveloped, single-stranded RNA human pathogenic virus from the *Paramyxoviridae* family [89]. A hallmark of many *Paramyxovirus* infections is the formation of syncytia in tissues harboring high-levels of virus [56]. Studies to develop antivirals have elucidated the structural components responsible for virus-host and host-host fusion observed in MeV infection. The MeV fusogenic membrane glycoprotein is a hetero-oligomeric protein composed of a tetramer of the H domain (attachment domain) complexed with a trimer of the F domain (fusion domain) [56]. Once associated in the endoplasmic reticulum, this complex of proteins is trafficked to the cell surface where it mediates both virus-host and host-host cell membrane fusion. The H domain is responsible for the MeV FMG cell specificity, and has two cellular protein targets: signaling lymphocyte activation molecule (SLAM, also known as CD150), or Nectin-4 (N4) [90]. Once the H domain binds to either of these targets, it mediates an irreversible conformational change in the F domain, which induces membrane curvature and ultimately membrane fusion [56].

In 1990, Alkhatib et al. published a study on their efforts to express the F domain of MeV from a non-replicating Ad [71]. The goal of the study was to explore the biochemical mechanism for fusion of the H/F FMG, however this was also the first proof-of-concept that heterologous expression of a viral FMG could generate functional fusogenic units and initiate cell-cell fusion in treated cells. Although

the vector encoded only the F domain, expression of this protein induced syncytium formation in 293 and HeLa cell lines. Studies using plasmid-mediated expression of both the H and F domains showed that fusion could be achieved in a variety of glioma cell lines, and was most potent when the plasmids were administered at a 1:1 ratio [72]. To translate this work in vivo, two Ad vectors were constructed, AdF and AdH, which were subsequently tested in a U87 xenograft glioma tumor model in nude mice [72]. Virus was administered once daily for three days and, at the end of the study period (70 days), 100% of the AdH/AdF-treated mice were alive, compared to only 20% of the mice treated with Ad-green fluorescent protein (AdGFP). Interestingly, these researchers also tested the efficacy of a lentiviral vector expressing the GaLV FMG, and saw no significant reduction in tumor burden or increase in lifespan. However, of note, the dose of lentiviral vector was much lower than that of the Ad, due to the difficulty in generating high-titer lentivirus stocks. This observation highlights one of the advantages of Ad vectors—the ability to easily generate high-titer stocks of vector.

Due to the ubiquitous expression of SLAM and CD40 throughout the human body, several research groups have attempted to target the MeV FMG H domain to cells expressing particular cell surface markers. Single chain antibodies (scFvs) targeting carcinoembryonic antigen (CEA) or the myeloma marker CD38 were fused to the H domain of MeV FMG [91,92]. These mutant H domains conferred specificity upon ablation of the native H binding domains, and MeV expressing the mutant proteins were able to selectively fuse cells expressing the targeted binding proteins. In a follow-up study, the group fused a scFv recognizing EGF receptor (EGFR) to the H domain, and expressed this protein from a non-replicating Ad vector also expressing the F domain [90]. Upon infection in EGFR(−) Chinese Hamster Ovary (CHO) cells, this virus did not induce cell fusion. However, infection of genetically engineered EGFR(+) CHO cells, resulted in widespread fusion. Taken together, these studies clearly show that some fusogenic proteins can be altered to decrease “generalized” fusion, and achieve cell selective fusion using hybrid proteins.

To enhance immune responses to the treated tumor cells, and essentially promote in situ vaccination, an Ad vector expressing the MeV H/F FMGs (Ad.MV-H/F) was tested alone and in combination with Ad vectors expressing various cytokines IL-2, IL-12, IL-18, IL-21 or granulocyte-macrophage colony-stimulating factor (GM-CSF) [73]. Previous studies had shown that expression of an FMG could improve the immunogenicity of a weakly allogenic melanoma vaccine [93], possibly due to the enhanced release of syncytiosomes from the fused cells, and subsequent cross-presentation of tumor-associated antigens to dendritic cells [61]. Mice bearing MC38 syngeneic colorectal tumors in both left and right hind flanks received intratumoral injection of Ad.MV-H/F in combination with the various cytokines in only one of the two tumors [73]. Combination treatment led to an 87%–98% reduction in injected tumor size at the end of the study, compared to treatment with Ad.MV-H/F (51% reduction) or Ad-cytokines alone (1%–40% reduction). Impressively, contralateral, uninjected tumors showed similar response rates, with a significantly greater response in mice treated with Ad.MV-H/F in combination with cytokines (85% reduction in tumor size) compared with treated with either Ad.MV-H/F (54% reduction) or Ad expressing cytokines alone (8% to 46%). Both the injected and non-injected tumors showed high levels of macrophage infiltration when Ad.MV-H/F was combined with the cytokine-expressing vectors.

4.3. Other Fusogenic Envelope Proteins

In 2007, Hoffman et al. examined the therapeutic efficacy of Ad vectors expressing the RSV fusion peptide RSV-F or vesicular stomatitis virus (VSV) fusion peptide VSV-G [77,78]. Reduction in tumor growth in mice treated with Ad.RSV-F was similar to that observed for Ad.MV-H/F noted above. Although Ad.VSV-G also mediated a significant anti-tumor effect, it was less effective than Ad vector expressing either of the other two fusogenic proteins. Another potential FMG for immune stimulation is the simian virus SV5-F protein. The SV5-F protein is a potent antigen, and displayed promise in early cell-culture models of cancer [79]; however, no follow up experiments were performed to further explore its anticancer capacity in vivo. Considering these results, the measles virus fusion proteins,

as well as other similar fusogenic glycoproteins, offer considerable promise in arming oncolytic Ad, but this remains to be tested experimentally.

4.4. Fusion Associated Small Transmembrane Proteins

Unlike the FMGs discussed above, the FAST proteins of the *Reoviridae* family were discovered in non-enveloped viruses [58]. The primary function of all of the FMGs described above is to mediate fusion of the viral membrane to the host membrane during virus internalization. Non-enveloped viruses do not enter the cell through fusion, therefore the primary function of the non-structural FAST proteins is believed to be to aid in lateral transmission of the virus.

As their name implies, FAST proteins are small in size, and include the 10 kDa proteins from Nelson Bay and avian reoviruses, the 14 kDa reptilian reovirus protein and the 15 kDa baboon reovirus protein [58]. Their small size makes them excellent candidates for use in oncolytic viruses, since they readily fall within the cloning capacity of most vector systems. In the context of Ad, use of such small fusogenic proteins means that there would be sufficient remaining cloning capacity to accommodate a tissue-specific promoter to confer tumor selectivity or a second transgene (e.g., a cytokine to enhance immune stimulation) in the same vector. Early studies showed that transfection of a plasmid expressing the p14 FAST protein induced extensive syncytium formation and apoptosis, once again suggesting that these proteins may be ideal as anti-cancer therapeutics [63].

To evaluate the ability of the p14 FAST protein to elicit an anti-cancer effect, Brown et al. published work examining expression of the p14 FAST protein from an otherwise wild-type VSV [94]. In cell culture, this VSV/FAST recombinant virus induced the formation of large syncytia with no deleterious effect on VSV replication. After intravenous injection, the VSV/FAST virus entered the central nervous system of BALB/c mice more rapidly and replicated to a higher titer than VSV/GFP (1 to 4 logs higher between 24 and 96 h post infection). When delivered intranasally, VSV/FAST also caused clinical pathogenesis (hind-limb paralysis) at a much lower dose than VSV/GFP (1×10^7 plaque-forming unit (PFU) vs. 5×10^8 PFU per mouse, respectively). These results indicate that the p14 FAST protein can act as a virulence factor in non-endogenous viruses. The efficacy of FAST was also examined in a VSV derivative that is selective for replication in cancer cells, VSVΔ51 [95,96]. VSVΔ51/FAST, was tested for oncolytic potency in combination with VVDD, a double-deleted vaccinia virus whose replication is restricted to cells that overexpress the E2F transcription factor [96]. The two viruses were chosen for their complementary effects; VVDD naturally inhibits production of type I interferons (IFNs), while VSVΔ51 is sensitive to this antiviral pathway [95,97]. Compared to treatment with VVDD/VSVΔ51, VVDD/VSVΔ51FAST caused increased cell killing (shown by crystal violet assay) and a 100-fold increase in VVDD production. These results suggest that the p14 FAST protein can be used in non-endogenous oncolytic viruses to enhance efficiency.

Due to the attractive properties of p14 FAST, our lab created a non-replicating Ad, AdFAST, expressing the p14 FAST protein from the high-activity cytomegalovirus immediate early enhancer/promoter (CMV) replacing the E1 region [62]. In A549 lung adenocarcinoma cells, AdFAST efficiently caused formation of syncytia at high multiplicity of infection (MOI). Syncytium formation was shown to reduce metabolic activity compared to a control Ad vector lacking a transgene. AdFAST-treated cells showed a significant increase in cleaved caspase-3 compared to cells treated with control virus, suggesting the AdFAST induced apoptosis in the treated cells. Unfortunately, these promising in vitro results did not translate in vivo, as immunodeficient mice bearing subcutaneous A549 tumors did not show improved tumor regression or prolonged lifespan when treated with AdFAST compared to animals treated with control virus or vehicle. AdFAST was also tested in an immunocompetent, syngeneic mouse model of cancer but, unfortunately, no increase in treated mouse survival or reduction in tumor growth was observed [80]. Studies in vitro in 293 cells, in which the AdFAST virus can replicate due to complementation of the E1-deletion in this cell line, showed that extensive fusion and a decrease in metabolic activity could be achieved at a very low MOI [62]. These observations suggest that the ineffectiveness of AdFAST in the A549 model may

be due to inefficient levels of FAST protein expression, and that FAST may be more effective when included in an oncolytic Ad vector. Replication of the oncolytic Ad would dramatically increase the template copy number of the FAST gene within the tumor cell, resulting in enhanced FAST protein expression and, likely, improved tumor regression.

5. Trans-Complementing Adenovirus Systems for Expression of Fusion Proteins

Despite the relatively large cloning capacity of Ad, it can be difficult to encode traditional FMGs in replication competent Ad due to DNA packaging limits [98,99]. The Ad capsid can accommodate about 105% of the normal genome length, giving a cloning capacity of about 1.8 kb in a vector that does not contain any compensatory deletions. Most traditional FMGs are rather large proteins and often require multiple subunits [55,71], and either can not be accommodated in a single Ad vector (especially if the vector retains the E1 region, such as in most oncolytic Ad) or preclude the ability to incorporate additional therapeutic genes (such as immune modulators). Chen et al. (2011) circumvented this issue by using tumors based on 293 cells, which complement the E1-deletion in traditional Ad vectors permitting replication in these E1+ tumors [67]. The authors explored the efficacy of an E1-deleted Ad vector encoding the GaLV FMG under regulation by the human endothelial receptor tyrosine kinase (eTie1) promoter, which is a vascular endothelial specific promoter activated in proliferating endothelium, such as that found during tumor angiogenesis [67]. The study sought to improve Ad escape from the vasculature of the tumor following intravenous (i.v.) injection. Quantitative polymerase chain reaction (qPCR) analysis of xenograft tissue extracted from tumor-bearing mice treated with Ad-eTie1-GALV showed three-fold higher genome copy numbers compared to animals that had received a control Ad lacking the fusion gene. Furthermore, hybrid cells derived from the fusion of the 293 cells and endothelial cells were observed in the tumor periphery, suggesting improved escape of Ad from the vasculature mediated by virus-induced fusion.

Vile et al. overcame the cloning limitations in Ad vectors by using plasmid-mediated expression of the FMG in combination with delivery of a therapeutic, oncolytic Ad [68]. Plasmid-mediated expression of GaLV FMG in combination with delivery of Ad5 wild-type was able to eradicate slow-growing tumors in 4 of 10 treated mice, leading to long term survival [68]. In faster-growing tumors, expression of the FMG and viral replication could not keep tumor growth in check and survival increases were minimal. This study also showed that co-expression of the FMG with replicating Ad increased viral release and spread through a monolayer of cells in vitro. In this co-culture experiment, human glioma U87 tumor cells were infected with AdGFP and Ad5 wild-type (WT), and subsequently transfected with a plasmid expressing GaLV FMG. When these cells were co-cultured with mouse B16 cells, which lack the PiT-1 receptor for GaLV (and therefore can not fuse to U87 cells), AdGFP spread to the B16 cells at a higher frequency relative to un-transfected cells, suggesting a fusion-independent increase in viral spread induced by enhanced release of the virus in the FMG-expressing cells.

Another method to circumvent the cloning restrictions of Ad is through *trans*-complementation. When a host cell is infected with multiple Ad strains, the proteins from both viruses are expressed, and complementation of missing genes can occur. Thus, when cells are co-infected with an E1-deleted Ad carrying a copy of an FMG gene and a replication competent Ad carrying the E1 sequences necessary for replication, viral progeny can be produced from both viruses, and viable Ad carrying the FMG transgene can spread to new cells [100]. An E1-deleted Ad expressing the MeV fusion proteins (Ad.H/F) was tested in combination with the replication-restricted vector Ad.COX-MK [74,75]. This latter vector put two regions critical for Ad replication, the E1 and the E4 regions, under the control of the cyclooxygenase-2 and midkine promoters, respectively, restricting replication of the virus to pancreatic and colon cancer cells. This combination of vectors was tested both in xenograft models of pancreatic and colorectal cancer, and in combination with multiple chemotherapeutics. In human pancreatic subcutaneous xenografts (CFPAC-1), the triple-treatment combination of Ad.H/F, Ad.COX-MK and gemcitabine, an antineoplastic chemotherapeutic, resulted in the greatest reduction in tumor volume compared to all other combinations. In intraperitoneal xenografts, which mimic

metastatic disease, the combination of Ad.H/F and Ad.COX:MK resulted in one long-term survivor (alive at the end of the observation period, 120 days) of eight treated mice, while the triple combination resulted in three long-term survivors. No other treatment group had survivors. When tested in a colorectal cancer xenograft model in mice, a similar trend was observed when the viruses were combined with the chemotherapeutic FOLFOX (folinic acid, 5-fluorouracil and oxaliplatin). The group also found a synergistic effect between an Ad5 mutant bearing Ad35 fiber (for improved tumor penetrance), and Ad.H/F in melanoma models of cancer [76]. Taken together, these results show that expression of fusogenic proteins can be used to increase the potency of replication-competent Ad.

6. Expression of Fusogenic Proteins from Replication Competent Adenovirus

To date, few groups have successfully shown expression of a fusogenic protein from a replication competent Ad, mainly due to two barriers to vector propagation. First, as discussed above, DNA packaging limitations of the Ad capsid limits the amount of “extra” DNA that can be included in the vector, and genes for fusogenic proteins tend to be large. Second, high-level expression of the fusogenic protein during vector propagation may lead to premature cell death, resulting in an inability to effectively grow stocks of the virus. In 1989, Dewar et al. used an E1-competent, E3-deleted Ad to express the human immunodeficiency virus (HIV) envelope glycoproteins (Ad5env) [81], which was the first evidence that Ad replication was compatible with cell–cell fusion. Twelve years later, Li et al. showed that expression of the HIV envelope glycoprotein from this same virus increased viral release, spread, and replication, compared to wildtype Ad5, in an HeLa-CD4+ cell culture model, which is permissive for HIV envelope protein-mediated fusion [82].

As mentioned, incorporating GaLV and other FMGs into conditionally replicating Ad has proven difficult, as FMGs often cause cell death prior to generation of high titers of virus. Some evidence suggests that tetracycline regulation of toxic transgenes could help to bypass this problem by restricting expression of the transgene during viral propagation *in vitro* [101]. Another strategy to circumvent this issue is to place the FMG under regulation by the Ad MLP, thus allowing the virus to begin replication prior to expression of the lethal fusogenic protein [69]. This strategy was used to generate a conditionally replicating virus containing the GaLV FMG under regulation of the MLP, with E1 expression controlled by a tumor-selective promoter containing eight E2F-binding sites and one specificity protein 1 (Sp1)-binding, designated ICOVIR16. This virus showed potent anti-tumor activity in mouse xenograft subcutaneous models of pancreatic cancer (NP18) and melanoma (SkMe128) [70]. ICOVIR16 was significantly better than its parent virus, ICOVIR15, which contains only the regulated E1 expression and lacks an FMG, at reducing tumor volume following intratumoral injection. In addition, the virus also showed anti-tumor efficacy following systemic administration, which would be important for potentially reaching undetected metastases. As mouse cells are non-permissive for Ad replication, a Syrian hamster model was used to assess toxicity of the armed virus. Importantly, arming of ICOVIR16 did not increase the systemic toxicity of the virus, suggesting that the presence of the fusogenic protein did not affect tumor-specific replication, thus offering a promising platform for future studies. Therefore, equipping selectively replicating Ad with fusogenic proteins is a powerful strategy to increase antitumor activity with no apparent increase in toxicity.

7. Conclusions

The promising results from early experiments with armed Ad in the fight against inoperable tumors has led to the development of a variety of strategies to combat this devastating disease. Expression of fusogenic proteins from viral vectors can not only act as an effective sole therapeutic, directly resulting in cancer cell killing, but can also enhance virus spread between cancer cells in a tumor mass, and promote release of syncytiosomes that may augment the anti-tumor immune response. It is likely that these fusogenic proteins are best suited for use in the context of conditionally replicating Ad vectors, to take full advantage of the ability of fusogenic proteins to aid in virus spread and dispersion in a tumor. Continued research into new and more potent fusogenic proteins, as well as

development of more specific and active conditionally replicating Ad vectors will undoubtedly further enhance this very promising area of anti-cancer therapeutic research.

Acknowledgments: We thank Bratati Saha and Leslie Nash, and other members of the Parks lab, for critical evaluation of the manuscript and valuable discussions. Research in the Parks laboratory is supported by grants from the Canadian Institutes of Health Research (CIHR), the National Sciences and Engineering Research Council (NSERC) and the Cancer Research Society. Joshua Del Papa was supported by a Queen Elizabeth II Graduate Scholarship in Science and Technology from the Ontario government.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Garcia, M.; Jemal, A.; Ward, E.; Center, M.; Hao, Y.; Siegel, R.; Thun, M. Global cancer facts & figures 2007. *Atlanta GA Am. Cancer Soc.* **2007**, *1*, 1–52.
2. Torre, L.A.; Bray, F.; Siegel, R.L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A. Global cancer statistics, 2012. *CA Cancer J. Clin.* **2015**, *65*, 87–108. [[CrossRef](#)] [[PubMed](#)]
3. Schwab, M. *Encyclopedia of Cancer*; Schwab, M., Ed.; Springer: Heidelberg, Germany, 2008.
4. Green, D.M.; Kun, L.E.; Matthay, K.K.; Meadows, A.T.; Meyer, W.H.; Meyers, P.A.; Spunt, S.L.; Robison, L.L.; Hudson, M.M. Relevance of historical therapeutic approaches to the contemporary treatment of pediatric solid tumors. *Pediatr. Blood Cancer* **2013**, *60*, 1083–1094. [[CrossRef](#)] [[PubMed](#)]
5. Asghar, U.; Witkiewicz, A.K.; Turner, N.C.; Knudsen, E.S. The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nat. Rev. Drug Discov.* **2015**, *14*, 130–146. [[CrossRef](#)] [[PubMed](#)]
6. García-Cao, I.; García-Cao, M.; Martín-Caballero, J.; Criado, L.M.; Klatt, P.; Flores, J.M.; Weill, J.C.; Blasco, M.A.; Serrano, M. ‘Super p53’ mice exhibit enhanced DNA damage response, are tumor resistant and age normally. *EMBO J.* **2002**, *21*, 6225–6235. [[CrossRef](#)] [[PubMed](#)]
7. Gottesman, M.M. Mechanisms of cancer drug resistance. *Annu. Rev. Med.* **2002**, *53*, 615–627. [[CrossRef](#)] [[PubMed](#)]
8. Bauerschmitz, G.J.; Barker, S.D.; Hemminki, A. Adenoviral gene therapy for cancer: From vectors to targeted and replication competent agents (review). *Int. J. Oncol.* **2002**, *21*, 1161–1174. [[CrossRef](#)] [[PubMed](#)]
9. Heise, C.; Kim, D.H. Replication-selective adenoviruses as oncolytic agents. *J. Clin. Investig.* **2000**, *105*, 847–851. [[CrossRef](#)] [[PubMed](#)]
10. Kim, D.H.; Wang, Y.; Le Boeuf, F.; Bell, J.; Thorne, S.H. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. *PLoS Med* **2007**, *4*, e353. [[CrossRef](#)] [[PubMed](#)]
11. Senzer, N.N.; Kaufman, H.L.; Amatruda, T.; Nemunaitis, M.; Reid, T.; Daniels, G.; Gonzalez, R.; Glaspy, J.; Whitman, E.; Harrington, K. Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J. Clin. Oncol.* **2009**, *27*, 5763–5771. [[CrossRef](#)] [[PubMed](#)]
12. Lichty, B.D.; Power, A.T.; Stojdl, D.F.; Bell, J.C. Vesicular stomatitis virus: Re-inventing the bullet. *Trends Mol. Med.* **2004**, *10*, 210–216. [[CrossRef](#)] [[PubMed](#)]
13. Khuri, F.R.; Nemunaitis, J.; Ganly, I.; Arseneau, J.; Tannock, I.F.; Romel, L.; Gore, M.; Ironside, J.; MacDougall, R.; Heise, C. A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat. Med.* **2000**, *6*, 879–885. [[CrossRef](#)] [[PubMed](#)]
14. Greig, S.L. Talimogene laherparepvec: First global approval. *Drugs* **2016**, *76*, 147–154. [[CrossRef](#)] [[PubMed](#)]
15. Peng, Z. Current status of gendicine in China: Recombinant human Ad-p53 agent for treatment of cancers. *Hum. Gene Ther.* **2005**, *16*, 1016–1027. [[CrossRef](#)] [[PubMed](#)]
16. Xia, Z.; Chang, J.; Zhang, L.; Jiang, W.; Guan, Z.; Liu, J.; Zhang, Y.; Hu, X.; Wu, G.; Wang, H. Phase III randomized clinical trial of intratumoral injection of E1B gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus. *Chin. J. Cancer* **2004**, *23*, 1666–1670.
17. Edelstein, M. Gene Therapy Clinical Trials Worldwide. Available online: <http://www.abedia.com/wiley/> (accessed on 25 July 2016).

18. DeWeese, T.L.; van der Poel, H.; Li, S.; Mikhak, B.; Drew, R.; Goemann, M.; Hamper, U.; DeJong, R.; Detorie, N.; Rodriguez, R. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* **2001**, *61*, 7464–7472. [[PubMed](#)]
19. Vecil, G.G.; Lang, F.F. Clinical trials of adenoviruses in brain tumors: A review of Ad-p53 and oncolytic adenoviruses. *J. Neuro-Oncol.* **2003**, *65*, 237–246. [[CrossRef](#)]
20. Sauthoff, H.; Hu, J.; Maca, C.; Goldman, M.; Heitner, S.; Yee, H.; Pipiya, T.; Rom, W.N.; Hay, J.G. Intratumoral spread of wild-type adenovirus is limited after local injection of human xenograft tumors: Virus persists and spreads systemically at late time points. *Hum. Gene Ther.* **2003**, *14*, 425–433. [[CrossRef](#)] [[PubMed](#)]
21. Harrison, D.; Sauthoff, H.; Heitner, S.; Jagirdar, J.; Rom, W.N.; Hay, J.G. Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved—deletion of the viral E1b-19-kD gene increases the viral oncolytic effect. *Hum. Gene Ther.* **2001**, *12*, 1323–1332. [[CrossRef](#)] [[PubMed](#)]
22. Guedan, S.; Rojas, J.J.; Gros, A.; Mercade, E.; Cascallo, M.; Alemany, R. Hyaluronidase expression by an oncolytic adenovirus enhances its intratumoral spread and suppresses tumor growth. *Mol. Ther.* **2010**, *18*, 1275–1283. [[CrossRef](#)] [[PubMed](#)]
23. Toth, K.; Djeha, H.; Ying, B.; Tollefson, A.E.; Kuppuswamy, M.; Doronin, K.; Krajcsi, P.; Lipinski, K.; Wrighton, C.J.; Wold, W.S. An oncolytic adenovirus vector combining enhanced cell-to-cell spreading, mediated by the ADP cytolytic protein, with selective replication in cancer cells with deregulated Wnt signaling. *Cancer Res.* **2004**, *64*, 3638–3644. [[CrossRef](#)] [[PubMed](#)]
24. Yumul, R.; Richter, M.; Lu, Z.Z.; Saydaminova, K.; Wang, H.; Wang, C.H.; Carter, D.; Lieber, A. Epithelial Junction Opener Improves Oncolytic Adenovirus Therapy in Mouse Tumor Models. *Hum. Gene Ther.* **2016**, *27*, 325–337. [[CrossRef](#)] [[PubMed](#)]
25. Dmitriev, I.; Krasnykh, V.; Miller, C.R.; Wang, M.; Kashentseva, E.; Mikheeva, G.; Belousova, N.; Curiel, D.T. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J. Virol.* **1998**, *72*, 9706–9713. [[PubMed](#)]
26. Li, Y.; Pong, R.-C.; Bergelson, J.M.; Hall, M.C.; Sagalowsky, A.I.; Tseng, C.-P.; Wang, Z.; Hsieh, J.-T. Loss of adenoviral receptor expression in human bladder cancer cells: A potential impact on the efficacy of gene therapy. *Cancer Res.* **1999**, *59*, 325–330. [[PubMed](#)]
27. Vigne, E.; Mahfouz, I.; Dedieu, J.-F.; Brie, A.; Perricaudet, M.; Yeh, P. RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J. Virol.* **1999**, *73*, 5156–5161. [[PubMed](#)]
28. Poulin, K.L.; Lanthier, R.M.; Smith, A.C.; Christou, C.; Quiroz, M.R.; Powell, K.L.; O'Meara, R.W.; Kothary, R.; Lorimer, I.A.; Parks, R.J. Retargeting of adenovirus vectors through genetic fusion of a single-chain or single-domain antibody to capsid protein IX. *J. Virol.* **2010**, *84*, 10074–10086. [[CrossRef](#)] [[PubMed](#)]
29. Poulin, K.L.; Tong, G.; Vorobyova, O.; Pool, M.; Kothary, R.; Parks, R.J. Use of Cre/loxP recombination to swap cell binding motifs on the adenoviral capsid protein IX. *Virology* **2011**, *420*, 146–155. [[CrossRef](#)] [[PubMed](#)]
30. Nwanegbo, E.; Vardas, E.; Gao, W.; Whittle, H.; Sun, H.; Rowe, D.; Robbins, P.D.; Gambotto, A. Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. *Clin. Diagn. Lab. Immunol.* **2004**, *11*, 351–357. [[CrossRef](#)] [[PubMed](#)]
31. Mast, T.C.; Kierstead, L.; Gupta, S.B.; Nikas, A.A.; Kallas, E.G.; Novitsky, V.; Mbebe, B.; Pitisuttithum, P.; Schechter, M.; Vardas, E. International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: Correlates of high Ad5 titers and implications for potential HIV vaccine trials. *Vaccine* **2010**, *28*, 950–957. [[CrossRef](#)] [[PubMed](#)]
32. Tsai, V.; Johnson, D.E.; Rahman, A.; Wen, S.F.; LaFace, D.; Philopena, J.; Nery, J.; Zepeda, M.; Maneval, D.C.; Demers, G.W. Impact of human neutralizing antibodies on antitumor efficacy of an oncolytic adenovirus in a murine model. *Clin. Cancer Res.* **2004**, *10*, 7199–7206. [[CrossRef](#)] [[PubMed](#)]
33. Martin, M.; Knipe, D.; Fields, B.; Howley, P.; Griffin, D.; Lamb, R. Adenoviridae: The viruses and their replication. *Fields' Virology* **2007**, *2*, 2355–2395.
34. Kojaghlanian, T.; Flomenberg, P.; Horwitz, M.S. The impact of adenovirus infection on the immunocompromised host. *Rev. Med. Virol.* **2003**, *13*, 155–171. [[CrossRef](#)] [[PubMed](#)]

35. Giberson, A.N.; Davidson, A.R.; Parks, R.J. Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Res.* **2012**, *40*, 2369–2376. [[CrossRef](#)] [[PubMed](#)]
36. Davison, A.J.; Benkő, M.; Harrach, B. Genetic content and evolution of adenoviruses. *J. Gen. Virol.* **2003**, *84*, 2895–2908. [[CrossRef](#)] [[PubMed](#)]
37. Nash, L.A.; Parks, R.J. Adenovirus Biology and Development as a Gene Delivery Vector. In *Gene Therapy with Adenoviral Based Vectors*; Ng, P., Brunetti-Pierri, N., Eds.; Taylor and Francis: New York, NY, USA, 2016.
38. Smith, A.C.; Poulin, K.L.; Parks, R.J. DNA genome size affects the stability of the adenovirus virion. *J. Virol.* **2009**, *83*, 2025–2028. [[CrossRef](#)] [[PubMed](#)]
39. Christensen, J.B.; Byrd, S.A.; Walker, A.K.; Strahler, J.R.; Andrews, P.C.; Imperiale, M.J. Presence of the adenovirus IVa2 protein at a single vertex of the mature virion. *J. Virol.* **2008**, *82*, 9086–9093. [[CrossRef](#)] [[PubMed](#)]
40. O'Malley, R.P.; Mariano, T.M.; Siekierka, J.; Mathews, M.B. A mechanism for the control of protein synthesis by adenovirus VA RNA I. *Cell* **1986**, *44*, 391–400. [[CrossRef](#)]
41. Aparicio, O.; Razquin, N.; Zaratiegui, M.; Narvaiza, I.; Fortes, P. Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *J. Virol.* **2006**, *80*, 1376–1384. [[CrossRef](#)] [[PubMed](#)]
42. Saha, B.; Wong, C.M.; Parks, R.J. The adenovirus genome contributes to the structural stability of the virion. *Viruses* **2014**, *6*, 3563–3583. [[CrossRef](#)] [[PubMed](#)]
43. Nelson, J.E.; Kay, M.A. Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J. Virol.* **1997**, *71*, 8902–8907. [[PubMed](#)]
44. Wolf, J.K.; Bodurka, D.C.; Gano, J.B.; Deavers, M.; Ramondetta, L.; Ramirez, P.T.; Levenback, C.; Gershenson, D.M. A phase I study of Adp53 (INGN 201; ADVEXIN) for patients with platinum- and paclitaxel-resistant epithelial ovarian cancer. *Gynecol. Oncol.* **2004**, *94*, 442–448. [[CrossRef](#)] [[PubMed](#)]
45. Miller, G.; Lahrs, S.; DeMatteo, R.P. Overexpression of interleukin-12 enables dendritic cells to activate NK cells and confer systemic antitumor immunity. *FASEB J.* **2003**, *17*, 728–730. [[CrossRef](#)] [[PubMed](#)]
46. Rodriguez, R.; Schuur, E.R.; Lim, H.Y.; Henderson, G.A.; Simons, J.W.; Henderson, D.R. Prostate attenuated replication competent adenovirus (ARCA) CN706: A selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* **1997**, *57*, 2559–2563. [[PubMed](#)]
47. Ding, M.; Cao, X.; Xu, H.-N.; Fan, J.-K.; Huang, H.-L.; Yang, D.-Q.; Li, Y.-H.; Wang, J.; Li, R.; Liu, X.-Y. Prostate cancer-specific and potent antitumor effect of a DD3-controlled oncolytic virus harboring the PTEN gene. *PLoS ONE* **2012**, *7*, e35153. [[CrossRef](#)] [[PubMed](#)]
48. Bandara, L.R.; La Thangue, N.B. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* **1991**, *351*, 494–497. [[CrossRef](#)] [[PubMed](#)]
49. Jiang, H.; Alemany, R.; Gomez-Manzano, C.; Medrano, D.R.; Lemoine, M.G.; Olson, M.V.; Alonso, M.M.; Lee, O.-H.; Conrad, C.C.; Yung, W.A. Downmodulation of E1 A Protein Expression as a Novel Strategy to Design Cancer-Selective Adenoviruses. *Neoplasia* **2005**, *7*, 723–729. [[CrossRef](#)] [[PubMed](#)]
50. Nevins, J.R. The Rb/E2F pathway and cancer. *Hum. Mol. Genet.* **2001**, *10*, 699–703. [[CrossRef](#)] [[PubMed](#)]
51. Lu, W.; Zheng, S.; Li, X.-F.; Huang, J.-J.; Zheng, X.; Li, Z. Intra-tumor injection of H101, a recombinant adenovirus, in combination with chemotherapy in patients with advanced cancers: A pilot phase II clinical trial. *World J. Gastroenterol.* **2004**, *10*, 3634–3638. [[CrossRef](#)] [[PubMed](#)]
52. Heise, C.C.; Williams, A.; Olesch, J.; Kim, D.H. Efficacy of a replication-competent adenovirus (ONYX-015) following intratumoral injection: Intratumoral spread and distribution effects. *Cancer Gene Ther.* **1999**, *6*, 499–504. [[CrossRef](#)] [[PubMed](#)]
53. Lang, F.F.; Bruner, J.M.; Fuller, G.N.; Aldape, K.; Prados, M.D.; Chang, S.; Berger, M.S.; McDermott, M.W.; Kunwar, S.M.; Junck, L.R. Phase I trial of adenovirus-mediated p53 gene therapy for recurrent glioma: Biological and clinical results. *J. Clin. Oncol.* **2003**, *21*, 2508–2518. [[CrossRef](#)] [[PubMed](#)]
54. Smith, E.; Breznik, J.; Lichty, B.D. Strategies to enhance viral penetration of solid tumors. *Hum. Gene Ther.* **2011**, *22*, 1053–1060. [[CrossRef](#)] [[PubMed](#)]
55. Delassus, S.; Sonigo, P.; Wain-Hobson, S. Genetic organization of gibbon ape leukemia virus. *Virology* **1989**, *173*, 205–213. [[CrossRef](#)]
56. Plattet, P.; Alves, L.; Herren, M.; Aguilar, H.C. Measles Virus Fusion Protein: Structure, Function and Inhibition. *Viruses* **2016**, *8*, 1–30. [[CrossRef](#)] [[PubMed](#)]

57. Zhao, X.; Singh, M.; Malashkevich, V.N.; Kim, P.S. Structural characterization of the human respiratory syncytial virus fusion protein core. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 14172–14177. [[CrossRef](#)] [[PubMed](#)]
58. Shmulevitz, M.; Duncan, R. A new class of fusion-associated small transmembrane (FAST) proteins encoded by the non-enveloped fusogenic reoviruses. *EMBO J.* **2000**, *19*, 902–912. [[CrossRef](#)] [[PubMed](#)]
59. Chernomordik, L.V.; Kozlov, M.M. Mechanics of membrane fusion. *Nat. Struct. Mol. Biol.* **2008**, *15*, 675–683. [[CrossRef](#)] [[PubMed](#)]
60. Bateman, A.; Bullough, F.; Murphy, S.; Emilusen, L.; Lavillette, D.; Cosset, F.L.; Cattaneo, R.; Russell, S.J.; Vile, R.G. Fusogenic membrane glycoproteins as a novel class of genes for the local and immune-mediated control of tumor growth. *Cancer Res.* **2000**, *60*, 1492–1497. [[PubMed](#)]
61. Bateman, A.R.; Harrington, K.J.; Kottke, T.; Ahmed, A.; Melcher, A.A.; Gough, M.J.; Linardakis, E.; Riddle, D.; Dietz, A.; Lohse, C.M.; et al. Viral fusogenic membrane glycoproteins kill solid tumor cells by nonapoptotic mechanisms that promote cross presentation of tumor antigens by dendritic cells. *Cancer Res.* **2002**, *62*, 6566–6578. [[PubMed](#)]
62. Wong, C.M.; Poulin, K.L.; Tong, G.; Christou, C.; Kennedy, M.A.; Falls, T.; Bell, J.C.; Parks, R.J. Adenovirus-Mediated Expression of the p14 Fusion-Associated Small Transmembrane Protein Promotes Cancer Cell Fusion and Apoptosis In Vitro but Does Not Provide Therapeutic Efficacy in a Xenograft Mouse Model of Cancer. *PLoS ONE* **2016**, *11*, e0151516. [[CrossRef](#)] [[PubMed](#)]
63. Salsman, J.; Top, D.; Boutilier, J.; Duncan, R. Extensive syncytium formation mediated by the reovirus FAST proteins triggers apoptosis-induced membrane instability. *J. Virol.* **2005**, *79*, 8090–8100. [[CrossRef](#)] [[PubMed](#)]
64. Tan, L.; Jia, H.; Liu, R.; Wu, J.; Han, H.; Zuo, Y.; Yang, S.; Huang, W. Inhibition of NF- κ B in fusogenic membrane glycoprotein causing HL-60 cell death: Implications for acute myeloid leukemia. *Cancer Lett.* **2009**, *273*, 114–121. [[CrossRef](#)] [[PubMed](#)]
65. Allen, C.; McDonald, C.; Giannini, C.; Peng, K.W.; Rosales, G.; Russell, S.J.; Galanis, E. Adenoviral vectors expressing fusogenic membrane glycoproteins activated via matrix metalloproteinase cleavable linkers have significant antitumor potential in the gene therapy of gliomas. *J. Gene Med.* **2004**, *6*, 1216–1227. [[CrossRef](#)] [[PubMed](#)]
66. Brade, A.M.; Szmitko, P.; Ngo, D.; Liu, F.F.; Klamut, H.J. Heat-directed tumor cell fusion. *Hum. Gene Ther.* **2003**, *14*, 447–461. [[CrossRef](#)] [[PubMed](#)]
67. Chen, H.H.; Cawood, R.; El-Sherbini, Y.; Purdie, L.; Bazan-Peregrino, M.; Seymour, L.W.; Carlisle, R.C. Active adenoviral vascular penetration by targeted formation of heterocellular endothelial-epithelial syncytia. *Mol. Ther.* **2011**, *19*, 67–75. [[CrossRef](#)] [[PubMed](#)]
68. Ahmed, A.; Jevremovic, D.; Suzuki, K.; Kottke, T.; Thompson, J.; Emery, S.; Harrington, K.; Bateman, A.; Vile, R. Intratumoral expression of a fusogenic membrane glycoprotein enhances the efficacy of replicating adenovirus therapy. *Gene Ther.* **2003**, *10*, 1663–1671. [[CrossRef](#)] [[PubMed](#)]
69. Guedan, S.; Gros, A.; Cascallo, M.; Vile, R.; Mercade, E.; Alemany, R. Syncytia formation affects the yield and cytotoxicity of an adenovirus expressing a fusogenic glycoprotein at a late stage of replication. *Gene Ther.* **2008**, *15*, 1240–1245. [[CrossRef](#)] [[PubMed](#)]
70. Guedan, S.; Grases, D.; Rojas, J.J.; Gros, A.; Vilardell, F.; Vile, R.; Mercade, E.; Cascallo, M.; Alemany, R. GALV expression enhances the therapeutic efficacy of an oncolytic adenovirus by inducing cell fusion and enhancing virus distribution. *Gene Ther.* **2012**, *19*, 1048–1057. [[CrossRef](#)] [[PubMed](#)]
71. Alkhatib, G.; Richardson, C.; Shen, S.H. Intracellular processing, glycosylation, and cell-surface expression of the measles virus fusion protein (F) encoded by a recombinant adenovirus. *Virology* **1990**, *175*, 262–270. [[CrossRef](#)]
72. Galanis, E.; Bateman, A.; Johnson, K.; Diaz, R.M.; James, C.D.; Vile, R.; Russell, S.J. Use of viral fusogenic membrane glycoproteins as novel therapeutic transgenes in gliomas. *Hum. Gene Ther.* **2001**, *12*, 811–821. [[CrossRef](#)] [[PubMed](#)]
73. Hoffmann, D.; Bayer, W.; Wildner, O. In situ tumor vaccination with adenovirus vectors encoding measles virus fusogenic membrane proteins and cytokines. *World J. Gastroenterol.* **2007**, *13*, 3063–3070. [[PubMed](#)]
74. Hoffmann, D.; Wildner, O. Enhanced killing of pancreatic cancer cells by expression of fusogenic membrane glycoproteins in combination with chemotherapy. *Mol. Cancer Ther.* **2006**, *5*, 2013–2022. [[CrossRef](#)] [[PubMed](#)]
75. Hoffmann, D.; Bangen, J.M.; Bayer, W.; Wildner, O. Synergy between expression of fusogenic membrane proteins, chemotherapy and facultative virotherapy in colorectal cancer. *Gene Ther.* **2006**, *13*, 1534–1544. [[CrossRef](#)] [[PubMed](#)]

76. Hoffmann, D.; Bayer, W.; Heim, A.; Potthoff, A.; Nettelbeck, D.M.; Wildner, O. Evaluation of twenty-one human adenovirus types and one infectivity-enhanced adenovirus for the treatment of malignant melanoma. *J. Investig. Dermatol.* **2008**, *128*, 988–998. [[CrossRef](#)] [[PubMed](#)]
77. Hoffmann, D.; Bayer, W.; Grunwald, T.; Wildner, O. Intratumoral expression of respiratory syncytial virus fusion protein in combination with cytokines encoded by adenoviral vectors as in situ tumor vaccine for colorectal cancer. *Mol. Cancer Ther.* **2007**, *6*, 1942–1950. [[CrossRef](#)] [[PubMed](#)]
78. Hoffmann, D.; Bayer, W.; Wildner, O. Therapeutic immune response induced by intratumoral expression of the fusogenic membrane protein of vesicular stomatitis virus and cytokines encoded by adenoviral vectors. *Int. J. Mol. Med.* **2007**, *20*, 673–681. [[PubMed](#)]
79. Gomez-Trevino, A.; Castel, S.; Lopez-Iglesias, C.; Cortadellas, N.; Comas-Riu, J.; Mercade, E. Effects of adenovirus-mediated SV5 fusogenic glycoprotein expression on tumor cells. *J. Gene Med.* **2003**, *5*, 483–492. [[CrossRef](#)] [[PubMed](#)]
80. Wong, C.M.; Nash, L.A.; Del Papa, J.; Poulin, K.L.; Falls, T.; Bell, J.C.; Parks, R.J. Expression of the fusogenic p14 FAST protein from a replication-defective adenovirus vector does not provide a therapeutic benefit in an immunocompetent mouse model of cancer. *Cancer Gene Ther.* **2016**, *23*, 355–364. [[CrossRef](#)] [[PubMed](#)]
81. Dewar, R.L.; Vasudevachari, M.; Natarajan, V.; Salzman, N. Biosynthesis and processing of human immunodeficiency virus type 1 envelope glycoproteins: Effects of monensin on glycosylation and transport. *J. Virol.* **1989**, *63*, 2452–2456. [[PubMed](#)]
82. Li, H.; Haviv, Y.S.; Derdeyn, C.A.; Lam, J.; Coolidge, C.; Hunter, E.; Curiel, D.T.; Blackwell, J.L. Human immunodeficiency virus type 1-mediated syncytium formation is compatible with adenovirus replication and facilitates efficient dispersion of viral gene products and de novo-synthesized virus particles. *Hum. Gene Ther.* **2001**, *12*, 2155–2165. [[CrossRef](#)] [[PubMed](#)]
83. Van Zeijl, M.; Johann, S.V.; Closs, E.; Cunningham, J.; Eddy, R.; Shows, T.B.; O'Hara, B. A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 1168–1172. [[CrossRef](#)] [[PubMed](#)]
84. Kavanaugh, M.P.; Kabat, D. Identification and characterization of a widely expressed phosphate transporter/retrovirus receptor family. *Kidney Int.* **1996**, *49*, 959–963. [[CrossRef](#)] [[PubMed](#)]
85. Forestell, S.P.; Dando, J.S.; Chen, J.; de Vries, P.; Bohnlein, E.; Rigg, R.J. Novel retroviral packaging cell lines: Complementary tropisms and improved vector production for efficient gene transfer. *Gene Ther.* **1997**, *4*, 600–610. [[CrossRef](#)] [[PubMed](#)]
86. Fielding, A.K.; Chapel-Fernandes, S.; Chadwick, M.P.; Bullough, F.J.; Cosset, F.L.; Russell, S.J. A hyperfusogenic gibbon ape leukemia envelope glycoprotein: Targeting of a cytotoxic gene by ligand display. *Hum. Gene Ther.* **2000**, *11*, 817–826. [[CrossRef](#)] [[PubMed](#)]
87. Johnson, K.J.; Peng, K.W.; Allen, C.; Russell, S.J.; Galanis, E. Targeting the cytotoxicity of fusogenic membrane glycoproteins in gliomas through protease-substrate interaction. *Gene Ther.* **2003**, *10*, 725–732. [[CrossRef](#)] [[PubMed](#)]
88. Wust, P.; Hildebrandt, B.; Sreenivasa, G.; Rau, B.; Gellermann, J.; Riess, H.; Felix, R.; Schlag, P.M. Hyperthermia in combined treatment of cancer. *Lancet Oncol.* **2002**, *3*, 487–497. [[CrossRef](#)]
89. Katz, S.L.; Yanagi, Y.; Kemper, C.; Navaratnarajah, C.K.; Rima, B.K. *Measles: History and Basic Biology*, 1st ed.; Griffin, D.E., Oldstone, M.B., Eds.; Springer: Heidelberg, Germany, 2008.
90. Nakamura, T.; Peng, K.W.; Vongpunswad, S.; Harvey, M.; Mizuguchi, H.; Hayakawa, T.; Cattaneo, R.; Russell, S.J. Antibody-targeted cell fusion. *Nat. Biotechnol.* **2004**, *22*, 331–336. [[CrossRef](#)] [[PubMed](#)]
91. Hammond, A.L.; Plemper, R.K.; Zhang, J.; Schneider, U.; Russell, S.J.; Cattaneo, R. Single-chain antibody displayed on a recombinant measles virus confers entry through the tumor-associated carcinoembryonic antigen. *J. Virol.* **2001**, *75*, 2087–2096. [[CrossRef](#)] [[PubMed](#)]
92. Peng, K.W.; Donovan, K.A.; Schneider, U.; Cattaneo, R.; Lust, J.A.; Russell, S.J. Oncolytic measles viruses displaying a single-chain antibody against CD38, a myeloma cell marker. *Blood* **2003**, *101*, 2557–2562. [[CrossRef](#)] [[PubMed](#)]
93. Linardakis, E.; Bateman, A.; Phan, V.; Ahmed, A.; Gough, M.; Olivier, K.; Kennedy, R.; Errington, F.; Harrington, K.J.; Melcher, A.; et al. Enhancing the efficacy of a weak allogeneic melanoma vaccine by viral fusogenic membrane glycoprotein-mediated tumor cell-tumor cell fusion. *Cancer Res.* **2002**, *62*, 5495–5504. [[PubMed](#)]

94. Brown, C.W.; Stephenson, K.B.; Hanson, S.; Kucharczyk, M.; Duncan, R.; Bell, J.C.; Lichty, B.D. The p14 FAST protein of reptilian reovirus increases vesicular stomatitis virus neuropathogenesis. *J. Virol.* **2009**, *83*, 552–561. [[CrossRef](#)] [[PubMed](#)]
95. Stojdl, D.F.; Lichty, B.D.; Paterson, J.M.; Power, A.T.; Knowles, S.; Marius, R.; Reynard, J.; Poliquin, L.; Atkins, H.; Brown, E.G. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **2003**, *4*, 263–275. [[CrossRef](#)]
96. Le Boeuf, F.; Diallo, J.-S.; McCart, J.A.; Thorne, S.; Falls, T.; Stanford, M.; Kanji, F.; Auer, R.; Brown, C.W.; Lichty, B.D. Synergistic interaction between oncolytic viruses augments tumor killing. *Mol. Ther.* **2010**, *18*, 888–895. [[CrossRef](#)] [[PubMed](#)]
97. Symons, J.A.; Alcamí, A.; Smith, G.L. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell* **1995**, *81*, 551–560. [[CrossRef](#)]
98. Bett, A.; Prevec, L.; Graham, F. Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* **1993**, *67*, 5911–5921. [[PubMed](#)]
99. Kennedy, M.A.; Parks, R.J. Adenovirus virion stability and the viral genome: Size matters. *Mol. Ther.* **2009**, *17*, 1664–1666. [[CrossRef](#)] [[PubMed](#)]
100. Imler, J.-L.; Bout, A.; Dreyer, D.; Dieterlé, A.; Schultz, H.; Valerio, D.; Mehtali, M.; Pavirani, A. Trans-complementation of E1-deleted adenovirus: A new vector to reduce the possibility of codissemination of wild-type and recombinant adenoviruses. *Hum. Gene Ther.* **1995**, *6*, 711–721. [[CrossRef](#)] [[PubMed](#)]
101. Tietge, U.J.; Kozarsky, K.F.; Donahee, M.H.; Rader, D.J. A tetracycline-regulated adenoviral expression system for in vivo delivery of transgenes to lung and liver. *J. Gene Med.* **2003**, *5*, 567–575. [[CrossRef](#)] [[PubMed](#)]



© 2017 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

Appendix 2: Published Manuscript

An Oncolytic Adenovirus Vector Expressing p14 FAST Protein Induces Widespread Syncytium Formation and Reduces Tumor Growth Rate *In Vivo*

Josh Del Papa,^{1,2,3} Julia Petryk,⁴ John C. Bell,^{2,3,4} and Robin J. Parks^{1,2,3,5}

¹Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON K1H 8L6, Canada; ²Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON K1N 6N5, Canada; ³Department of Medicine, University of Ottawa, Ottawa, ON K1N 6N5, Canada; ⁴Cancer Therapeutics Program, Ottawa Hospital Research Institute, Ottawa, ON K1H 8L6, Canada; ⁵Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON K1N 6N5, Canada

Intratumoral injection of oncolytic viruses provides a direct means of tumor cell destruction for inoperable tumors. Unfortunately, oncolytic vectors based on human adenovirus (HAdV) typically do not spread efficiently throughout the tumor mass, reducing the efficacy of treatment. In this study, we explore the efficacy of a conditionally replicating HAdV vector expressing the p14 Fusion-Associated Small Transmembrane (FAST) protein (CRAdFAST) in both immunocompetent and immunodeficient mouse models of cancer. The p14 FAST protein mediates cell-cell fusion, which may enhance spread of the virus-mediated, tumor cell-killing effect. In the murine 4T1 model of cancer, treatment with CRAdFAST resulted in enhanced cell death compared to vector lacking the p14 FAST gene, but it did not reduce the tumor growth rate *in vivo*. In the human A549 lung adenocarcinoma model of cancer, CRAdFAST showed significantly improved oncolytic efficacy *in vitro* and *in vivo*. In an A549 xenograft tumor model *in vivo*, CRAdFAST induced tumor cell fusion, which led to the formation of large acellular regions within the tumor and significantly reduced the tumor growth rate compared to control vector. Our results indicate that expression of p14 FAST from an oncolytic HAdV can improve vector efficacy for the treatment of cancer.

INTRODUCTION

Despite continuing efforts to curb the immense burden of cancer through treatment, research, and prevention, approximately 40% of the human population will be diagnosed with some form of cancer during their lifetime.¹ Approximately half of these patients will succumb to the disease, underscoring the need for new therapeutic options. Oncolytic viruses, which are designed to specifically replicate in and kill cancer cells, have shown tremendous efficacy in preclinical studies.^{2–4} Currently, there are a large number of oncolytic viruses in phase I, II, and III clinical trials, based on a diverse array of viruses, including adenovirus, reovirus, and vaccinia, among others, while one, the herpes simplex virus type 1 (HSV-1)-based T-Vec, is approved for use in patients by the United States Food and Drug Administration, the European Medical Agency, and the Australian

Therapeutic Goods Administration.^{5–9} The development and improvement of oncolytic viruses continues to be an important field of study.

Human adenovirus (HAdV) has been explored extensively as an anti-cancer agent, using two main strategies. Replication-deficient HAdV vectors can be used to deliver anti-cancer genes (e.g., p53), immunomodulators (e.g., interleukin [IL]-12), or other potentially therapeutic genes to tumor cells.^{10,11} Indeed, a non-replicating HAdV encoding p53, termed Gendicine, is widely available to patients in China.¹² Alternatively, oncolytic HAdV can be engineered to replicate specifically in cancer cells while leaving normal tissue relatively unaffected,¹³ and Oncorine, a conditionally replicating HAdV, is commercially available to patients in China.¹⁴ Unfortunately, when injected intratumorally, HAdV typically does not disperse far from the injection tract, leaving much of the tumor unaffected.^{15,16} Indeed, although HAdV-5 (serotype 5) encodes proteins within the early region 3 (E3) specifically dedicated to enhancing release of virus from the infected cell at very late times post-infection,¹⁷ much of the virus still remains cell associated.

For this reason, a number of approaches have been explored to improve HAdV dispersion in a tumor, including the expression of extracellular matrix-degrading enzymes, junction-opening peptides, and proteins intended to improve cytolysis.^{18–21} Alternatively, several research groups have evaluated the ability of fusogenic proteins, such as those encoded by the gibbon ape leukemia virus (GALV) or measles virus, to enhance spread of virus-associated cell death through the tumor mass through direct cell-cell fusion.^{22–25} However, one issue with the use of many of these fusion proteins is their size: inclusion of the coding sequences for these fusogenic proteins, such as the GALV fusion protein (2,004 bp), in an oncolytic HAdV approaches

Received 25 October 2018; accepted 1 May 2019;
<https://doi.org/10.1016/j.omto.2019.05.001>.

Correspondence: Robin J. Parks, Regenerative Medicine Program, Ottawa Hospital Research Institute, 501 Smyth, Ottawa, ON K1H 8L6, Canada.
E-mail: rparks@ohri.ca



Molecular Therapy: Oncolytics Vol. 14 September 2019 © 2019 The Authors. 107
This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

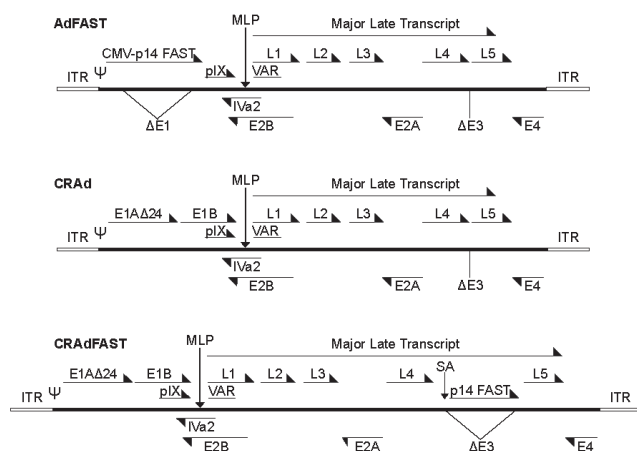


Figure 1. Adenovirus Constructs

Schematic diagrams of HAdV vectors used in this study: AdFAST, an E1- and E3-deleted non-replicating HAdV vector expressing p14 FAST from the cytomegalovirus immediate early enhancer-promoter in the E1; CRAd, an E1Δ24, E3-deleted conditionally replicating vector; and CRAdFAST, an E1Δ24, E3-deleted conditionally replicating vector expressing p14 FAST from within the E3 region through the inclusion of a major late promoter splice acceptor (SA).

or exceeds the upper limits for DNA packaging in the viral capsid.^{26,27} This DNA-packaging limit may preclude the use of other DNA elements designed to enhance vector efficacy, such as tumor-specific promoters to confer selective viral replication or inclusion of other therapeutic genes designed to arm the oncolytic HAdV vector. Although inclusion of the E3 can increase oncolytic efficacy, its inclusion further limits the ability of such viruses to harbor ancillary regulatory elements or genes.^{28,29}

The p14 Fusion-Associated Small Transmembrane (p14 FAST) protein is a small (14-kDa, 375-bp) fusogenic protein from a reptilian reovirus.³⁰ Unlike most fusogenic proteins, p14 FAST is non-structural (i.e., not a component of an infecting virion), but it likely enhances lateral virus spread between host cells through its ability to promote cell-cell fusion. The exact mechanism of p14 FAST protein-associated fusion has yet to be elucidated; however, due to its small size and relatively minute ectodomain, p14 FAST protein likely does not act through a mechanism similar to typical structural viral fusion proteins.^{31,32} Early studies showing that plasmid-mediated expression of p14 FAST protein induced syncytium formation and, subsequently, apoptosis in the fused cells suggested that p14 FAST protein may show efficacy as a sole therapeutic or could be used to enhance the efficacy of oncolytic viruses.³³ Indeed, expression of p14 FAST protein from an oncolytic vesicular stomatitis virus (VSV) (VSVΔ51FAST) enhanced the therapeutic efficacy of the virus, and co-delivery of VSV-FAST with an oncolytic vaccinia virus also provided a synergistic effect in cell culture and *ex vivo* models of cancer.^{34,35}

In previous studies, our lab explored the efficacy of an early region 1 (E1)-deleted, replication-deficient HAdV expressing the p14 FAST protein (AdFAST) in both immunodeficient human A549 lung adenocarcinoma xenograft-bearing CD-1 nude mice and immuno-

competent BALB/c 4T1 tumor models.^{36,37} In cell culture, AdFAST induced significant cell fusion and cell death relative to control vector. Higher levels of virus were needed to achieve this effect in the mouse 4T1 cell line, in part due to lower infectivity and p14 FAST protein expression relative to the human A549 cell line. Unfortunately, these very promising *in vitro* results did not translate *in vivo*, where no effects on tumor growth or mouse survival were observed. Interestingly, during the course of our *in vitro* studies, we showed that, under conditions in which the E1-deleted AdFAST virus could replicate (i.e., in the E1-complementing 293 cell line), p14 FAST protein caused significantly more efficient cell fusion and killing with greatly reduced quantity of vector. This observation suggests that p14 FAST protein may provide the greatest efficacy when expressed from a replicating, oncolytic HAdV. In this context, expression of the p14 FAST protein would amplify as the virus replicates, potentially providing greater cell fusion and enhanced induction of apoptosis, in addition to facilitating vector spread through the tumor.

In this study, we have investigated the efficacy of an oncolytic, conditionally replicating HAdV (CRAd) vector encoding the p14 FAST protein in tissue culture and animal models of cancer.

RESULTS

CRAdFAST Can Replicate and Express Viral Genes but Does Not Produce Progeny Virions in Mouse 4T1 Cells

Previously, we showed that vectors based on HAdV-5 infected 4T1 mouse mammary carcinoma cells at an efficiency approximately one-sixth that of A549 cells, a cell line that infects well with HAdV and is used commonly in many HAdV studies.³⁷ HAdV can replicate its DNA and express both early and late genes in many mouse cell lines, but typically infection is not productive (i.e., progeny virions are not produced). We therefore examined whether HAdV infection was productive in 4T1 cells.

4T1 cells were infected at an MOI of 3 with AdFAST (E1 deleted, replication defective) or CRAdFAST (E1Δ24, replication competent), and total DNA was harvested for qPCR analysis of viral genome content 4–72 h later. The HAdV vector structures are shown in Figure 1. As shown in Figure 2A, as expected, infection of 4T1 cells with the replication-defective AdFAST vector did not result in an increase

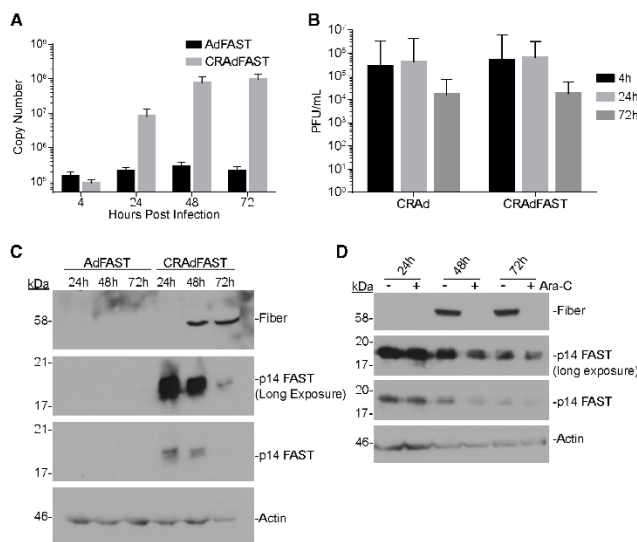


Figure 2. Replication of HAdV-5 Oncolytic Vectors in 4T1 Cells Is Non-productive

(A) 4T1 cells were infected with either AdFAST or CRAdFAST at an MOI of 10. At 4, 24, 48, and 72 hpi, media were removed and the cells were harvested in SDS-Proteinase K buffer for DNA extraction. qPCR was performed on 200 ng total isolated DNA for the Ad hexon genome region. Error bars represent mean $\pm$ SD. (B) 4T1 cells were infected with either CRAd or CRAdFAST at an MOI of 10. Cells and medium were collected at 4, 24, and 72 hpi. Viral titers were determined by plaque assay on 293 cells. Error bars represent mean $\pm$ SD. (C) 4T1 cells were infected with either AdFAST or CRAdFAST at an MOI of 3 and harvested at 24, 48, and 72 hpi. Immunoblot analysis was performed to examine HAdV fiber and HA (p14 FAST) protein levels, and actin was used as a loading control. (D) 4T1 cells were infected at an MOI of 50 with CRAdFAST for 1 h, and they were overlaid with medium lacking (–) or containing (+) 20 μ g/mL cytosine arabinoside (Ara-C) to inhibit DNA replication. Crude protein samples were harvested at 24, 48, and 72 hpi, and they were assayed by immunoblot for the expressions of fiber protein, p14 FAST protein, and actin (loading control).

in viral genome copy number over the course of the experiment. In contrast, we observed an approximate 1,000-fold increase in viral genome copy number in CRAdFAST-treated 4T1 cells between 4 and 72 h post-infection (hpi), clearly showing that the virus can replicate its DNA in this cell line.

To determine if infection of 4T1 cells was productive, cells were infected at an MOI of 10 with CRAd or CRAdFAST, and the cells were harvested into the medium at various times post-infection and titered for virus. As shown in Figure 2B, we did not observe an increase in the amount of recovered virus between 4 and 72 hpi, suggesting that CRAd and CRAdFAST did not give rise to progeny virions during this time period. However, the increase in genome copy number as the virus replicates its DNA should effectively amplify viral gene expression, leading to the accumulation of high levels of virus-encoded proteins, including the p14 FAST protein, within the infected cells.

We next evaluated the expression of virus-encoded proteins, specifically the native viral fiber gene and p14 FAST. CRAdFAST contains the p14 FAST cDNA preceded by a splice acceptor (SA) site replacing the E3 deletion, which should allow for high-level expression of p14 FAST only when the virus replicates, a strategy that we and others have employed previously.^{38–40} 4T1 cells were infected with AdFAST (E1 deleted, replication defective) or CRAdFAST, and protein expression was examined at 24, 48, and 72 hpi. As expected, treatment of cells with AdFAST did not result in a detectable expression of fiber protein (Figure 2C). At this low MOI of 3, p14 FAST protein expression was also not detectable in AdFAST-treated cells, consistent with

our previous observation that a high MOI (>100) is required to visualize FAST expression in this cell line.³⁷ In contrast, likely due to the increase in template copy number within the cells infected with CRAdFAST (Figure 2A), expressions of both p14 FAST protein and the late fiber protein were easily detectable for this virus (Figure 2C).

Interestingly, we detected the expression of p14 FAST protein at a much earlier time point compared to fiber protein. If the onset of fiber protein expression correlates with active virus DNA replication, it suggests that p14 FAST protein expression may precede replication (i.e., is under early regulation) in 4T1 cells. To assess this possibility, we examined the expression of fiber and p14 FAST protein in the presence or absence of cytosine arabinoside (cytarabine, Ara-C), a compound that blocks viral DNA replication. Since the major late promoter is not activated until viral genome replication begins,⁴¹ treatment with Ara-C should prevent the expression of fiber and p14 FAST protein if they are indeed under late regulation. 4T1 cells were infected with CRAdFAST at an MOI of 50 in the absence or presence of 20 μ g/mL Ara-C, and they were harvested at 24, 48, and 72 hpi. As shown in Figure 2D, while fiber expression was indeed inhibited by treatment with Ara-C, p14 FAST expression was unaffected by this treatment. Thus, although CRAdFAST can provide very high-level expression of p14 FAST protein in the 4T1 mouse cell line, unlike endogenous viral genes (i.e., fiber), its expression does not appear to be conditionally tied to active virus replication.

Expression of p14 FAST Protein from CRAd Enhances 4T1 Cell Killing *In Vitro*

We next examined whether the elevated expression of p14 FAST protein from CRAdFAST enhanced the ability of the oncolytic

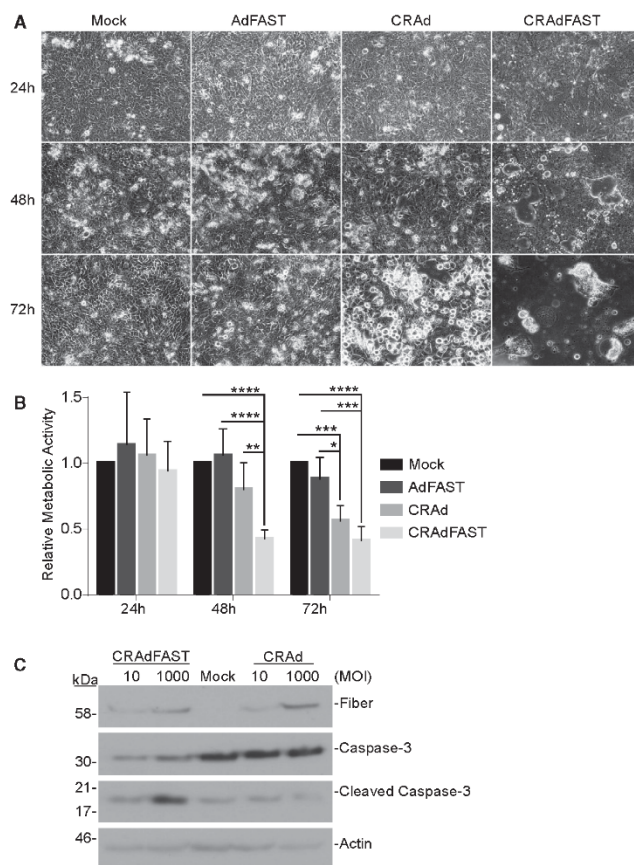


Figure 3. CRAdFAST Induces Cellular Fusion and Cell Death in 4T1 Cells More Efficiently Than Non-replicating AdFAST

(A) 4T1 cells were mock infected or infected with AdFAST, CRAd, or CRAdFAST at an MOI of 100. Phase-contrast images were obtained at 24, 48, and 72 hpi. (B) 4T1 cells were mock infected or infected with AdFAST, CRAd, or CRAdFAST at an MOI of 1,000 in triplicate wells ($n = 6$ technical replicates, $n = 2$ independent experimental replicates). At 48 hpi, metabolic activity was assessed by MTS assay, and the values are reported relative to metabolic activity in mock-infected cells. Mean values reported $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (C) 4T1 cells were mock infected or infected with CRAd or CRAdFAST at MOIs of 10 and 1,000, and protein samples were harvested at 48 hpi. Protein lysates from infected cells were subjected to immunoblot for HAdV fiber, full-length caspase-3, and cleaved caspase-3, with actin used as a loading control.

fused and exhibited extensive CPE. Thus, treatment of 4T1 cells with the replication-competent CRAdFAST results in greatly enhanced fusion relative to the replication-defective AdFAST.

To determine whether enhanced cell fusion mediated by CRAdFAST was associated with 4T1 cell death, we first examined metabolic activity of the treated cells over time. 4T1 cells were infected at an MOI of 1,000 with AdFAST, CRAd, or CRAdFAST or mock infected with PBS, and metabolic activity was examined at 24-h intervals by MTS assay. Treatment of cells with AdFAST did not result in a decrease in metabolic activity at any of the time points examined (Figure 3B).

In contrast, CRAdFAST significantly reduced metabolic activity of the cells as early as 48 hpi. By 72 hpi, treatment with CRAd also induced a decrease in metabolic activity compared to AdFAST- and mock-treated cells.

We also examined the cells for evidence of apoptotic cell death by immunoblot analysis for cleaved caspase-3 (Figure 3C). Treatment of 4T1 cells with CRAd did not result in enhanced levels of cleaved caspase-3 compared to untreated cells. In contrast, treatment of cells with CRAdFAST did result in enhanced cleavage of caspase-3, but only at a high MOI. Taken together, these results indicate that treatment of 4T1 cells with CRAdFAST results in high-level expression of p14 FAST protein and significant cell fusion, resulting in a reduction in cellular metabolic activity and the onset of apoptosis-like cell death. Importantly, all of these effects are enhanced relative to 4T1 cells

vector to mediate cell fusion and cause cancer cell death in 4T1 cells in culture. 4T1 cells were infected with AdFAST, CRAd, or CRAdFAST at an MOI of 100 or mock infected with PBS, and the cells were analyzed visually for cell fusion by phase-contrast microscopy at varying times post-infection. We did not observe evidence of fusion in mock- or AdFAST-treated cells at any of the time points (Figure 3A), consistent with our previous work showing that a very high MOI of AdFAST was required to achieve visible fusion in 4T1 cells.³⁷ Cells treated with CRAd showed evidence of cytopathic effect (CPE), but only at the 72-h time point, and no cell fusion occurred. However, CRAdFAST-treated 4T1 cells showed clear evidence of cell fusion as early as 24 hpi, which increased over the 72-h time course. By 72 hpi, the entire monolayer of CRAdFAST-treated 4T1 cells had

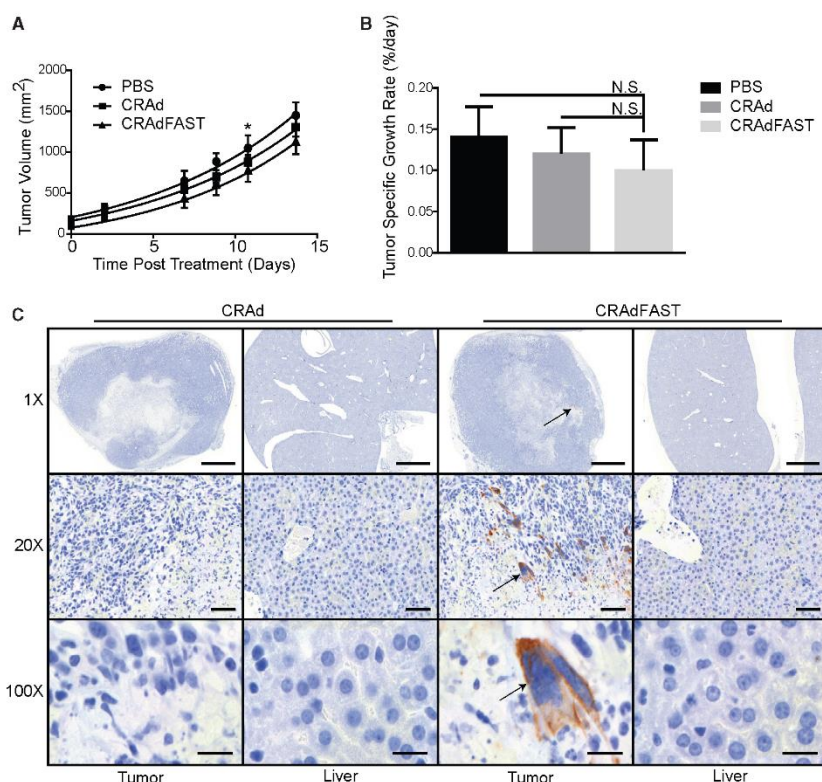


Figure 4. Expression of p14 FAST Protein Does Not Improve Oncolytic Efficacy of CRAd in the Murine 4T1 Model of Cancer

Tumor-bearing mice were injected intratumorally with PBS or 7×10^9 PFU CRAd or CRAdFAST, and tumor volumes were measured for eight mice per group three times weekly. (A) Symbols represent mean calculated tumor volumes over time. Lines represent non-linear regression model of tumor growth rates. At day 11, PBS-treated tumors were significantly larger than CRAdFAST-treated tumors ($p = 0.03$). Error bars represent mean $\pm$ SEM; * $p = 0.033$ for CRAdFAST compared to PBS. (B) Tumor-specific growth rates were calculated for each tumor and averaged; error bars represent SD. (C) Sections of tumor and liver from mice sacrificed at day 5 post-injection were probed with anti-HA antibody (p14 FAST) and counterstained with hematoxylin to assess p14 FAST expression. At 1 $\times$, 20 $\times$, and 100 $\times$ magnifications, scale bars represent 2,000, 100, and 20 μ m, respectively.

treated with AdFAST¹ or CRAd, suggesting that arming an oncolytic HAdV vector with p14 FAST protein enhances the anti-neoplastic potential of the vector.

Expression of p14 FAST Protein from CRAd Does Not Improve Therapeutic Efficacy in the 4T1 Murine Model of Cancer in Immunocompetent Mice

We next examined whether p14 FAST protein expression could improve the efficacy of CRAd in the immunocompetent 4T1 mouse model of cancer. BALB/c mice bearing 4T1 subcutaneous tumors were injected intratumorally with PBS or 7×10^9 plaque-forming

units (PFU) of CRAd or CRAdFAST¹, and tumor size was measured by caliper three times weekly. As shown in Figures 4A and 4B, treatment of tumors with either CRAd or CRAdFAST¹ resulted in a trend toward a reduced growth rate, but it did not reach statistical significance. Tumor-specific growth rates of PBS-treated tumors (0.159%/day) were not significantly different from those of CRAd- (0.143%/day, $p = 0.74$) or CRAdFAST¹-treated tumors (0.110%/day, $p = 0.097$) (Figure 4B). We did not observe tumor regression or stasis, and all animals reached endpoint and were euthanized at day 14. Tumor tissues removed at day 5 post-treatment were stained for hemagglutinin (HA) to detect p14 FAST protein and

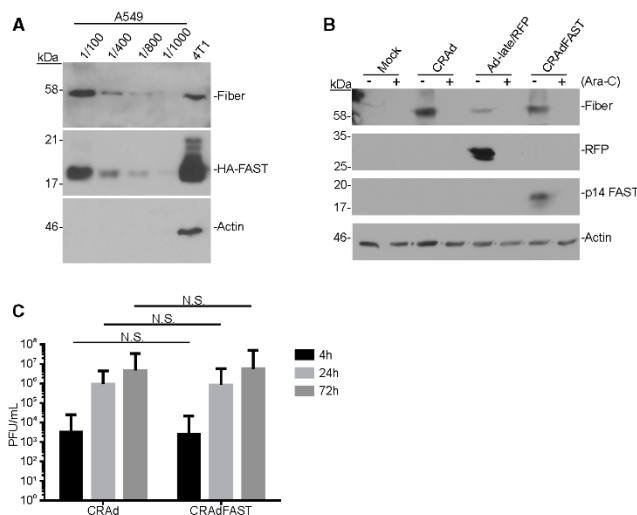


Figure 5. Analysis of Protein Expression and Virus Yield of CRAdFAST in A549 Cells In Vitro

(A) A549 and 4T1 cells were infected at an MOI of 10 and harvested at 48 hpi. A549 samples were diluted to 1/100, 1/400, 1/800, and 1/1,000 to compare protein levels to those obtained in 4T1 cells. (B) A549 cells were infected at an MOI of 3 with CRAd, AdRFP, or CRAdFAST or mock infected, and they were overlaid with medium lacking (–) or containing (+) 20 μ g/mL Ara-C to inhibit viral DNA replication. Cells were harvested at 24 hpi, and immunoblot analysis was performed to examine HAdV fiber, RFP, and HA (p14 FAST) protein levels, with actin used as a loading control. (C) A549 cells were infected at an MOI of 10 with either CRAd or CRAdFAST. Cells and media were harvested at 4, 24, and 72 hpi. Viral titers were determined by plaque assay. Error bars represent mean $\pm$ SD.

examined visually for evidence of syncytia. As expected, CRAd-treated tumors showed no evidence of p14 FAST protein or syncytial formation. p14 FAST protein was detected in tumors from CRAdFAST-treated mice, and syncytia were observed in isolated regions within the treated tumors (Figure 4C). Livers from mice injected with the CRAd or CRAdFAST showed no obvious pathology. Thus, CRAdFAST can infect 4T1 tumors, giving rise to the expression of p14 FAST protein, resulting in syncytium formation, but, ultimately, it does not reduce the tumor growth rate or enhance animal survival.

CRAdFAST Replicates Efficiently in Human A549 Cells

In general, HAdV has an enhanced ability to infect human cell lines compared to mouse cell lines. To examine this in our system, A549 and 4T1 cells were infected at an MOI of 10 with CRAdFAST; crude protein samples were collected 48 hpi, and they were subjected to immunoblot for the relative levels of HAdV fiber and p14 FAST protein (Figure 5A). We compared the undiluted 4T1-infected cell sample to a dilution series of the A549-infected cell sample, to get a more accurate estimation of the relative viral protein expression. As shown in Figure 5A, the quantity of HAdV fiber protein in the 4T1 sample was between 1/100 and 1/400 that present in A549 cells, which would reflect the cumulative effect of differences in infection efficiency, genome replication, and gene expression between the two cell lines. Interestingly, the ratio of p14 FAST protein present in the CRAdFAST-infected 4T1 cells compared to A549 cells appeared proportionately higher than that of fiber. Nevertheless, CRAdFAST can achieve higher levels of infection and gene expression in A549 cells compared to 4T1 cells.

To confirm that p14 FAST protein expression was tied to virus replication in human A549 cells, we again examined gene expression in the presence or absence of the DNA replication inhibitor Ara-C. A549 cells were infected at an MOI of 10 with CRAd, Ad-late/red fluorescent protein (RFP), or CRAdFAST, in the presence or absence of 20 μ g/mL Ara-C, and protein expression was examined 24 h later. Ad-late/RFP is similar in structure to CRAdFAST, but it contains a wild-type E1 and a SA-RFP expression cassette instead of SA-p14 FAST within the E3.³⁹ As shown in Figure 5B, treatment of cells with Ara-C abrogated the expression of the late fiber protein for all of the viruses, and it also inhibited the expression of RFP and p14 FAST from Ad-late/RFP and CRAdFAST, respectively. Thus, inclusion of an expression cassette consisting of a SA and transgene replacing the E3 of an HAdV-5 vector allows for replication-dependent expression of the transgene in human A549 cells, but not the murine 4T1 cell line.

We also examined whether expression of p14 FAST protein altered the replication kinetics of the virus by examining virus yield at varying times post-infection. A549 cells were infected at an MOI of 10, and the cells were harvested into the medium at varying times post-infection and, subsequently, assayed for virus titer. As shown in Figure 5C, infection of A549 cells with both CRAd and CRAdFAST resulted in an $\sim$ 300-fold increase in viral titer between 4 and 24 hpi, which further increased by 72 hpi. Importantly, there was no difference in virus recovery between CRAd containing or lacking the p14 FAST gene, suggesting that the expression of p14 FAST protein did not adversely affect virus growth and recovery.

CRAdFAST Shows Enhanced Cancer Cell Killing In Vitro in Human A549 Cells

We next examined whether expression of p14 FAST protein from CRAdFAST led to fusion and cell death of A549 cells in culture. A549 cells were infected at MOIs 1, 0.1, and 0.01 with AdFAST, CRAd, and CRAdFAST, and phase-contrast images were obtained

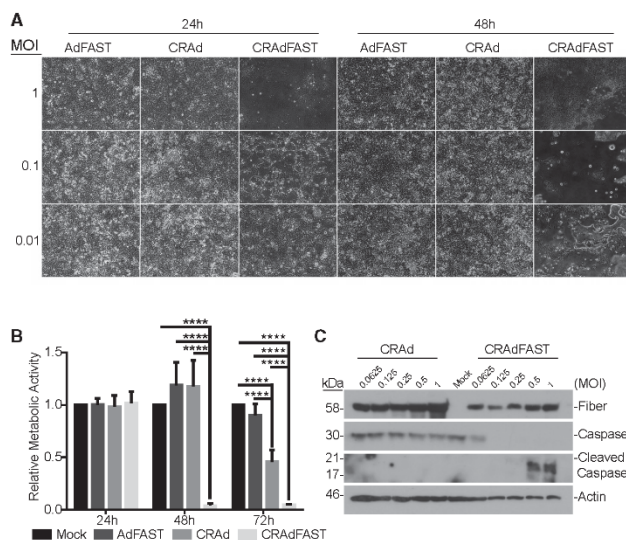


Figure 6. CRAdFAST Mediates Enhanced Cell Fusion and Cell Death in A549 Cells *In Vitro*

(A) Phase-contrast images taken at 24 and 48 hpi of A549 cells infected at MOIs of 0.01, 0.1, and 1 with AdFAST, CRAd, or CRAdFAST. (B) A549 cells were mock infected or infected with AdFAST, CRAd, or CRAdFAST at an MOI of 1 in triplicate ($n = 6$ technical replicates, $n = 2$ independent experimental replicates). At 24, 48, and 72 hpi, metabolic activity was assessed by MTS assay; values reported are relative to mock-infected cells and represent mean $\pm$ SD (**** $p < 0.0001$). (C) A549 cells were mock infected or infected at MOIs of 0.0625, 0.125, 0.25, 0.5, and 1 with CRAd or CRAdFAST, and they were harvested for immunoblot analysis at 48 hpi. Blots were probed for HAdV fiber, full-length caspase-3, or cleaved caspase-3, and actin was used as a loading control.

at 24 and 48 hpi. No fusion was observed in AdFAST-infected cells (Figure 6A), which is consistent with our previous studies showing that an MOI of ~ 50 of this replication-defective virus is required to achieve fusion in this cell line.³⁶ Only minor evidence of CPE was observed in CRAd-infected cells at the highest MOI (MOI = 1) and at the latest time point (48 hpi) examined. In contrast, syncytia were observed in cells treated with CRAdFAST at an MOI as low as 0.1 and as early as 24 hpi. By 48 hpi, even an MOI as low as 0.01 yielded complete monolayer fusion, suggesting that CRAdFAST is very efficient at inducing fusion in A549 cells.

To determine if CRAdFAST-mediated cell fusion impacted A549 cell viability, A549 cells were infected with AdFAST, CRAd, or CRAdFAST or mock infected with PBS, and metabolic activity was assayed at varying times post-infection. Treatment of A549 cells with CRAdFAST caused a dramatic decrease in metabolic activity relative to cells treated with AdFAST or CRAd (Figure 6B). At 48 hpi, cells treated with CRAdFAST showed a metabolic activity of only 4% of untreated cells, which was also significantly lower than that observed for CRAd and AdFAST (117% and 118%, respectively, compared to 4% for CRAdFAST, $p < 0.0001$). By 72 hpi, metabolic activity of CRAdFAST-infected cells remained significantly lower than that of cells treated with the control viruses.

To determine whether this decrease in metabolic activity was associated with cell death, we examined treated cells for the presence of cleaved caspase-3 by immunoblot (Figure 6C). A549 cells were either mock infected or infected with varying amounts of virus (MOI 0.0625–1), and crude protein extracts were prepared 48 h

later. Infection of cells with CRAd did not lead to a decline in the cellular quantity of full-length caspase-3 or formation of the active cleavage product. In contrast, in cells treated with CRAdFAST, full-length caspase-3 was only detectable at the lowest MOI tested, suggesting processing of the protein to its active form, and cleaved caspase-3 was readily detectable at MOIs of 0.5 and higher. Taken together, these data indicate that expression of p14 FAST protein from a CRAd enhances the oncolytic potential of the virus, resulting in significant cell fusion and a decline in cellular metabolic activity due to the induction of apoptotic-like cell death in A549 cells.

Expression of p14 FAST Protein Improves CRAd Efficacy in the Human A549 Xenograft Tumor Model *In Vivo*

As CRAdFAST showed significant efficacy as an oncolytic virus in A549 cells in culture, we tested its potency *in vivo* using an A549 xenograft model of cancer. CD-1 nude mice bearing A549 tumors were injected with a single dose of vehicle (PBS) or 1×10^9 PFU of CRAd or CRAdFAST, and treated tumors were measured by caliper three times weekly. A statistically significant decrease in tumor size was achieved with both CRAd (beginning at day 27) and CRAdFAST (beginning at day 24) relative to animals treated with vehicle alone (Figure 7A). Although CRAdFAST-treated tumors trended toward a smaller size than those treated with CRAd, there was no statistically significant difference between these treatments by day 50 when mice were euthanized. However, tumors treated with CRAdFAST showed a statistically significant reduction in growth rate (CRAdFAST = 3.9 ± 0.45 mm³/day) compared to tumors treated with either PBS (19.2 ± 1.95 mm³/day, $p < 0.0001$) or CRAd (6.7 ± 0.92 mm³/day, $p = 0.0013$) (Figure 7B). Thus, arming an oncolytic HAdV with p14 FAST enhanced oncolytic efficacy of the vector *in vivo* in A549 tumors.

We next examined tumor sections histologically. Since vectors based on HAdV tend to accumulate in the liver, even following intratumoral

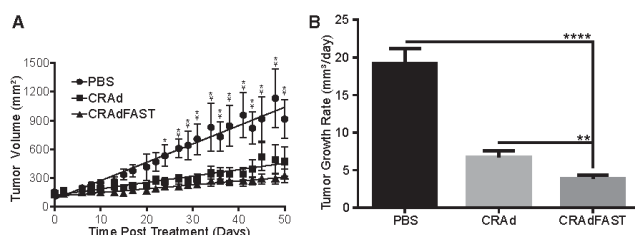


Figure 7. Expression of p14 FAST Protein Significantly Improves Oncolytic Efficacy of CRAdFAST following Intratumoral Injection in a Human A549 Xenograft Model of Cancer

Mice bearing A549 tumors were injected intratumorally with PBS, CRAd, or CRAdFAST. Tumor volumes were measured for five mice per group, three times weekly. (A) Symbols represent calculated tumor volumes over time. Lines represent linear regression model of tumor growth rates. Beyond day 29, PBS-treated tumors are significantly larger than both CRAd- and CRAdFAST-treated tumors. Error bars represent mean $\pm$ SEM; * $p < 0.05$ for CRAdFAST compared to PBS; Y represents $p < 0.05$ for CRAd compared to PBS. (B) Tumor growth rates were calculated for each tumor and averaged; error bars represent SD (** $p = 0.0013$, **** $p < 0.0001$).

injection,⁴² we also examined the livers from treated animals for signs of syncytia or other pathology. Tumor and liver tissue were harvested at 5 and 50 days post-treatment, processed, and stained with anti-HAdV antibody to detect viral gene expression and anti-HA antibody to detect the expression of FAST protein. Anti-HAdV antibody primarily detects structural (late) proteins, which are only expressed after DNA replication has initiated, and it is thus a surrogate marker for active virus replication.⁴³

At 5 days post-injection, both CRAd and CRAdFAST appeared to have replicated efficiently in A549 tumors, as evidenced by the presence of viral late proteins; however, the two viruses showed strikingly different patterns of spatial spread (Figure 8A). While CRAd-treated tumors appeared to have intense HAdV staining localized to pockets in the interior of the tumor, sections from CRAdFAST-treated tumors showed more diffuse staining spread throughout the tumor mass. As expected, immunohistochemistry for the HA-tagged p14 FAST protein was negative in CRAd-treated tumors, and there was no evidence of multinucleated syncytia at either day 5 or day 50 (Figure 8B). In contrast, in CRAdFAST-treated animals, expression of the HA-tagged p14 FAST protein was readily detected in tumors at day 5 and, to a much lesser extent, also at day 50 (Figure 8B). Importantly, HA-positive areas of the tumor showed clear evidence of syncytium formation. Interestingly, although livers from mice injected with CRAdFAST stained positive with anti-HAdV at day 5 post-vector injection, no anti-HAdV staining was observed for CRAd (Figure 8C). Staining of liver sections for the HA-tagged p14 FAST protein showed small regions of positive staining around blood vessels at day 5 (Figure 8D), accompanied by apparent syncytium formation, which was resolved by day 50, suggesting this transient effect resolved without intervention and produced no obvious long-term effect on liver health.

Taken together, our results indicate that treatment of A549 cells with a CRAd vector expressing p14 FAST protein induces dramatic cell fusion, with a concomitant decline in cellular metabolic activity and induction of apoptosis, resulting in a statistically significant reduction in tumor growth rate *in vivo*, relative to CRAd alone.

DISCUSSION

Oncolytic viruses have shown tremendous potential in preclinical studies, which, in many cases, has not translated to significant efficacy in clinical trials. Like many oncolytic viruses, HAdV has a poor ability to home to the tumor mass following systemic delivery, and, instead, it is primarily sequestered in the liver.^{44,45} Although the amount of virus delivered to the tumor can be increased through direct injection of the vector into the tumor, HAdV typically does not transit far from the injection tract, leaving much of the tumor mass unaffected by the virus.^{15,16,46} One approach to increase the fraction of the tumor impacted by the gene therapy vector is to engineer the virus to express fusion proteins.²⁵ In this manner, the few tumor cells that are initially infected with the virus will fuse with adjacent cells, producing multinucleated syncytia, thus directly spreading the area of the tumor impacted by the virus. This approach can be applied to both non-replicating vectors, allowing a co-expressed therapeutic transgene (e.g., p53) to spread between cells, and replicating oncolytic vectors. For oncolytic vectors, the virus can not only be spread through classical release of progeny virions and uptake by adjacent cells but also through fusion of the infected cell with adjacent naive cells.

In this study, we armed an oncolytic HAdV vector with the p14 FAST protein, and we evaluated the efficacy of the vector in tissue culture and animal models of cancer. Our oncolytic HAdV vector contained the E1A Δ 24 mutation, which confers selective replication in cancer cells that are deficient in the Rb pathway.⁴⁷ To restrict expression of the p14 FAST protein only to cancer cells, we placed it within the late transcription unit, under control by the HAdV major late promoter (MLP) (Figure 1). Transcripts under the control of the MLP are expressed poorly at early times of infection, but they increase to a great extent following the initiation of HAdV DNA replication.⁴⁸ Previously, we used this approach to achieve high-level expression of proteins for retargeting HAdV infection⁴⁰ and to design a reporter virus for high-throughput screening of small molecules affecting HAdV replication.³⁹

Under conditions in which CRAdFAST could replicate, very high-level expression of the p14 FAST protein was achieved (Figures 2 and 5). Interestingly, in 4T1 cells in culture, we observed different

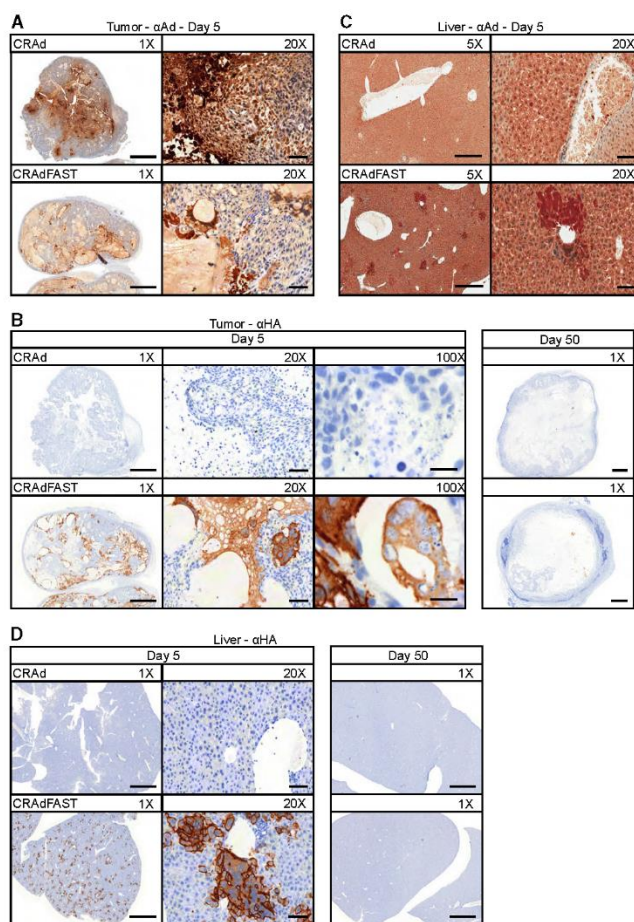


Figure 8. Treatment with CRAdFAST Induces Syncytium Formation in A549 Tumors *In Vivo*

(A) Sections of A549 xenograft tumors from mice treated with CRAd or CRAdFAST and sacrificed at day 5 post-injection were probed with anti-HAdV-5 antibody and counterstained with hematoxylin to assess HAdV replication. Images were taken at 1× and 20× magnifications. (B) Tumor sections from mice treated with CRAd or CRAdFAST and sacrificed at day 5 and day 50 post-injection were probed with anti-HA (p14 FAST) antibody and counterstained with hematoxylin to assess p14 FAST expression. Images were taken at 1×, 20×, and 100× magnifications. (C) Liver sections from mice treated with CRAd or CRAdFAST and sacrificed at day 5 and day 50 post-injection were probed with anti-HA (p14 FAST) and counterstained with hematoxylin to assess p14 FAST expression. Images were taken at 1× and 20× magnifications. (D) Liver sections from mice treated with CRAd or CRAdFAST and sacrificed at day 5 post-injection were probed with anti-HAdV-5 and counterstained with hematoxylin to assess HAdV replication. Images were taken at 5× and 20× magnifications. At 1×, 5×, 20×, and 100×, scale bars represent 2,000, 500, 100, and 20 μm, respectively.

expression for both the late fiber and p14 FAST proteins (Figure 5). Thus, there appear to be fundamental differences in promoter utilization and regulation in the murine 4T1 and human A549 cell lines. It is likely that these differences contribute to species-specific replication of HAdV.

The efficacy of oncolytic vectors is attributed in part to their ability to directly replicate in and kill cancer cells and also through their ability to act as an adjuvant to induce antitumor immunity.^{3,49} Thus, we initially tested CRAdFAST in the 4T1 immunocompetent mouse model of cancer. However, in general, HAdV-5 has a relatively poor ability to infect mouse cells, including 4T1 cells,⁴⁷ and infection is typically not productive (Figure 2). Although treatment of 4T1 cells in

culture with CRAdFAST led to cell fusion, a reduction in cellular metabolic activity, and induction of apoptosis (Figure 3), no beneficial effect was achieved *in vivo* (Figure 4). However, we did observe regions of syncytium formation in CRAdFAST-treated 4T1 tumors, which was not observed in CRAd-treated animals, suggesting that p14 FAST protein could mediate cell fusion *in vivo* (Figure 4).

While inclusion of retargeting ligands can increase infection efficiency,^{40,50–54} the inability of HAdV to productively replicate in mouse cells is a distinct limitation to testing HAdV in mouse models of cancer. Researchers have identified other animal models that are more permissive to HAdV replication, such as the Syrian hamster

temporal expression patterns between p14 FAST and fiber protein (Figure 2B). The p14 FAST protein was detected at an earlier time point (24 hpi) and declined at later time points (72 hpi), whereas HAdV fiber was first detected at 48 hpi and remained constant at 72 hpi. This led us to perform experiments with Ara-C, a DNA replication inhibitor that allows the differentiation between early and late gene expression. In the 4T1 cell line, p14 FAST expression from CRAdFAST appears to be under early, rather than late, transcriptional control (Figure 2). Although the majority of the E3 region is deleted in this viral construct, the early E3 promoter is retained, and it may be influencing the expression of p14 FAST. In human A549 cells, this same vector showed DNA replication-dependent

and cotton rat,^{55,56} which may be more amenable to studying CRAdFAST in the context of an intact innate immune system. The mechanism for species-specific restriction of HAdV replication is unclear, although all aspects of the virus life cycle appear affected (i.e., early gene expression, DNA replication, and late gene expression).^{57,58} HAdV is reported to productively replicate in mouse CMT-64 cells,^{28,59–61} suggesting that this cell line may be a more appropriate model in which to assess efficacy of oncolytic HAdV in immunocompetent animals.

We next evaluated CRAdFAST in a human A549 xenograft model of cancer in immunodeficient mice. In this model, CRAd and CRAdFAST would be expected to replicate and spread to adjacent cells, but the effect of these vectors on antitumor immunity cannot be evaluated. In tissue culture, a very low quantity of CRAdFAST was required to achieve complete fusion of the cell monolayer, which was accompanied by a dramatic decline in cellular metabolic activity and induction of apoptotic-like cell death, as indicated by cleavage of caspase-3 (Figure 6). *In vivo*, treatment with CRAdFAST caused a statistically significant decline in tumor growth rate (Figure 7).

ICOVIR16, a conditionally replicating HAdV expressing the GALV fusion protein, induced a similar reduction in SkMel-28 tumor growth rates following a single intratumoral injection.²² Indeed, inclusion of the GALV fusion protein in ICOVIR16 appeared to have a greater therapeutic effect than we observed for p14 FAST. It is possible that the efficiency with which HAdV can replicate and spread within A549 cells may mask some of the benefits induced by intratumoral syncytium formation in our experiments. Immunohistological examination of tumors treated with CRAd showed intense staining primarily in the center regions of the tumor, whereas CRAdFAST showed less intense but more diffuse staining (Figure 8A). In addition, CRAdFAST-treated tumors had large acellular pockets bordered by regions of significant anti-HA (p14 FAST) staining (Figure 8B). Multinucleated syncytia were also noted in CRAdFAST-treated A549 tumors. Nuclear fragmentation, which is a hallmark of apoptotic cell death, was evident in both CRAd- and CRAdFAST-treated tumors (Figure 8A, 100 $\times$). Interestingly, not all fusogenic proteins induce apoptosis,^{62,63} suggesting that different fusogenic proteins may induce alternate forms of cell death.

Since a large proportion of the initial injectate can escape the tumor and infect the liver,^{42,44} we also examined pathology of the liver in treated animals. Interestingly, although the livers of all animals did not show any gross abnormal pathology at 5 days post-treatment, we did observe significant anti-HAdV staining primarily adjacent to blood vessels in the liver of CRAdFAST-treated animals, which was not present in animals treated with CRAd (Figure 8C). Similarly, anti-HA (p14 FAST) staining revealed the formation of small syncytia in regions adjacent to blood vessels in the liver of CRAdFAST-treated animals (Figure 8D).

The lack of anti-HAdV staining in the liver of mice treated with CRAd suggests that p14 FAST conferred some property that allowed for the

accumulation of viral proteins in the liver of CRAdFAST-treated animals. p14 FAST protein may allow for enhanced replication of CRAd in mouse hepatocytes, although the mechanism by which this would occur is unclear. Cell fusion or syncytium formation within the CRAdFAST-infected tumor may allow for enhanced release of progeny virus into circulation, resulting in enhanced uptake of virus in the liver, which would not occur in CRAd-treated animals. In this scenario, the presence of HAdV proteins in the liver would be due to *de novo* replication within the liver, which we find unlikely given the cancer-specific replication conferred by the E1A Δ 24 mutation.

Alternatively, we and others have shown that expression of fusion proteins, including p14 FAST protein,³⁷ can lead to the enhanced release of exosome-like particles termed syncytiosomes.^{64,65} Syncytiosomes are hypothesized to have the capacity to transport tumor-associated antigen outside of the tumor micro-environment. Thus, the HAdV and p14 FAST proteins present in the liver in CRAdFAST-treated animals may have been released from infected tumor cells in syncytiosomes, which were subsequently taken up by the liver. Consistent with this scenario, we have observed enhanced release of exosome-like particles from CRAdFAST-infected cells (data not shown). Syncytiosomes are also extremely efficient at delivering their contents to dendritic cells, thereby enhancing tumor-associated antigen cross-presentation and activation of T cells.^{64,65} This latter point would obviously be extremely beneficial for the formation of anti-tumor immunity to help mediate tumor regression, thus improving CRAd efficacy in permissive and immunocompetent animal models of cancer and, ultimately, human patients.

MATERIALS AND METHODS

Cell Culture

Human A549 (ATCC CRM-CCL-185)⁶⁶ and 293 cells (ATCC CRL-1573)⁶⁷ were maintained in minimal essential medium (MEM) (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM GlutaMAX (Invitrogen, Waltham, MA, USA), and 1 $\times$ antibiotic-antimycotic (Invitrogen). Mouse 4T1 (ATCC CRL-2539)⁶⁸ cells were maintained in DMEM-high glucose (Sigma-Aldrich) with identical supplements.

Adenoviral Constructs

Schematic representations of all HAdV vectors are shown in Figure 1. All HAdV vectors are based on serotype 5 (HAdV-5), and they were generated by a combination of traditional and RecA-mediated cloning.⁶⁹ AdFAST-HA is an E1- and E3-deleted HAdV vector, it contains the p14 FAST gene under regulation of the human cytomegalovirus (CMV) immediate early enhancer-promoter and bovine growth hormone polyadenylation sequence (BGHpA) replacing the E1 region, and it has been described previously.³⁶ For simplicity, this vector was referred to as AdFAST. HAdV vectors CRAd and CRAdFAST used in this study contain the E1A Δ 24 mutation and are also deleted of the E3 region. E1A Δ 24 prevents the E1A protein from binding the retinoblastoma (Rb) protein, which is required for efficient replication in normal tissues, thus conferring selective replication in cancer cells defective in the Rb pathway.⁴⁷

To link p14 FAST protein expression to virus replication, we placed an expression cassette comprising an SA site-p14 FAST coding sequence replacing the E3 deletion, which places p14 FAST expression under regulation of the viral MLP.^{38–40} Since the MLP is only active at appreciable levels following active virus DNA replication,⁴⁸ p14 FAST protein is only expressed in cells in which the virus can replicate. The p14 FAST proteins expressed from AdFAST and CRAdFAST contain a C-terminal HA epitope tag, as previously described.³⁶ Ad-late/RFP is similar in structure to CRAdFAST, but it contains a wild-type E1 and encodes an SA-monomeric RFP expression cassette within the E3, rather than SA-p14 FAST, and it has been described previously.³⁹ HAdV vector stocks were amplified in 293N3S cells, which were a kind gift of Dr. Frank Graham (McMaster University),⁷⁰ with the exception that CRAdFAST was amplified in A549 cells in suspension culture. Viruses were purified and titered as described previously.⁷¹

Analysis of CRAdFAST Growth Kinetics

To determine whether expression of p14 FAST altered the growth kinetics of the vector, 35-mm dishes of A549 and 4T1 cells were infected at an MOI of 10 PFU/cell with either CRAd or CRAdFAST. At 2, 24, and 72 hpi, the cells were collected into the medium and subjected to two rounds of freeze-thaw to release progeny virions. Viral titers in the initial inoculum and at the various time points were determined by plaque assay.⁷¹

To quantify HAdV genome replication in 4T1 cells, 35-mm dishes of 4T1 cells were infected at an MOI of 3 in 200 μ L PBS with either AdFAST or CRAdFAST for 1 h, washed with PBS, and medium was replaced. At 4, 24, 48, and 72 hpi, medium was removed and the cells were incubated with 500 μ L SDS-Proteinase K buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, 1% SDS [w/v], and 1 mg/mL proteinase K) overnight at 37°C. DNA extraction was performed as described previously,⁷¹ and qPCR was performed using 200 ng isolated DNA and the following primer sets: Hexon (5'-CTT ACCCCCAACGAGTTTGA-3' and 5'-GGAGTACATGCGGTCCT TGT-3') and actin (5'-CCGTACAGGCAGCTCATAGCTCTTC-3' and 5'-CTGAACCCCTAAGGCCAACCGT-3'). qPCR for the actin gene was performed to verify that equal quantities of DNA were present in the samples (data not shown). In parallel, a standard curve of known viral genome quantities was prepared, and it was used to calculate genome concentrations in each sample. Copy numbers were calculated by the method previously described by Whelan et al.⁷²

Immunoblot Analysis

Cells were seeded in 35-mm culture dishes at a density of 6.0×10^5 cells/well. The next day, the cells were infected with virus at varying MOIs for 1 h and the medium was replaced. For experiments including Ara-C, medium containing 20 μ g/mL drug was placed onto the cells after the 1-h infection. At varying times post-infection, the medium was removed, the cells were harvested in $2 \times$ Laemmli buffer (62.5 mM Tris HCl [pH 6.8], 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, and 5% 2-mercaptoethanol), and the samples were stored at -20°C . Harvested samples were boiled for 5 min at

100°C, separated by SDS-PAGE, and transferred using a semi-dry transfer protocol onto polyvinylidene fluoride (PVDF) membrane (Millipore, Etobicoke, ON, Canada). Membranes were probed for β -actin (1:10,000, Clone AC-15 A1978, Sigma-Aldrich), HAdV-5 fiber (1/10,000 dilution, MS-1027-P0, Neomarkers [Fremont, CA, USA]), caspase-3 (1:10,000, 9662, Cell Signaling Technology [Beverly, MA, USA]), cleaved caspase-3 (1:1,000, 9664, Cell Signaling Technology), or HA epitope (1:3,000, 2367, Cell Signaling Technology). Immunoblots were developed using Luminata Classico horseradish peroxidase (HRP) substrate (Millipore) and visualized by autoradiography.

Microscopy

Phase-contrast images were acquired using a Zeiss Axio Observer Microscope (Carl Zeiss Canada, North York, ON, Canada) and analyzed using Zen 2 (Blue Edition) software (Carl Zeiss Canada). Images were processed using GNU Image Manipulation Program (GIMP)⁷³ and compiled using Inkscape software.⁷⁴

Metabolic Activity Assay

Metabolic activity was quantified using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). 4T1 and A549 cells were seeded at 2×10^4 cells/well in flat-bottom 96-well cell culture dishes (Thermo Scientific, Waltham, MA, USA). The next day, the cells were infected with varying MOIs of AdFAST, CRAd, or CRAdFAST in 25 μ L PBS for 1 h, and medium was replaced. Metabolic activity was evaluated at 24, 48, and 72 hpi. 20 μ L MTS substrate was added to each well and incubated in the dark for 1 h. Color development was analyzed in the 96-well plate using a SpectraMax 190 plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

4T1 Syngeneic Cancer Model

All mouse experiments were approved by the Animal Care Committee at the University of Ottawa. For the 4T1 mouse mammary carcinoma tumor model, 30 BALB/c (Charles River Laboratories, Wilmington, MA, USA) mice were injected subcutaneously with 1×10^5 cells in the right hind flank. When tumors reached approximately 5×5 mm (5 days post-injection), the mice received an intratumoral injection of 50 μ L PBS or 7×10^9 PFU CRAd or CRAdFAST diluted in PBS to a total volume of 50 μ L. Then 3 days after vector delivery, two mice from each group were euthanized for histological analysis of tumor and liver samples. For the remainder of the animals, tumor size was measured three times weekly via electronic calipers: two measurements were taken, one at the largest diameter and another perpendicular to the first, and volumes were calculated as $(\text{length} \times \text{width}^2)/2$. The experiment achieved endpoint when any tumor reached 15×15 mm, had a volume of approximately $1,687.5 \text{ mm}^3$, or the mice showed signs of ulceration.

A549 Xenograft Tumor Model

For the A549 human lung adenocarcinoma tumor model, 21 CD-1 nude mice (Charles River Laboratories) were injected subcutaneously with 1×10^6 A549 cells in the right hind flank. Then 15 days later,

when the tumors reached approximately 5×5 mm, the mice received an intratumoral injection of 50 μ L PBS or 1.0×10^9 PFU CRAd or CRAdFAST diluted to a volume of 50 μ L in PBS. Mice were monitored for tumor growth and survival as described above.

Tumor Histology and Immunohistochemistry

Immediately upon removal, tumors and livers were placed in 4% paraformaldehyde (PFA) solution. Then 48 h later, the samples were transferred to 70% ethanol, and they were further processed, embedded, sectioned, and stained with H&E at the University of Ottawa Histology Core Facility. Formalin-fixed, paraffin-embedded slides were deparaffinized in xylene and rehydrated through a 100%–70% ethanol gradient. Heat-induced epitope retrieval was performed at 110°C for 12 min with citrate buffer (pH 6.0), and 3% H₂O₂ was used to block endogenous peroxidases. Sections were further blocked with Background Sniper blocking reagent (BioCare Medical, CA, USA) and incubated with rabbit anti-HA antibody (Cell Signaling Technology, C29F4; 1:1,000 liver, 1:2,000 tumor) and/or rabbit anti-HAdV-5 (Abcam, ab6982; 1:2,000) for 1 h at room temperature. Antibody binding was visualized using the MAHC4TM + DAB detection system according to the manufacturer's instructions (BioCare Medical). Slides were then counterstained with hematoxylin, and images were obtained using the Zeiss Mirax Midi whole-slide digital scanner. Images were analyzed using Pannoramic Viewer software (3DHISTECH, build 1.15.4.43061, Budapest, Hungary), and they were compiled using Inkscape software.⁷⁴

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (version 6.01; GraphPad, San Diego, CA, USA). All bar graphs represent mean, while error bars represent SD. MTS data presented in bar graphs were compared by two-way ANOVA, followed by Tukey's honestly significant difference (HSD) post hoc analysis. 4T1 tumor growth rates presented in bar graphs were modeled using tumor-specific growth rate, as described by Mehrara et al.,⁷⁵ and compared by one-way ANOVA. For the A549 xenograft tumor model, a linear growth model was chosen to analyze growth, as the r^2 values overall were higher for linear regression (0.53 for PBS, 0.33 for CRAd, and 0.42 for CRAdFAST) than for non-linear regression using an exponential model (0.38 for PBS, 0.27 for CRAd, and 0.34 for CRAdFAST). There is significant evidence that tumors often take on a linear growth pattern.⁷⁶

AUTHOR CONTRIBUTIONS

The project was conceived by J.C.B. and R.J.P. and designed by J.D.P. and R.J.P. Data were collected and analyzed by J.D.P., J.P. and J.C.B. contributed to the design and implementation of animal trials. The manuscript was written by J.D.P. and R.J.P. and edited by all authors.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

We thank Kathy Poulin for excellent technical assistance and advice and Dr. Christina Addison (Ottawa Hospital Research Institute) for

providing cytosine arabinoside. Funding was provided by grants to R.J.P. from the Cancer Research Society, Canada (grant number 19363), Canadian Institutes of Health Research, Canada (grant numbers MOP-142316 and MOP-136898), and the Natural Sciences and Engineering Research Council of Canada, Canada (grant number RGPIN-2014-04810). J.D.P. is a recipient of a Queen Elizabeth II Graduate Scholarship in Science and Technology, Ontario, Canada and an Ontario Graduate Scholarship, Ontario, Canada.

REFERENCES

- Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. *CA Cancer J. Clin.* 65, 87–108.
- Stojdl, D.F., Lichty, B., Knowles, S., Marius, R., Atkins, H., Sonenberg, N., and Bell, J.C. (2000). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat. Med.* 6, 821–825.
- Parato, K.A., Senger, D., Forsyth, P.A., and Bell, J.C. (2005). Recent progress in the battle between oncolytic viruses and tumours. *Nat. Rev. Cancer* 5, 965–976.
- Lawler, S.E., Speranza, M.-C., Cho, C.-F., and Chiocca, E.A. (2017). Oncolytic viruses in cancer treatment: a review. *JAMA Oncol.* 3, 841–849.
- DeWeese, T.L., van der Poel, H., Li, S., Mikhak, B., Drew, R., Goemann, M., Hamper, U., DeJong, R., Dettori, N., Rodriguez, R., et al. (2001). A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* 61, 7464–7472.
- Vidal, L., Pandha, H.S., Yap, T.A., White, C.L., Twigger, K., Vile, R.G., Melcher, A., Coffey, M., Harrington, K.J., and DeBono, J.S. (2008). A phase I study of intravenous oncolytic reovirus type 3 Dearing in patients with advanced cancer. *Clin. Cancer Res.* 14, 7127–7137.
- Eder, J.P., Kantoff, P.W., Roper, K., Xu, G.X., Bubley, G.J., Boyden, J., Gritz, L., Mazzara, G., Oh, W.K., Arlen, P., et al. (2000). A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer. *Clin. Cancer Res.* 6, 1632–1638.
- Andrbacka, R.H., Kaufman, H.L., Collichio, F., Amatruda, T., Senzer, N., Chesney, J., Delman, K.A., Spitzer, L.E., Puzanov, I., Agarwala, S.S., et al. (2015). Talmogene laherparepvec improves durable response rate in patients with advanced melanoma. *J. Clin. Oncol.* 33, 2780–2788.
- Rehman, H., Silk, A.W., Kane, M.P., and Kaufman, H.L. (2016). Into the clinic: Talmogene laherparepvec (T-VEC), a first-in-class intratumoral oncolytic viral therapy. *J. Immunother. Cancer* 4, 53.
- Lang, F.F., Bruner, J.M., Fuller, G.N., Aldape, K., Prados, M.D., Chang, S., Berger, M.S., McDermott, M.W., Kunwar, S.M., Junck, L.R., et al. (2003). Phase I trial of adenovirus-mediated p53 gene therapy for recurrent glioma: biological and clinical results. *J. Clin. Oncol.* 21, 2508–2518.
- Narvaiza, I., Mazzolini, G., Barajas, M., Duarte, M., Zaratiegui, M., Qian, C., Melero, I., and Prieto, J. (2000). Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN- γ -inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. *J. Immunol.* 164, 3112–3122.
- Peng, Z. (2005). Current status of gene therapy in China: recombinant human Ad-p53 agent for treatment of cancers. *Hum. Gene Ther.* 16, 1016–1027.
- Khuri, F.R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I.F., Romel, L., Gore, M., Ironside, J., MacDougall, R.H., Heise, C., et al. (2000). A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat. Med.* 6, 879–885.
- Russell, S.J., Peng, K.-W., and Bell, J.C. (2012). Oncolytic virotherapy. *Nat. Biotechnol.* 30, 658–670.
- Sauthoff, H., Hu, J., Maca, C., Goldman, M., Heitner, S., Yee, H., Pipiya, T., Rom, W.N., and Hay, J.G. (2003). Intratumoral spread of wild-type adenovirus is limited after local injection of human xenograft tumors: virus persists and spreads systemically at late time points. *Hum. Gene Ther.* 14, 425–433.

16. Heise, C.C., Williams, A., Olesch, J., and Kirn, D.H. (1999). Efficacy of a replication-competent adenovirus (ONYX-015) following intratumoral injection: intratumoral spread and distribution effects. *Cancer Gene Ther.* 6, 499–504.
17. Tollefson, A.E., Scaria, A., Hermiston, T.W., Ryerse, J.S., Wold, L.J., and Wold, W.S. (1996). The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J. Virol.* 70, 2296–2306.
18. Barton, K.N., Paielli, D., Zhang, Y., Koul, S., Brown, S.L., Lu, M., Seely, J., Kim, J.H., and Freytag, S.O. (2006). Second-generation replication-competent oncolytic adenovirus armed with improved suicide genes and ADP gene demonstrates greater efficacy without increased toxicity. *Mol. Ther.* 13, 347–356.
19. Al-Zaher, A.A., Moreno, R., Fajardo, C.A., Arias-Badia, M., Farrera, M., de Sostoa, J., Rojas, L.A., and Alemany, R. (2018). Evidence of anti-tumoral efficacy in an immune competent setting with an iRGD-modified hyaluronidase-armed oncolytic adenovirus. *Mol. Ther. Oncolytics* 8, 62–70.
20. Guedan, S., Rojas, J.J., Gros, A., Mercade, E., Cascallo, M., and Alemany, R. (2010). Hyaluronidase expression by an oncolytic adenovirus enhances its intratumoral spread and suppresses tumor growth. *Mol. Ther.* 18, 1275–1283.
21. Yumul, R., Richter, M., Lu, Z.Z., Saydamina, K., Wang, H., Wang, C.H., Carter, D., and Lieber, A. (2016). Epithelial Junction Opener Improves Oncolytic Adenovirus Therapy in Mouse Tumor Models. *Hum. Gene Ther.* 27, 325–337.
22. Guedan, S., Grases, D., Rojas, J.J., Gros, A., Vilardell, F., Vile, R., Mercade, E., Cascallo, M., and Alemany, R. (2012). GALV expression enhances the therapeutic efficacy of an oncolytic adenovirus by inducing cell fusion and enhancing virus distribution. *Gene Ther.* 19, 1048–1057.
23. Guedan, S., Gros, A., Cascallo, M., Vile, R., Mercade, E., and Alemany, R. (2008). Syncytia formation affects the yield and cytotoxicity of an adenovirus expressing a fusogenic glycoprotein at a late stage of replication. *Gene Ther.* 15, 1240–1245.
24. Li, H., Haviv, Y.S., Derdeyn, C.A., Lam, J., Coolidge, C., Hunter, E., Curiel, D.T., and Blackwell, J.L. (2001). Human immunodeficiency virus type 1-mediated syncytium formation is compatible with adenovirus replication and facilitates efficient dispersion of viral gene products and de novo-synthesized virus particles. *Hum. Gene Ther.* 12, 2155–2165.
25. Del Papa, J., and Parks, R.J. (2017). Adenoviral Vectors Armed with Cell Fusion-Inducing Proteins as Anti-Cancer Agents. *Viruses* 9, 13.
26. Saha, B., Wong, C.M., and Parks, R.J. (2014). The adenovirus genome contributes to the structural stability of the virion. *Viruses* 6, 3563–3583.
27. Bett, A.J., Prevec, L., and Graham, F.L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* 67, 5911–5921.
28. Wang, Y., Hallden, G., Hill, R., Anand, A., Liu, T.-C., Francis, J., Brooks, G., Lemoine, N., and Kirn, D. (2003). E3 gene manipulations affect oncolytic adenovirus activity in immunocompetent tumor models. *Nat. Biotechnol.* 21, 1328–1335.
29. Suzuki, K., Alemany, R., Yamamoto, M., and Curiel, D.T. (2002). The presence of the adenovirus E3 region improves the oncolytic potency of conditionally replicative adenoviruses. *Clin. Cancer Res.* 8, 3348–3359.
30. Shmulevitz, M., and Duncan, R. (2000). A new class of fusion-associated small transmembrane (FAST) proteins encoded by the non-enveloped fusogenic reoviruses. *EMBO J.* 19, 902–912.
31. Corcoran, J.A., Salsman, J., de Antueno, R., Touhami, A., Jericho, M.H., Clancy, E.K., and Duncan, R. (2006). The p14 fusion-associated small transmembrane (FAST) protein effects membrane fusion from a subset of membrane microdomains. *J. Biol. Chem.* 281, 31778–31789.
32. Ciechonska, M., and Duncan, R. (2014). Reovirus FAST proteins: virus-encoded cellular fusogens. *Trends Microbiol.* 22, 715–724.
33. Salsman, J., Top, D., Bouillier, J., and Duncan, R. (2005). Extensive syncytium formation mediated by the reovirus FAST proteins triggers apoptosis-induced membrane instability. *J. Virol.* 79, 8090–8100.
34. Le Boeuf, F., Gebremeskel, S., McMullen, N., He, H., Greenshields, A.L., Hoskin, D.W., Bell, J.C., Johnston, B., Pan, C., and Duncan, R. (2017). Reovirus FAST Protein Enhances Vesicular Stomatitis Virus Oncolytic Virotherapy in Primary and Metastatic Tumor Models. *Mol. Ther. Oncolytics* 6, 80–89.
35. Le Boeuf, F., Diallo, J.-S., McCart, J.A., Thorne, S., Falls, T., Stanford, M., Kanji, F., Auer, R., Brown, C.W., Lichty, B.D., et al. (2010). Synergistic interaction between oncolytic viruses augments tumor killing. *Mol. Ther.* 18, 888–895.
36. Wong, C.M., Poulin, K.L., Tong, G., Christou, C., Kennedy, M.A., Falls, T., Bell, J.C., and Parks, R.J. (2016). Adenovirus-Mediated Expression of the p14 Fusion-Associated Small Transmembrane Protein Promotes Cancer Cell Fusion and Apoptosis In Vitro but Does Not Provide Therapeutic Efficacy in a Xenograft Mouse Model of Cancer. *PLoS ONE* 11, e0151516.
37. Wong, C.M., Nash, L.A., Del Papa, J., Poulin, K.L., Falls, T., Bell, J.C., and Parks, R.J. (2016). Expression of the fusogenic p14 FAST protein from a replication-defective adenovirus vector does not provide a therapeutic benefit in an immunocompetent mouse model of cancer. *Cancer Gene Ther.* 23, 355–364.
38. Carette, J.E., Graat, H.C., Schagen, F.H., Abou El Hassan, M.A., Gerritsen, W.R., and van Beusechem, V.W. (2005). Replication-dependent transgene expression from a conditionally replicating adenovirus via alternative splicing to a heterologous splice-acceptor site. *J. Gene Med.* 7, 1053–1062.
39. Saha, B., and Parks, R.J. (2019). Histone deacetylase inhibitor SAHA suppresses human adenovirus gene expression and replication. *J. Virol.* Published online April 2019. <https://doi.org/10.1128/JVI.00088-19>.
40. Poulin, K.L., Lanthier, R.M., Smith, A.C., Christou, C., Risco Quiroz, M., Powell, K.L., O'Meara, R.W., Kothary, R., Lorimer, L.A., and Parks, R.J. (2010). Retargeting of adenovirus vectors through genetic fusion of a single-chain or single-domain antibody to capsid protein IX. *J. Virol.* 84, 10074–10086.
41. Gaynor, R.B., Tsukamoto, A., Montell, C., and Berk, A.J. (1982). Enhanced expression of adenovirus transforming proteins. *J. Virol.* 44, 276–285.
42. Bramson, J.L., Hitt, M., Gaudie, J., and Graham, F.L. (1997). Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. *Gene Ther.* 4, 1069–1076.
43. Saha, B., and Parks, R.J. (2017). Human adenovirus type 5 vectors deleted of early region 1 (E1) undergo limited expression of early replication E2 proteins and DNA replication in non-permissive cells. *PLoS ONE* 12, e0181012.
44. Bernt, K.M., Ni, S., Gaggari, A., Li, Z.Y., Shayakhmetov, D.M., and Lieber, A. (2003). The effect of sequestration by nontarget tissues on anti-tumor efficacy of systemically applied, conditionally replicating adenovirus vectors. *Mol. Ther.* 8, 746–755.
45. Ross, P.J., and Parks, R.J. (2003). Oncolytic adenovirus: getting there is half the battle. *Mol. Ther.* 8, 705–706.
46. Wang, Y., Hu, J.K., Krol, A., Li, Y.-P., Li, C.-Y., and Yuan, F. (2003). Systemic dissemination of viral vectors during intratumoral injection. *Mol. Cancer Ther.* 2, 1233–1242.
47. Fueyo, J., Gomez-Manzano, C., Alemany, R., Lee, P.S., McDonnell, T.J., Mitlianga, P., Shi, Y.X., Levin, V.A., Yung, W.K., and Kyritsis, A.P. (2000). A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene* 19, 2–12.
48. Thomas, G.P., and Mathews, M.B. (1980). DNA replication and the early to late transition in adenovirus infection. *Cell* 22, 523–533.
49. Lindenmann, J., and Klein, P.A. (2012). Immunological Aspects of Viral Oncolysis (Springer Science & Business Media).
50. Smith, T.A., Idamakanti, N., Marshall-Neff, J., Rollence, M.L., Wright, P., Kaloss, M., King, L., Mech, C., Dinges, L., Iverson, W.O., et al. (2003). Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. *Hum. Gene Ther.* 14, 1595–1604.
51. Tao, N., Gao, G.-P., Parr, M., Johnston, J., Baradet, T., Wilson, J.M., Barsoum, J., and Fawell, S.E. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol. Ther.* 3, 28–35.
52. Zhang, Z., Krimmel, J., Zhang, Z., Hu, Z., and Seth, P. (2011). Systemic delivery of a novel liver-detargeted oncolytic adenovirus causes reduced liver toxicity but maintains the antitumor response in a breast cancer bone metastasis model. *Hum. Gene Ther.* 22, 1137–1142.
53. Coughlan, L., Vallath, S., Gros, A., Giménez-Alejandro, M., Van Rooijen, N., Thomas, G.J., Baker, A.H., Cascallo, M., Alemany, R., and Hart, I.R. (2012). Combined fiber modifications both to target $\alpha(v)\beta(6)$ and detarget the coxsackievirus-adenovirus

- receptor improve virus toxicity profiles in vivo but fail to improve antitumoral efficacy relative to adenovirus serotype 5. *Hum. Gene Ther.* 23, 960–979.
54. Poulin, K.L., Tong, G., Vorobyova, O., Pool, M., Kothary, R., and Parks, R.J. (2011). Use of Cre/loxP recombination to swap cell binding motifs on the adenoviral capsid protein IX. *Virology* 420, 146–155.
 55. Li, X., Wang, P., Li, H., Du, X., Liu, M., Huang, Q., Wang, Y., and Wang, S. (2017). The efficacy of oncolytic adenovirus is mediated by T-cell responses against virus and tumor in Syrian hamster model. *Clin. Cancer Res.* 23, 239–249.
 56. Steel, J.C., Morrison, B.J., Mannan, P., Abu-Asab, M.S., Wildner, O., Miles, B.K., Yim, K.C., Ramanan, V., Prince, G.A., and Morris, J.C. (2007). Immunocompetent syngeneic cotton rat tumor models for the assessment of replication-competent oncolytic adenovirus. *Virology* 369, 131–142.
 57. Silverstein, G., and Strohl, W.A. (1986). Restricted replication of adenovirus type 2 in mouse Balb/3T3 cells. *Arch. Virol.* 87, 241–264.
 58. Blair, G.E., Dixon, S.C., Griffiths, S.A., and Zajdel, M.E.B. (1989). Restricted replication of human adenovirus type 5 in mouse cell lines. *Virus Res.* 14, 339–346.
 59. Halldén, G., Hill, R., Wang, Y., Anand, A., Liu, T.-C., Lemoine, N.R., Francis, J., Hawkins, L., and Kirn, D. (2003). Novel immunocompetent murine tumor models for the assessment of replication-competent oncolytic adenovirus efficacy. *Mol. Ther.* 8, 412–424.
 60. Cheong, S.C., Wang, Y., Meng, J.H., Hill, R., Sweeney, K., Kirn, D., Lemoine, N.R., and Halldén, G. (2008). E1A-expressing adenoviral E3B mutants act synergistically with chemotherapeutics in immunocompetent tumor models. *Cancer Gene Ther.* 15, 40–50.
 61. Liu, T.-C., Halldén, G., Wang, Y., Brooks, G., Francis, J., Lemoine, N., and Kirn, D. (2004). An E1B-19 kDa gene deletion mutant adenovirus demonstrates tumor necrosis factor-enhanced cancer selectivity and enhanced oncolytic potency. *Mol. Ther.* 9, 786–803.
 62. Bateman, A., Bullough, F., Murphy, S., Emiliussen, L., Lavilette, D., Cosset, F.L., Cattaneo, R., Russell, S.J., and Vile, R.G. (2000). Fusogenic membrane glycoproteins as a novel class of genes for the local and immune-mediated control of tumor growth. *Cancer Res.* 60, 1492–1497.
 63. Tan, L., Jia, H., Liu, R., Wu, J., Han, H., Zuo, Y., Yang, S., and Huang, W. (2009). Inhibition of NF-kappaB in fusogenic membrane glycoprotein causing HL-60 cell death: implications for acute myeloid leukemia. *Cancer Lett.* 273, 114–121.
 64. Errington, F., Jones, J., Merrick, A., Bateman, A., Harrington, K., Gough, M., O'Donnell, D., Selby, P., Vile, R., and Melcher, A. (2006). Fusogenic membrane glycoprotein-mediated tumour cell fusion activates human dendritic cells for enhanced IL-12 production and T-cell priming. *Gene Ther.* 13, 138–149.
 65. Linardakis, E., Bateman, A., Phan, V., Ahmed, A., Gough, M., Olivier, K., Kennedy, R., Errington, F., Harrington, K.J., Melcher, A., and Vile, R. (2002). Enhancing the efficacy of a weak allogeneic melanoma vaccine by viral fusogenic membrane glycoprotein-mediated tumor cell-tumor cell fusion. *Cancer Res.* 62, 5495–5504.
 66. Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., and Parks, W.P. (1973). In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst.* 51, 1417–1423.
 67. Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, 59–74.
 68. Dexter, D.L., Kowalski, H.M., Blazar, B.A., Fligiel, Z., Vogel, R., and Heppner, G.H. (1978). Heterogeneity of tumor cells from a single mouse mammary tumor. *Cancer Res.* 38, 3174–3181.
 69. Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., and Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J. Virol.* 70, 4805–4810.
 70. Graham, F.L. (1987). Growth of 293 cells in suspension culture. *J. Gen. Virol.* 68, 937–940.
 71. Ross, P.J., and Parks, R.J. (2009). Construction and characterization of adenovirus vectors. *Cold Spring Harb. Protoc.* 2009, pdb.prot5011.
 72. Whelan, J.A., Russell, N.B., and Whelan, M.A. (2003). A method for the absolute quantification of cDNA using real-time PCR. *J. Immunol. Methods* 278, 261–269.
 73. Kimball, S., and Mattis, P. (1995). GNU Image Manipulation Program, version 2.8.22. <https://www.gimp.org/>.
 74. Harrington, B. (2005). Inkscape. <https://inkscape.org/>.
 75. Mehrara, E., Forsell-Aronsson, E., Ahlman, H., and Bernhardt, P. (2007). Specific growth rate versus doubling time for quantitative characterization of tumor growth rate. *Cancer Res.* 67, 3970–3975.
 76. Brú, A., Albertos, S., Luis Subiza, J., García-Asenjo, J.L., and Brú, I. (2003). The universal dynamics of tumor growth. *Biophys. J.* 85, 2948–2961.

Curriculum Vitae

Josh Del Papa Curriculum Vitae

Education and Awards

2017-Present	University of Ottawa Ottawa, ON Canada Ph.D. Biochemistry
2015-2017	University of Ottawa Ottawa, ON Canada M.Sc. Biochemistry- Specialization in Human and Molecular Genetics-Transfer to PhD
2012-2014	University of Windsor Windsor, ON Canada B.Sc. Honors Biochemistry with Thesis Final average of 86.36%
2010-2012	Western University London, ON Canada B.Sc. Microbiology and Immunology

Awards

- \$15,000, Ontario Graduate Scholarship, Spring 2018 – Spring 2019
- \$600, International Oncolytic Virus Conference Top Ten Oral presentations, Oxford, UK—
April 12, 2018
- \$15,000, Queen Elizabeth II Graduate Scholarship in Science and Technology, Fall 2016 – Fall 2017
- \$500, Ottawa Hospital Research Institute Research Day, 1st Place Poster Competition—
November 2016
- \$15,000, University of Ottawa Entrance Scholarship, Fall 2015 – Present
- \$500, University of Windsor In-Course Scholarship, Winter 2014
- \$400, Peter Wyzinski Sr. Memorial Award for Excellence in Biochemistry, Winter 2013

Research Contributions

Publications

Del Papa, J., Petryk, J., Bell, J.C., Parks, R.J. *An Oncolytic Adenovirus Vector Expressing p14 FAST Protein Induces Widespread Syncytium Formation and Reduces Tumor Growth Rate In Vivo*, Molecular Therapy Oncolytics (2019), 14, 107-120

Del Papa, J., Parks, R.J. (2016) *Adenoviral Vectors Armed with Cell Fusion-Inducing Proteins as Anti-Cancer Agents*, Viruses (2017), 9(1), 13

Wong, M.C., Nash, A.L., **Del Papa, J.**, Poulin, K.L., Falls, T., Bell, J.C., Parks, R.J. (2016) *Expression of the fusogenic p14 FAST protein from a replication-defective adenovirus vector*

does not provide a therapeutic benefit in an immunocompetent mouse model of cancer, Cancer Gene Therapy (2016), 23, 355–364

Halfyard, E.A., Webber, D., **Del Papa, J.**, Leadley, T., Kessel, S.T., Colborne, S.F., Fisk, A.T. (2016) *Evaluation of an acoustic telemetry transmitter designed to identify predation events*, Methods in Ecology and Evolution (2017), doi: 10.1111/2041-210X.12726

Nawrocki, B., McLeod, A.M., Hussey, N.E., **Del Papa, J.**, Fisk, A.T. (2019) *Revisiting trophic positions of fish species from the top predator guild in the Huron-Erie corridor of the Laurentian Great Lakes*, submitted to Freshwater Biology, In Revision

Presentations

Seminar

Del Papa, J., Petryk, J., Bell, J.C. and Parks, R.J., Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency, presented at uOttawa Biochemistry, Microbiology and Immunology Seminar day, March 2019.

Del Papa, J., Petryk, J., Bell, J.C. and Parks, R.J., Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency, presented at International Oncolytic Virus Conference, Oxford, UK, April 2018.

Del Papa, J., Petryk, J., Bell, J.C. and Parks, R.J., Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency, presented at OHRI regenerative medicine work in progress meeting, November 2017.

Del Papa, J., Petryk, J., Bell, J.C. and Parks, R.J., Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency, presented at uOttawa Biochemistry, Microbiology and Immunology Seminar day, March 2017.

Del Papa, J., Wong, C.M., Bell, J.C., Parks, R.J., Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency, presented at OHRI regenerative medicine work in progress meeting, March 2017.

Del Papa, J., Wong, C.M., Bell, J.C., Parks, R.J., Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus for Enhanced Vector Efficacy, presented at OHRI regenerative medicine work in progress meeting, June 2016

Poster

Del Papa, J., Petryk, J., Bell, J.C. and Parks, R.J., *Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency*, presented at Canadian Society of Virology, Halifax, Canada, June 2018.

Del Papa, J., Petryk, J., Bell, J.C. and Parks, R.J., *Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency*, presented at Ottawa Hospital Resident Research Day, June 2017.

Del Papa, J., Wong, C.M., Bell, J.C., Parks, R.J., *Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus Improves Vector Efficiency*, presented at Ottawa Hospital Research Day, November 2016.

Del Papa, J., Wong, C.M., Bell, J.C., Parks, R.J., *Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus for Improved Vector Efficiency*, presented at Ottawa Hospital Resident Research Day, June 2016.

Del Papa, J., Wong, C.M., Bell, J.C., Parks, R.J., *Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus for Improved Vector Efficiency*, presented at uOttawa Biochemistry, Microbiology and Immunology Poster day, May 2016.

Del Papa, J., Wong, C.M., Bell, J.C., Parks, R.J., *Expression of p14 Fusion Associated Small Transmembrane Protein in an Oncolytic Adenovirus for Improved Vector Efficiency*, presented at Ottawa Hospital Research Day, November 2015.

Halfyard, E.A., Webber, D., **Del Papa, J.,** Leadley, T., Kessel, S., Fisk, A.T., *Performance of an Acoustic Telemetry Transmitter Designed to Detect Predation Events* (2015, July). Performance of an acoustic telemetry transmitter designed to detect predation events. Poster presented at the 3rd International Conference of Fish Telemetry, Halifax, Nova Scotia.

Previous Academic Experience

University of Windsor, Ontario (September 2014- December 2014)

Undergraduate Teaching Assistantship (Coordinator Dr. Julie Smit)

- Teaching lab portion of Genetics course (55-211)
- Involves lecturing, teaching proper lab technique, grading student assignments and proctoring exams
- Topics covered include DNA extraction, PCR, agarose gel electrophoresis, restriction enzyme analysis, microsatellite detection and transgenic organism detection

Great lakes Institute of Environmental Research, Windsor, Ontario (June 2013- August 2015)

Undergraduate Thesis (Available upon request)

- Sequenced and identified prey DNA within Walleye stomachs using next-generation Ion-Torrent sequencing
- Presented Thesis Defence seminar April 29th, 2014
- Worked closely with Dr. Aaron Fisk and Dr. Ryan Walter both of the University of Windsor, Ontario to troubleshoot and streamline novel techniques including primer optimization and computational analysis of next-generation sequence data

Lab Technician

- Continuation of Undergraduate Thesis under the supervision of Dr. Aaron Fisk to generate further sequence data from the stomach contents of select predatory fish species in the Great Lakes
- Extensive field work sampling fish of the great lakes including:
 - Organized and co-lead a team of six for two five-day long sampling trips to Mitchell's Bay, Ontario
 - Thorough knowledge and experience with many marine sampling techniques such as gill-netting, seine netting and electrofishing
 - Experience with all preparatory steps for stable isotope analysis by mass spectrometry

Relevant Skills

Lab Skills

- Thorough knowledge of all aspects of adenovirus handling and preparation, including large-scale growth and purification, infection and plaque assay
- Significant experience with mammalian cell culture and immunoblot preparation
- Extensive experience with high-throughput genetic techniques including primer design, extractions, plate-based PCR, DNA purification techniques and agarose gel electrophoresis
- Proficient with Excel, Word, Inkscape, Graphpad Prism, GNU image manipulator and LI-COR image studio
- Proficient with computational analysis, in particular of high-throughput next generation sequencing data
- Protein isolation, MALDI-TOF mass spectrometry and analysis experience
- Isotope-ratio mass spectrometry sample preparation and data analysis experience

Languages

Proficient in reading and writing in English, basic French