Generation and Functionality of a Novel, Cox2-Controlled, Conditionally-Replicating, Adenovirus Expressing Interferon Alpha for Pancreatic Cancer Therapy

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Leonard Hart Armstrong, MD

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Masato Yamamoto, M.D., Ph.D.

July 2011

© Leonard Hart Armstrong 2011

Acknowledgements

I thank my advisor Dr. Masato Yamamoto as well as Julia Davydova, MD, PhD, for their invaluable tutelage. I also have a debt of gratitude to Eric Brown, Shigenori Hoshino, Satoshi Yamasaki, Yoshiaki Miura, and Joohee Han for their help and camaraderie over the past two years. I also thank my examination committee members, Drs. Ameeta Kelekar, Greg Beilman, and Masato Yamamoto. In particular I especially thank Dr. Kelekar for her helpful editorial suggestions in revising the manuscript.

Dedication

This thesis is dedicated to my parents Phillip and Leila Armstrong, without whose parenting I would never have turned out so well. Not only that, but your example as role models and your advice I have never seen the equal of anywhere. Thank you for being such a profound influence on my life and character.

Abstract

The concept of virotherapy for cancer is not an outgrowth of recent advances, but surprisingly of experimentation over the past 60 years. Adenovirus, with its good safety profile, was briefly in vogue in the 1950s, but had low efficacy. In the 1990s, virotherapy was again at the forefront of experimental work in oncology and there has been resurgent interest in adenovirus for cancer therapy. It offers many notable advantages and is the most widely used viral vector for gene delivery.

Combining the unique characteristics of adenovirus with rational genetic modifications has resulted in an entirely new class of therapy. CRAds (conditionally replicative adenoviruses), controlled by tumor-specific promoters, replicate and have cytocidal effects only in the cell environment, but have the disadvantages of limited infection of cancer cells, limited intratumoral spread, limited specificity for cancer cells with off-target effects, and immunogenicity.

Pancreatic ductal adenocarcinoma is a highly lethal disease and it is uncommonly diagnosed at a localized and surgically treatable stage. Recently, alpha interferon (IFNα) has shown promising improvements in survival in multimodality adjuvant therapy. However, this regimen has up to a 95% incidence of side effects, and over 25% of patients cannot tolerate the systemic IFN component. IFN could be a powerful tool for the treatment of pancreas cancer. However, realization requires a means of limiting toxicity of IFN-based therapy.

A rationally designed, improved adenovirus for use in pancreatic cancer is the subject of this project. A vector optimized for infectivity to pancreas cancer, with enhanced virulence, controlled by the tumor-specific promoter Cox2, and expressing IFNα solely in the tumor environment is generated and tested *in vitro* and *in vivo*. An analogous virus for use in hamsters, a crucial small animal system allowing human adenovirus replication, is also tested against syngeneic hamster pancreas cancer and demonstrates a more powerful effect *in vivo* than that in nude mice due to host immune activation. It is hoped that this work will lead to gene therapy strategies to deliver IFNα to pancreas cancer patients to enhance its beneficial effects while limiting toxicity.

Table of Contents

	Page Number(s)
Acknowledgements	i
Dedication	ii
Abstract	iii
List of Figures	v
Chapter 1: Background	1-16
Chapter 2: The Novel Adenovirus Shows Strong Activity Against Pancreatic Cancer In Vitro and In Vivo	17-35
Chapter 3: The Structurally Analogous Vector RGDCox2CRAdΔE3 ADP-IFN Demonstrates Similarly Potent In Vivo Activity in Hamsters	36-47
Chapter 4: Discussion	48-55
Bibliography	56-61

List of Figures

		Page Number(s)
1-1.	Schematic of adenovirus genome showing early, delayed, and late genes.	4
1-2.	Illustration of adenovirus capsid proteins.	5
1-3.	Schematic of differential targeting by tumor-specific promoters.	9
1-4.	Diagram of pancreatic anatomy showing typical location of tumor.	14
2-1.	Schematic of E3-deleted ADP- and IFN-expressing adenoviruses.	24
2-2.	Superiority of Ad 5/Ad 3 fiber-knob chimeric adenoviruses for PDAc cell infection.	25
2-3.	Activity and selectivity of the Cox2 promoter in PDAc.	26
2-4.	Cytolytic effect of modified vectors *in vitro*.	27-28
2-5.	*In vitro* tumor cell killing ability of 5/3 chimeric E3-deleted, Cox2-controlled IFN-expressing adenovirus.	29
2-6.	IFN levels increase in a time- and replication-dependent fashion.	30
2-7.	Superiority of replication-competent, ADP- and IFN-expressing adenovirus in an *in vivo* human PDAc model.	31
3-1.	IFN production by viral infection in hamster cell line HP1.	42
3-2.	*In vitro c*ytocidal effect of differing infectious concentrations of RGDCox2CRAdΔE3ADP-IFN.	43
3-3.	Superiority of replication-competent, ADP- and IFN-expressing adenovirus in an *in vivo* immunocompetent hamster PDAc model	44

Chapter 1

Background

Virotherapy for Cancer

Despite our exponentially rising understanding of cancer, it remains a highly prevalent, incompletely understood, and serious malady for humankind. The concept of virotherapy for cancer, in which viruses are deliberately administered to attack and destroy cancer cells (oncolysis), is not an outgrowth of our recent advances in genetics and targeted molecular therapy, but surprisingly owes its origins to clinical observations made over one century ago, with active experimentation and human clinical trials taking place for over the past 60 years (1-3). In one widely cited report, viral infection with influenza was noted to induce (albeit temporarily) remission in a patient with acute leukemia (4). Subsequently, a variety of genetically unmodified viruses were given to cancer patients, some of whom did show a strong benefit. However, responders were in the minority, and additionally these trials suffered from poor experimental design as well as lack of oversight leading to dangerous conditions for subjects (1, 3). At times, highly pathogenic viruses including hepatitis B and West Nile virus were used, and serious side effects such as encephalitis were seen. Adenovirus, which had a much better safety profile, was briefly in vogue in the 1950s, but due to low efficacy overall despite impressive tumor oncolysis it faded from use (1).

In parallel, the development of experimental tools such as the ability to grow human cancer cells in culture has also helped to push the field forward. Rodent models of human cancer enabled researchers to investigate other viruses for activity and demonstrate proof of principle including tumor remission in treated animals and tumor-

tropic properties of several viruses. As early as the 1950s, it was recognized that the potential of viruses as cancer therapy would require modification of the genome. Experiments in which the adaptive ability of viruses could be exploited to design a more targeted and effective virus were undertaken, such as repeated passage of virus in tumor tissue to enhance its virulence. However, the results seen in human clinical trials were far inferior to the *in vivo* models which, combined with the poor conduct of some of the trials, served to greatly dampen enthusiasm (1). Overall, the early development and testing of virotherapy for cancer was notable as much for its failures and shortcomings as its novelty.

In the 1990s, virotherapy was again at the forefront of experimental work in oncology, pushed forward by advances in genetic engineering. This has created an era of great promise for this field, and has set the stage for work with recombinant adenovirus, the subject of this project.

Adenovirus: Its Characteristics and Use As a Therapeutic Oncolytic Virus

Adenovirus (Ad) is an unenveloped, 36 kb double-stranded DNA virus with overlapping transcriptional units on each strand. Over 50 proteins are produced due to extensive splicing, with 11 being structural proteins of the virion (5). In terms of the propagation cycle of the virus, there is an early and late phase which is demarcated by the onset of viral replication. There are three major groups of genes by time course of expression: early (E1A, E1B, E2, E3, E4), delayed (IX, IVa2), and the major late

transcription unit (L1-L5). The E1 region genes are critically important in viral replication: E1A is a transcriptional activator for other early genes as well as a component of the mechanism for cellular transformation along with E1B. Specifically, E1A plays a major role in activating the S phase of the cell replication cycle through binding to the E2F-pRB complex, which then releases E2F to activate multiple genes involved in cell cycle progression (6). The protein E1B55k binds p53 and inactivates it to prevent apoptosis of the target cell before viral replication can take place. The E2 region encodes proteins involved in DNA replication. The E3 region is involved in immune surveillance and suppression but is not essential for replication. E4 genes have a regulatory function on DNA replication (5, 7).

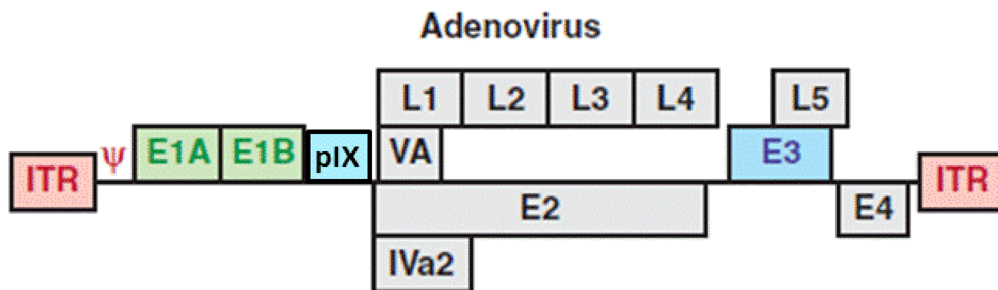


Figure 1-1. Schematic of adenovirus genome showing early (E), delayed (I), and late (L) genes. (Verma and Weitzman 2005)

The adenovirus capsid structure has functional relevance to the process of cell infection. The viral particle itself is an icosahedral shape with three important proteins: fiber/knob, hexon, and penton base. Initial infection is mediated by interaction between

the knob protein at the tip of the fibers which project outward from the capsid with the Coxsackie-adenovirus receptor (CAR) on the cell surface, and then the virus is internalized by interaction of the penton base arginine-glycine-aspartic acid (RGD) motif with cellular $\alpha_v\beta$ integrins, specifically $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (7-9).

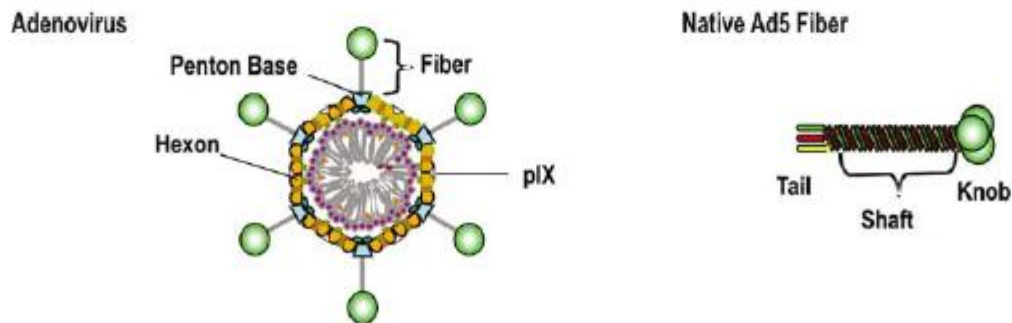


Figure 1-2. Illustration of adenovirus capsid proteins. (Mathis *et al.* 2005)

Adenovirus was identified in 1953 from adenoid tissue. Since then, over 50 serotypes have been described (1, 7). These viruses have a broad array of tropism including the respiratory tract, gastrointestinal tract, and bladder. Wild-type adenovirus, due to its better safety profile than comparable viruses which were in vogue in the 1950s for cancer therapy, led initially to great interest, but its lack of effect at improving survival despite histologic evidence of oncolysis in patients led to its near abandonment (1).

More recently, there has been resurgent interest in adenovirus as a gene delivery vector and specifically in cancer therapy. It offers many notable advantages including a high efficiency of *in vivo* gene delivery, the ability to infect and replicate in nondividing

cells, their naturally lytic replication cycle, and the lack of integration of viral DNA into the genome. Additionally, there is wide experience and understanding of genetic manipulation of adenovirus and it can be produced and purified to high titers (7, 8). Adenovirus is the most widely used viral vector for gene delivery, comprising over 25% of clinical trials, 75% of which are for cancer (8). Such experience with its use has contributed greatly to its well-understood safety profile, but it is highly immunogenic, capable of inducing severe host immune responses, and imprudent use has contributed to at least one highly publicized death (10).

Exploitation of the unique characteristics of adenovirus combined with rational genetic modifications has resulted in development of an entirely new class of therapy. A great conceptual leap was the recognition that the mutant adenovirus dl1520, which lacks the p53-binding protein E1B55k, could be used to selectively target cancer. The protein E1B55k functions to sequester cellular p53 in the infected cell which prevents apoptosis and in turn allows viral replication to proceed, so the mutant virus lacking this should therefore be unable to replicate in normal cells and only be able to propagate in p53-deficient cells, a frequent characteristic of cancer (6). Although clinical effectiveness with virus alone has been low, when tested in head and neck squamous cell carcinoma in combination with chemotherapy and/or radiotherapy, increased response rates have been seen. This has led to approval of the world's first oncolytic virus for cancer therapy (11).

Modification and Optimization of Adenovirus for Cancer Therapy

As the case of dl250 illustrates, virotherapy with adenovirus has broken from the early days of untargeted and unmodified virus use against cancer and highlights, at least conceptually, the ability to improve upon adenovirus's innate characteristics by rational design to create a selective and targeted vector. Much of the subsequent research in the field has been to achieve this very situation.

For all the advantages of adenovirus as a cancer therapeutic, it is hampered by several significant disadvantages. These include the following: limited infection of cancer cells, limited intratumoral spread, limited specificity for cancer cells with off-target effects, and immunogenicity (12, 13). Research has therefore been targeted towards overcoming these problems.

Early attempts at virotherapy for cancer used viruses which had a deleted E1 region. Since the E1A protein is required for expression of other proteins in the adenovirus transcription unit, and other E1 proteins are also critical for viral replication, these vectors are replication-incompetent and capable of expression of a transgene only (5, 7). They therefore serve as gene delivery vectors only. While these first-generation adenoviral vectors approach eliminated safety concerns related to widespread viral infection and replication within the body, they suffered from a low persistence in the body with transient gene expression (14, 15). Additionally, since these viruses are by design unable to replicate, they must be propagated on transcomplementing cell lines which express regions of viral DNA essential for replication. Homologous recombination producing replication-competent adenovirus (RCA) as a contaminant during production is a significant safety concern with this approach (7, 16).

Subsequent generations of adenovirus were also based on a nonreplicative backbone. Second-generation viruses were additionally deleted in E2 and E4 regions which expanded the insertional capacity for transgenes, and reduced immunogenicity and inflammation, but there is no conclusive evidence to suggest that this strategy prolonged transgene expression. Third generation vectors, also known by a variety of names including “gutless” vectors, have deletions of all viral genes except elements necessary for replication and packaging. This strategy does appear to be both less immunogenic and have prolonged transgene expression (7, 7, 16, 16).

However, it is important to point out that all of these strategies only produce, at best, temporary transgene expression and do so in a fashion that does not take advantage of the replicative capability of adenovirus. Solving the problem of allowing replication in tumor cells to attain the advantages of adenovirus’s lytic life cycle and ability to propagate through a tumor mass, while at the same time addressing safety concerns, requires a new concept: tissue-specific promoters.

Early adenoviral vectors targeted not only cancer tissues but also normal cells due to the fact that constitutive promoters such as cytomegalovirus (CMV) were used to drive transgene expression. To mitigate this toxicity, tissue-specific promoters have been used to replace these constitutive promoters and thereby restrict transgene expression, ideally only to the tumor tissue itself. Promoters have been selected to target such cancers as prostatic, breast, melanoma, myeloma, and pancreatic, and testing has shown the abrogation of systemic transgene expression as expected (17).

Taking this one step further, replicating vectors with tumor-specific promoters have been developed. These are replication-competent vectors but due to control of the E1 region (typically) by a promoter highly or exclusively expressed by cancer cells, these viruses behave as nonreplicating vectors everywhere in the body except the tumor environment (6, 15, 17, 18). Such adenoviruses are known as CRAds, conditionally replicative adenoviruses (8, 19). CRAds initially generated great excitement and have

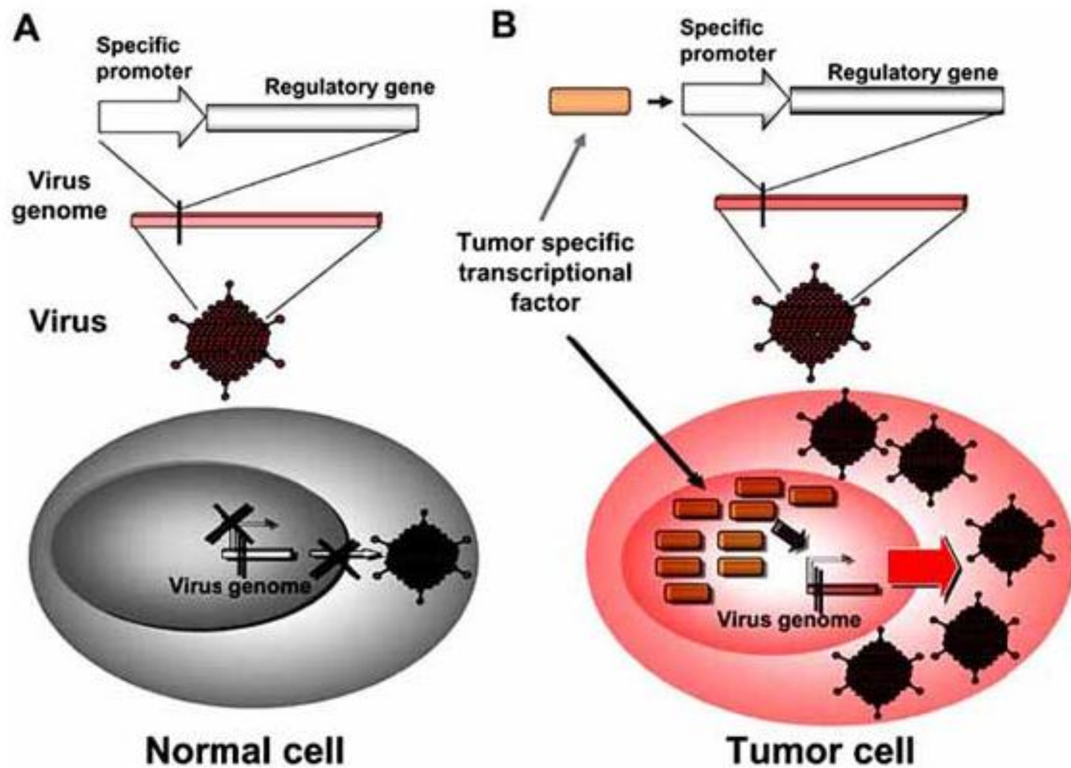


Figure 1-3. Schematic of differential targeting by tumor-specific promoters. In (A), a normal cell with absent or very low levels of the transcription factor is not permissive to viral replication, whereas in (B), high levels of the tumor-specific promoter allow efficient replication of adenovirus with subsequent lysis and spread. (Hardcastle *et al*. 2007).

been rapidly translated to the clinical setting. Tumor-specific promoters have been used to give enhanced selectivity to the virus, exemplified by a prostate-specific antigen (PSA)-controlled vector which by design can only replicate in prostatic cells (6, 15, 17, 19). However, such transcriptionally targeted adenovirus is only as specific as the promoter which is chosen, and human trials have uniformly demonstrated low efficacy with CRAd monotherapy (12). This again highlights the challenges with adenovirus use and the need to further optimize the vector structure.

Strategies for transductional targeting of adenovirus, improving its infectious nature through modifying its tropism, are an approach to one aspect of the overall problem of low efficacy. Wild type (unmodified) adenovirus requires interactions with the Coxsackie-adenovirus receptor for internalization, and many cancers including ovarian, hormone-refractory prostate cancer, pancreatic, and other gastrointestinal cancers lack CAR expression (12). Therefore, modifying adenovirus tropism is as important as modifying transcriptional control for a clinically usable virus.

Multiple strategies have been attempted. These include systemic delivery using carrier cells to sequester the virus from immune surveillance and achieve targeted delivery (7), an implantable silica matrix impregnated with adenovirus, adenoviral conjugation to polyethylene glycol (8), or heterologous targeting adapters (15). This specific strategy makes use of a targeted ligand which associates or binds with the viral particle such as an antibody against a component of the viral capsid conjugated to a retargeting moiety such as a cancer-specific surface marker such as the epidermal growth factor receptor. While such strategies have been studied and demonstrated to have

varying degrees of success and promise, genetic modification of the virus to alter its tropism, the strategy employed in this work, benefits from simplicity and the lack of another component to produce, purify, and test.

Increased knowledge of the steps required for adenovirus internalization into the target cell has facilitated attempts to genetically modify its tropism. It is known that the knob domain of the capsid fiber protein is critical for cell entry, and a simple modification to circumvent inefficient entry is to substitute a higher-affinity knob from another of the approximately 50 adenovirus serotypes known (15). Substitution of the fiber knob from adenovirus serotype 3 into adenovirus serotype 5 (5/3 chimera) (20) provides improved targeting to several gastrointestinal malignancies including pancreatic (21), as well as ovarian cancer and melanoma.

Structural modification of the capsid proteins themselves is another method of transductional retargeting. Interaction of the RGD moiety of penton base with target cell integrins occurs with adenovirus internalization, and it has been found that including the same RGD motif added to the C terminus of the fiber retargets adenovirus to multiple cell types which are resistant to wild-type adenovirus including pancreatic (22, 23). Additionally, other capsid proteins besides fiber/knob have also been modified to alter tropism. The hexon, penton base, and the small structural protein pIX have all been modified to express specific targeting moieties and all have been found to be functional (15).

Such modifications have gone a long way towards improving clinical usability of adenovirus. However, the problem of low efficiency of spread within a tumor mass and

low efficacy must be overcome by other means. Adenovirus can be “armed” to take advantage of adenovirus’s replicative nature and amplify virotherapy (6). Insertion of adenoviral death protein (ADP), an endogenous recently discovered adenoviral protein, can be inserted in such a way as to be overexpressed. ADP, typically inserted in the E3 region, greatly accelerates apoptosis and therefore viral spread (24). An approach known as suicide gene therapy has also been used in which tumor-targeted adenoviruses deliver a transgene which metabolizes a nontoxic prodrug into its active metabolite. Herpes simplex virus thymidine kinase delivery which metabolizes ganciclovir to its active form is a prime example (8). Adenoviruses can also be armed with cytokines or chemokines. One adenovirus has been designed to express granulocyte-macrophage colony stimulating factor which recruits macrophages and primes CD8+ T cells, and has had favorable results in phase I and II studies (8).

To deal with the next problem of adenovirus usage, the liver must be detargeted by transductional and/or transcriptional control. It is known from both in vitro and in vivo experiments that adenovirus concentrates in the liver when given systemically, and this has also been found in a human clinical trial upon autopsy of participants who received adenovirus (25). Not only does liver sequestration reduce efficacy, but the adenoviral E1A protein is toxic to hepatocytes (6). Thus, this is not just a problem of low efficacy but also of safety. Any possible clinical application of adenovirus will require a strategy to mitigate this. Various modifications of other adenoviral surface proteins has had some effect on reducing liver tropism including ablating CAR binding through fiber knob modification and the Ad 5/3 fiber/knob chimera (15). As Kuppfer cells in the liver

are involved in clearing circulating adenovirus, strategies to prevent uptake, deplete Kuppfer cells, or saturate their receptors have been employed. Circulating platelets bind Ad5 rapidly and Kuppfer cells clear the virus-platelet aggregates. Depletion of platelets before adenovirus administration has been found to increase circulating adenovirus, but this strategy clearly must be balanced with the risks of inducing thrombocytopenia. Kuppfer cells can also be depleted with GdCl3 or polyinosinic acid can be preinjected to saturate scavenger receptors and limit adenovirus uptake. Each of these strategies has been found to be useful in vivo (8). It is known that the hexon protein mediates liver targeting; novel adenoviruses designed to express hexon protein from a low-affinity serotype is one possible strategy (26).

Pancreatic Cancer: A Grave Problem but One Suitable for CRAd Therapy

Pancreatic ductal adenocarcinoma is a highly lethal disease, with an estimated 43,140 new cases and 36,800 deaths in 2010 (27). It is an epithelial malignancy arising from ductal cells of the exocrine pancreas. Owing to the location of the pancreas deep in the abdomen and the fact that early stage disease typically has no specific symptoms, it is uncommon for this cancer to be diagnosed at a localized and surgically treatable stage. Approximately 2/3 of tumors arise in the head of the pancreas, and growth which typically causes obstruction of the common bile duct with resultant jaundice is often the symptom leading to its discovery (28). Of the newly diagnosed cases, approximately 85-90% will have inoperable disease at presentation due to locally advanced stage or

metastases (29). Chemotherapy with gemcitabine is currently the standard of care for the adjuvant or post-surgical setting (30, 31), however overall survival remains poor with median survival of approximately 22-24 months in selected series (32).

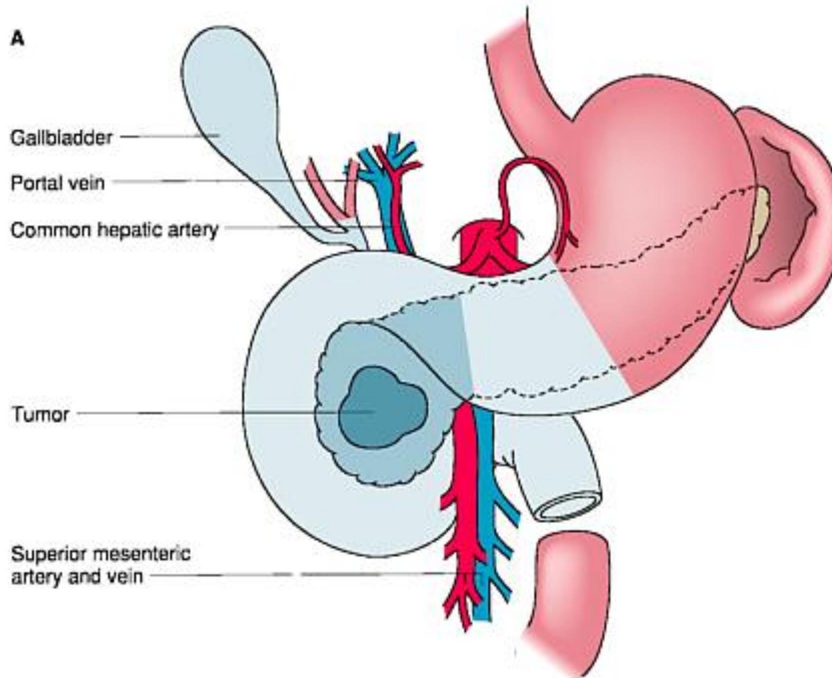


Figure 1-4. Diagram of pancreatic anatomy showing typical location of tumor. Shaded in blue are structures which require surgical resection for tumors of this location. (Nakeeb *et al.* 2006)

Recently alpha interferon (IFN), a cytokine with direct and indirect antitumor effects (33) has shown promising improvements in survival in multimodality adjuvant therapy. This was first reported by the Virginia Mason group (34) in which they found a statistically significant improvement in survival over gemcitabine-based adjuvant therapy at 26 months of followup, with 84% survival at 2 years. A subsequent phase II study by Linehan and colleagues using combination CRT with post-radiation gemcitabine instead

of 5-FU resulted in 56% 2 year actuarial survival (35), a survival rate identical to that reported by Picozzi and colleagues in the multicenter phase II ACOSOG Z05031 trial. (36) However, this regimen suffers from systemic side effects with an incidence as high as 95%, and over 25% of patients cannot tolerate the systemic IFN component (34-37). This indicates a pressing need for the development of highly active agents for the treatment of pancreatic cancer and IFN could be a powerful tool for the generation of such a modality. However, realization requires a means of limiting toxicity of IFN-based therapy.

In terms of virotherapy, pancreas cancer offers the advantage of several candidate tumor promoters. Specifically, like many other gastrointestinal cancers, pancreatic cancer cells highly overexpress cyclooxygenase-2 (Cox2) (22, 38). This appears to be specific to the tumor itself; studies using resected tumor tissue and adjacent normal pancreas found no induced upregulation of Cox2 in the normal gland (38).

Cox2 is an inducible enzyme of arachidonic acid metabolism and is an early response pro-inflammatory gene (39). It is not known to be constitutively expressed in the body including in the liver (40). In an acute inflammatory challenge, Cox2 production is almost totally absent from hepatocytes and does not become significant except in chronic pro-inflammatory conditions (41). However, as the major side effect of Cox2-selective inhibitors appears to be cardiac, this may suggest that constitutive expression does occur in cardiovascular tissue (39).

The hypothesis of this work is that an adenovirus, designed for optimal infectivity of pancreatic cancer, controlled by the tumor-specific promoter Cox2 to target pancreas

cancer while sparing toxicity to the liver, and with enhanced virulence through expression of ADP and IFN can be generated, and that this vector will possess superior attributes of cell infectivity, cell killing, and *in vivo* effect.

Chapter 2

The Novel Adenovirus 5/3Cox2CRAdΔE3ADP-IFN

Shows Strong Activity Against Pancreatic Cancer *In Vitro* and *In Vivo*

Introduction

Adenovirus (Ad) vector-based cancer gene therapy has been applied in humans with more than 3,000 patients. This vector has high *in vivo* infectivity but the conventional Ad vector is not suitable for pancreatic cancers. In order to overcome the weak points as cancer therapeutics, we have improved this vector system and generated a series of oncolytic Ads. We have developed a conditionally replicative adenoviral system (CRAds) (42) wherein viral replication is controlled by the Cox2 promoter, exploiting the known Cox2 overexpression in pancreatic tumors to drive viral replication, and its lack of expression in liver, the organ of most concern for replication-related toxicity, to mitigate side effects (22, 42-44). Modifications of the viral capsid proteins were made to dramatically improve pancreatic tumor cell infectivity over the wild type viral structure (21, 45). Work by our group and others (21) has demonstrated the practicality of this approach among many tumor types including pancreatic cancer. Investigators have previously deployed an earlier generation of IFN-expressing Ad vectors for pancreatic cancer therapy, however all of the described vectors have been of the nonreplicating type (46-49). Although these were effective at high concentrations, they lacked the ability to replicate and hence will have low *in vivo* persistence with temporally limited transgene expression. By combining the ability of modified, replication-competent Ad vectors to preferentially target cancer cells and to replicate within them, a therapeutic gene such as IFN can be locally delivered in massive amounts to augment the tumor-lytic viral effect while avoiding systemic toxicity. We hypothesize that a novel Cox2-controlled,

selectively replicating, CRAd which expresses IFN will be highly active both *in vitro* and *in vivo*, and will show superiority to nonreplicating, IFN-expressing Ad vectors previously tested.

Results

Confirmation of viral structure

PCR of viral DNA was used to confirm structure of the 5/3WtΔE3ADP-IFN with intact E1 region as well as the novel Cox-2 controlled virus 5/3Cox2CRAdΔE3ADP-IFN (Figure 2-1). Analysis was done for both the fiber region and for Cox2 promoter status as previously described (45). The structure of all experimental viruses was validated in this manner.

The 5/3 fiber modification is superior for targeting of PDAc

To analyze the effect of the 5/3 fiber modification on targeting of CRAds to pancreatic cancer cells, two identical replication-incompetent viral vectors encoding the CMV promoter-driven reporter gene Luc were used: AdCMVLuc, with wild type Ad5 fiber structure, and Ad5/3CMVLuc, expressing the 5/3 fiber-knob chimera. Statistically significant increases of reporter gene expression (5 -10 times) were detected in all four PDAc cell lines under investigation with the 5/3 modification as compared to the wild type fiber (Figure 2-2).

Functional status of Cox2 in PDAc cell lines

To determine the strength of the Cox2 promoter activity in PDAc cell lines, the human cell lines S2O13, S2VP10, ASPC-1, and MiaPaCa-2 were infected with two identical nonreplicating Luc-expressing vectors, AdCMVLuc, in which reporter gene expression is controlled by the ubiquitous CMV promoter, and AdCox2Luc, whereby Luc expression is dependent on cellular Cox2 promoter activity. A known Cox2 positive

(A549) and negative (BT474) cell line were used as controls. All PDAc cell lines tested were positive for Cox2 promoter activity, with two of them (S2VP10 and ASPC-1) found to be comparable in magnitude to the strong CMV promoter-driven activity and the others (S2O13 and MiaPaCa-2) significantly higher in Cox2 activity (Figure 2-3).

Increased oncolytic efficiency of IFN-expressing Ads in vitro

We generated Ads doubly modified for both increased cancer cell infectivity and enhanced cell killing ability with the 5/3 fiber modification and incorporation of the ADP-IFN expression cassette. We infected MiaPaCa-2 human PDAc cells with 5/3WtΔE3ADP-IFN at low titers (0.1 to 1 vp/cell) to allow for multiple rounds of replication. Crystal violet staining showed oncolysis among replicating vectors (Fig. 2-4A lanes 1-3) and minimal effect from nonreplicating vectors (lanes 4-5). The addition of ADP overexpression (5/3WtΔE3ADP-Luc) in comparison to 5/3Adwt, the infectivity-enhanced vector without ADP overexpression, showed increased oncolysis. Addition of IFN expression (5/3WtΔE3ADP-IFN) in comparison to 5/3WtΔE3ADP-Luc had the strongest effect, and furthermore the killing effect of the doubly modified virus was accelerated with a larger viral dose. A nonreplicating virus (5/3EasyIFN) with interferon expression alone showed no oncolysis upon infection with low titers (Figure 2-4A).

To evaluate the effect of Cox2 replication control on cancer cell oncolysis, we employed a similar virus incorporating both the 5/3 chimera and ADP-IFN expression cassette, but with control of viral replication under control of the Cox2 promoter (5/3Cox2CRAdΔE3ADP-IFN). In all cell lines tested, 5/3Cox2CRAdΔE3ADP-IFN was highly potent, at a level equaling or surpassing that of Adwt, the gold standard control

virus without selectivity. The novel virus was also equal or superior to 5/3Cox2CRAd, a powerful positive control, across all cell lines (Figure 2-4B-D). The replication-deficient IFN-expressing Ad (5/3EasyIFN) required approximately three orders higher titers to successfully kill the pancreatic cancer cells when compared to our ΔE3-based replication-competent Ads producing IFN (data not shown).

Additionally, to test for selective toxicity by Cox2 status, we infected human breast cancer BT474 cells, which are known to be Cox2-negative. Results of crystal violet staining at serial time points demonstrate no oncolysis from 5/3Cox2CRAdΔE3ADP-IFN or from a similar Cox2-controlled replicating virus (5/3Cox2CRAd) at day 8, and near complete oncolysis from replicating vectors without Cox2 specificity. These results indicate selective toxicity to Cox2-positive target cells with sparing of Cox2-negative cell populations (Figure 2-4E).

The replication and cytocidal effect of 5/3Cox2CRAdΔE3ADP-IFN were then quantitatively analyzed. In PDAc cell lines, 5/3Cox2CRAdΔE3ADP-IFN achieved comparable levels of cancer cell killing comparable to 5/3WtΔE3ADP-IFN after an early "lag" period owing to its replication control. In MiaPaCa-2, S2O13, and S2VP10 cells the percentage of surviving cells at the final time point was 22.9%, 29.8%, and 11.7% respectively compared to that of Adwt, the gold standard control, of 62.1%, 110%, and 102.4% respectively (p values for comparison 0.008, <0.005, and 0.008). Additionally, the killing ability of 5/3Cox2CRAdΔE3ADP-IFN and 5/3WtΔE3ADP-IFN trended toward equivalence in all cell lines and became so at the final timepoint in two out of three cell lines ($p<0.005$, p=NS, p=NS for MiaPaCa-2, S2O13, and S2VP10

respectively). Overall, the 5/3 modified Cox2-controlled IFN virus demonstrated a robust oncolytic effect (Figure 2-5A-C).

IFN expression is time- and replication-dependent

To understand the dynamics of IFN expression by 5/3Cox2CRAdΔE3ADP-IFN, IFN levels in cell culture supernatant after infection of S2O13 PDAc cells was assayed at serial time points. Similar to the results of the quantitative killing effect, 5/3Cox2CRAdΔE3ADP-IFN produced interferon in a lagging fashion behind 5/3WtΔE3ADP-IFN and increased in quantity in a time-dependent manner. 5/3WtΔE3ADP-IFN attained a level of 4349 units/ml at day 11 ($p<0.0005$), compared to 1897 units/ml for 5/3Cox2CRAdΔE3ADP-IFN. At day 11 5/3EasyIFN produced 59 units/ml (Figure 2-6).

Therapeutic efficacy of replication competent IFN-producing Ads in vivo

In vivo analysis of antitumor efficacy of replicating IFN producing viruses was performed using subcutaneous xenograft models in athymic nude mice. This experiment tested the *in vivo* antitumor efficacy of 5/3Cox2CRAdΔE3ADP-IFN in a subcutaneous xenograft model established using the aggressive metastatic-derived cell line S2O13. Established tumors were treated with a single intratumoral injection of 10^{10} vp of virus at day 0. At day 21, the 5/3Cox2CRAdΔE3ADP-IFN group showed an average relative tumor volume of 5.6, compared to 12.1 with 5/3Easy IFN, and 25.5 with PBS. Results demonstrate a statistically significant tumor suppression of 5/3Cox2CRAdΔE3ADP-IFN at day 21 when compared to saline and to 5/3EasyIFN ($p<0.05$ for both). (Figure 2-7).

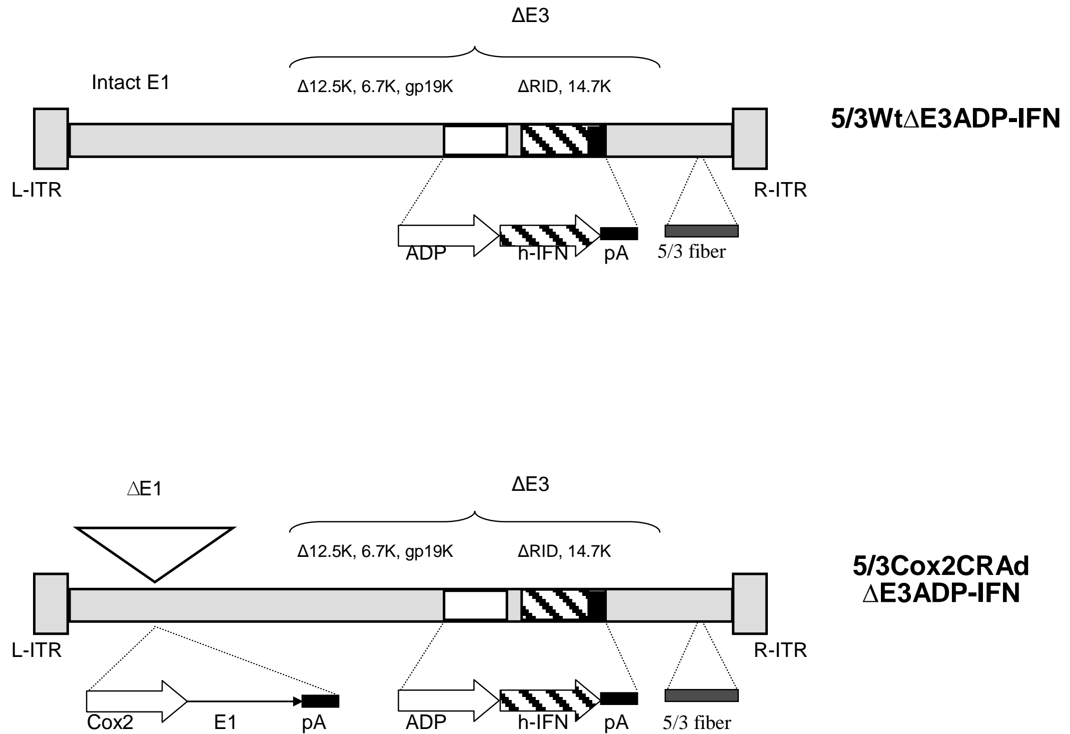


Figure 2-1. Schematic of E3-deleted ADP- and IFN-expressing adenoviruses. In both viruses, an expression cassette containing adenoviral death protein (ADP) and the human IFNα1 transgene was inserted into the viral E3 region. The cyclooxygenase (Cox)-2 controlled conditionally replicative adenovirus (CRAd) has the CoxL promoter inserted into the E1 region, whereas the wild type transgenic Ad has an intact E1 region. Both viruses are fiber modified with the Ad 5/Ad3 fiber-knob chimera.

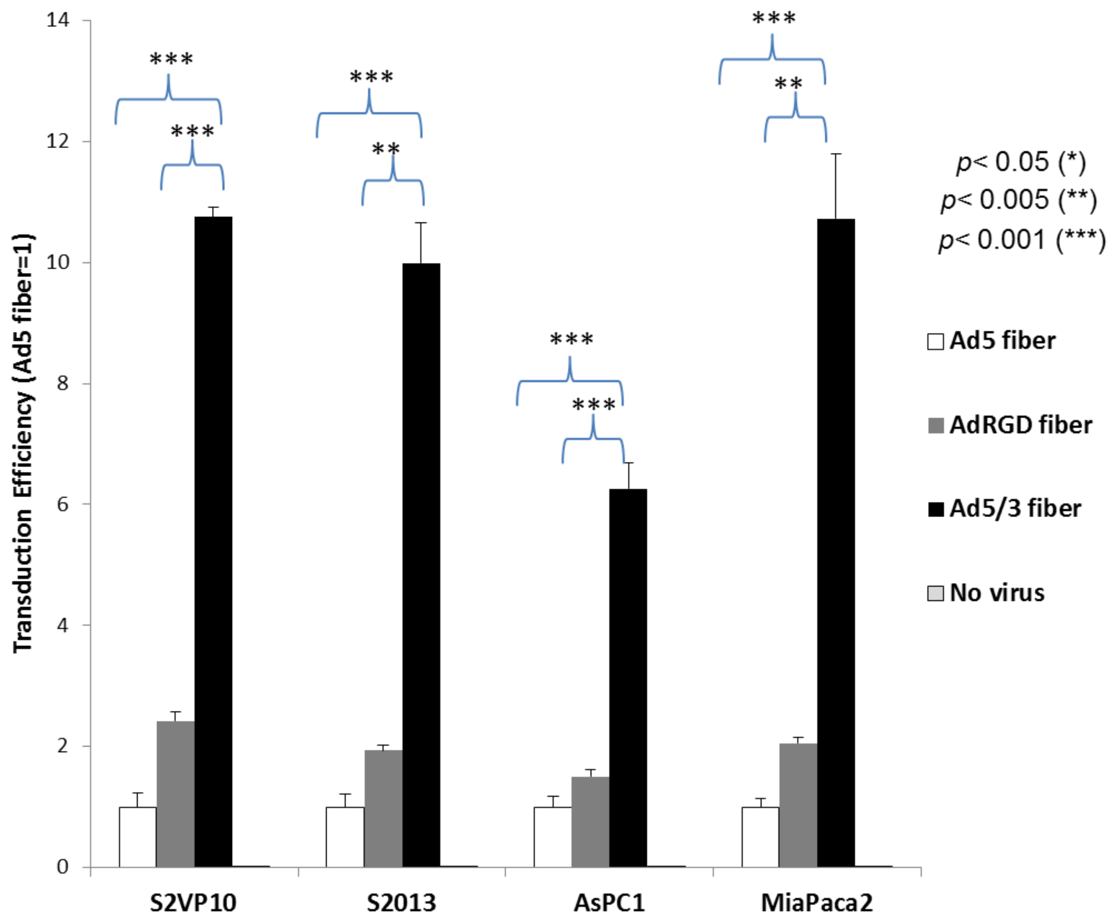
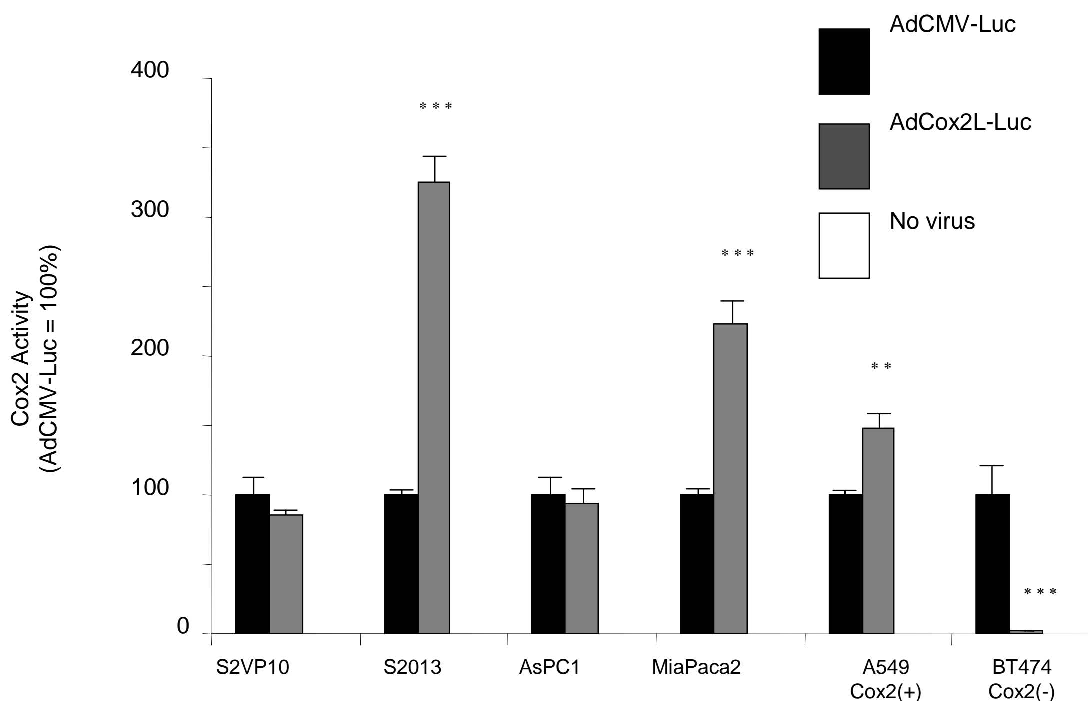


Figure 2-2. Superiority of Ad 5/Ad 3 fiber-knob chimeric adenoviruses for PDAc cell infection. Cancer cells were infected with a reporter vector under control of the ubiquitous CMV promoter with the wild type (Ad 5), Ad 5/Ad 3 fiber-knob chimeric (5/3) fiber, or RGD fiber Ads. Results are shown as relative light units (RLU) normalized to AdCMVLuc activity. The Ad5/3 fiber modification imparts significantly enhanced infectivity to all PDAc cell lines tested compared to RGD and unmodified wild type. Asterisks indicate signficance for the comparison of 5/3 fiber to Ad5 fiber or RGD.

Figure 3



p < 0.05 (*), *p* < 0.005 (**), *p* < 0.001 (***)

Figure 2-3. Activity and selectivity of the Cox2 promoter in PDAc. Human PDAc cell lines (S2O13, S2VP10, ASPC-1, MiaPaCa-2), Cox2 positive (A549), and Cox2 negative (BT474) cell lines were infected with AdCMVLuc or AdCox2Luc. Luc activity was measured 2 days after infection. Data are shown as percentages of relative light units (RLU) normalized to AdCMVLuc activity. The Cox2 promoters exhibited high levels of activity in all PDAc cell lines under investigation.

Figure 4

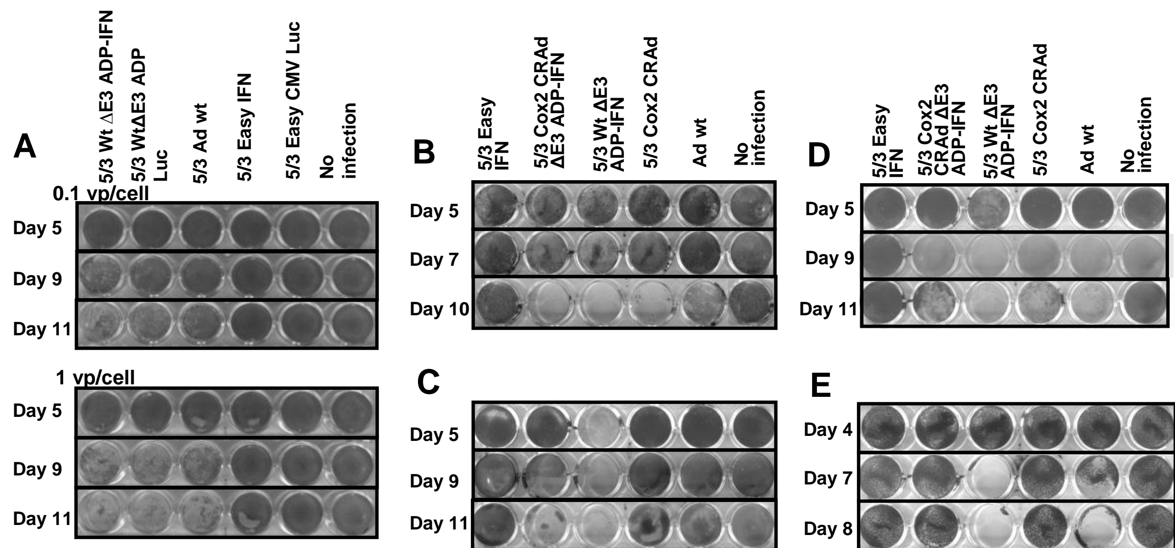


Figure 2-4. Cytolytic effect of modified vectors *in vitro*. **(A.)** MiaPaCa-2 cells were infected at 0.1 and 1 vp/cell with replication-deficient and replication-competent IFN-expressing vectors or controls, with surviving cells stained by crystal violet. Replication-incompetent viruses (lanes 4-5) show minimal effect compared to replication-competent viruses. The ADP- and IFN-expressing vector (lane 1) shows a superior killing effect in comparison to 5/3 Ad wt, the control virus with intact E3 region (lane 3) and to its identical counterpart expressing luciferase (lane 2). Furthermore, the superiority of 5/3WtΔE3ADP-IFN becomes more evident with increasing viral dose. Human PDAc cell lines MiaPaCa-2 **(B.)**, S2O13 **(C.)**, and S2VP10 **(D.)**, respectively, were infected with 1vp/cell using Cox2-controlled IFN-expressing replicating vectors or controls.

Across all three cell lines, the novel Cox2-controlled ADP- and IFN-expressing virus (lane 2) has superior potency to Ad wt, the gold standard control virus without cancer specificity (lane 5 in all), and was as good as its IFN-expressing counterpart without selectivity (lane 3 in all), as well as comparable or superior cytocidal effect to the positive control 5/3 Cox2 CRAd (lane 4 in all). **(E.)** BT474 cells, which are known to be Cox2-negative, were infected at 1vp/cell with Cox2-controlled vectors or controls. At day 8, the novel Cox2-controlled IFN vector (lane 2) as well as the powerful 5/3 Cox2 CRAd (lane 4) show no effect, however the remaining two replicating vectors (lanes 3 and 5) which are not controlled by the Cox2 promoter show near complete cytolysis.

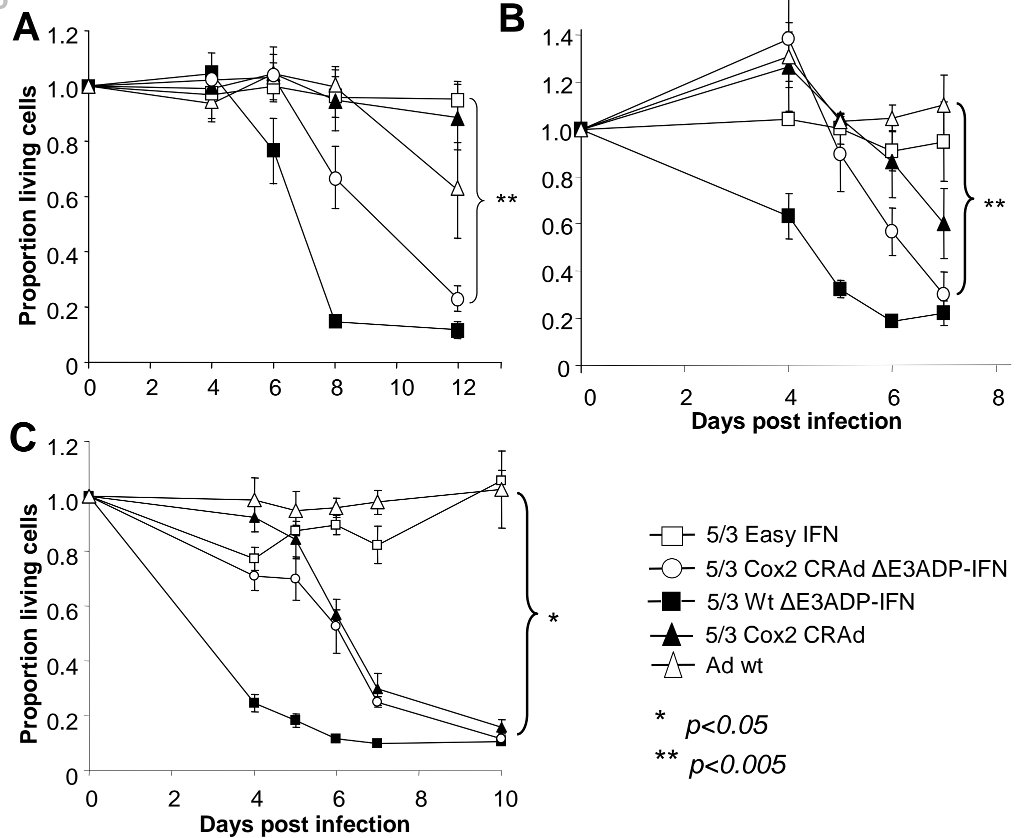


Figure 2-5. *In vitro* tumor cell killing ability of 5/3 chimeric E3-deleted, Cox2-controlled IFN-expressing adenovirus. Human PDAc cell lines MiaPaCa-2 **(A.)**, S2O13 **(B.)**, and S2VP10 **(C.)**, respectively, were infected at day 0. Cell viability was determined with a colorimetric cell proliferation assay. The results are shown as proportion of living cells remaining relative to unifected cells. 5/3Cox2CRAdΔE3ADP-IFN shows significantly higher cell killing ability compared to Ad wt, the gold standard control virus lacking cancer specificity, and is comparable in effect to the 5/3 chimeric ADP- and IFN-overexpressing adenovirus with unrestricted ability to replicate.

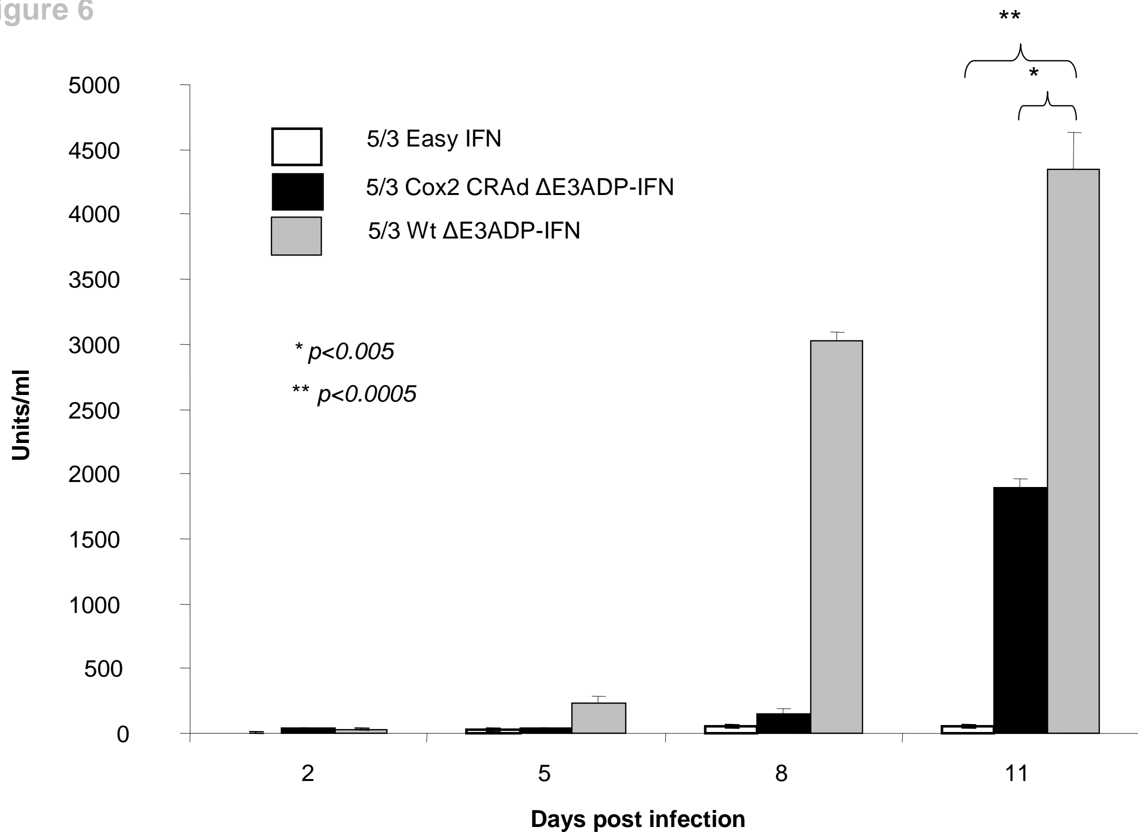


Figure 2-6. IFN levels increase in a time- and replication-dependent fashion. IFN levels were assayed from infected cell culture supernatant. 5/3Cox2CRAdΔE3ADP-IFN shows a robust production of IFN despite its control of replication by Cox2. As expected the 5/3WtΔE3ADP-IFN not under replication control by Cox2 attains higher levels of IFN production but is used for proof of principle only and is not suitable for use in vivo.

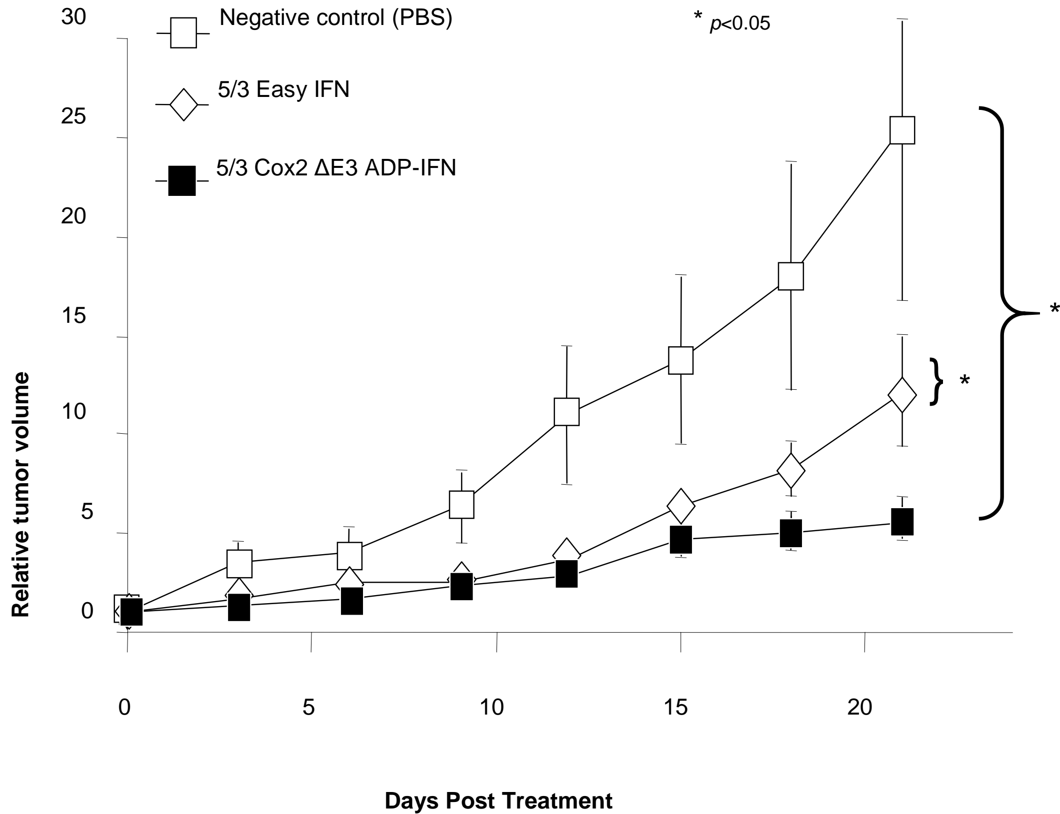


Figure 2-7. Superiority of replication-competent, ADP- and IFN-expressing adenovirus in an *in vivo* human PDAc model. S2O13 xenograft nude mice were treated with a single intratumoral injection of PBS or vector. Tumor size is shown as relative tumor volume compared to day 0. Error bars indicate standard error of the mean (SEM). On day 21 the 5/3Cox2CRAdΔE3ADPIFN showed significantly stronger antitumor effect than 5/3 Easy IFN and negative control ($p<0.05$ for both).

Materials and Methods

Cell lines and animals

The human pancreatic ductal adenocarcinoma (PDAc) cell lines MiaPaCa-2, S2O13, S2VP10, and ASPC-1, the Cox2-positive human nonsmall cell lung adenocarcinoma cell line A549, and the Cox2-negative human breast cancer cell line BT474 were obtained from the American Type Culture Collection (Manassas, VA). MiaPaCa-2, S2O13, S2VP10, A549, and ASPC-1 were maintained in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Herndon, VA) with 20% fetal bovine serum (FBS) for ASPC-1 (HyClone, Logan, UT) and 5% FBS for all other cell lines respectively. BT474 was maintained in Roswell Park Memorial Institute medium supplemented with 15% FBS and bovine insulin (0.01 mg/ml, Life Technologies, Rockville, MD). 911 cells (a kind gift of Dr. Van Der Eb, Leiden University, Netherlands (50)) were maintained in DMEM supplemented with 5% FBS. All media were supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were grown in a humidified incubator at 37ºC with a 5% CO_2 atmosphere.

Female athymic nude mice (NCr-nu/nu, National Cancer Institute at Frederick, Frederick, MD) at 6-8 weeks of age were used for *in vivo* studies. All animals received humane care based on the guidelines set by the American Veterinary Association. All experimental protocols involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Adenoviral vectors

Replication-deficient Ad vectors (AdCMVLuc, AdCox2Luc) encoding the firefly luciferase reporter gene (Luc) were generated as described previously (44). The 5/3 chimeric fiber-knob modification was incorporated into the adenoviral structure as we previously reported (45). To generate IFN-expressing vectors, an expression cassette containing adenoviral death protein (ADP), an enhancer of apoptosis and viral spread, and the gene for IFN were cloned into an E3 shuttle plasmid and introduced into the E3 region of the viral genome by homologous recombination in *E. coli* (51, 52). Cox2 promoter-controlled Ad vectors were generated using homologous recombination in *E. coli* as described previously (42, 52). All viruses were propagated in the 911 cell line and purified by double CsCl density gradient ultracentrifugation, followed by dialysis against phosphate-buffered saline (PBS) with 10% glycerol. The vectors were titrated by plaque assay, and viral particle (vp) number was measured spectrophotomectrically with absorbance at 260 nm (53). Vectors were stored at -80 ºC until ready for use. Viral structure was confirmed by PCR for Cox2 and 5/3 fiber structure as described previously (42).

In vitro analysis of infectivity and Cox2 promoter strength with Luciferase-expressing Ads

Cells ($5x10^4$ cells/well) were grown in 24 well plates were infected with 10 plaque forming units (pfu)/ml for 48 hours, followed by lysis with 100 µl of cell culture lysis buffer (Promega, Madison, WI) and Luc activity was determined with the Luciferase Assay System (Promega). All experiments were performed in triplicate.

In vitro quantitative analysis of cancer cell killing ability

Cells were seeded in 96-well plates at 3000vp/cell (1500vp/cell for S2O13, S2VP10) then infected with Ad vectors at 1vp/cell (MiaPaCa-2) or 10vp/cell (S2O13, S2VP10) in 100 µl of DMEM 5% medium. The cells were incubated under standard conditions and the number of living cells was measured colorimetrically at serial time points using the Cell Titer Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. The proportion of living cells at each time point was normalized to the number of living uninfected cells. All experiments were done in triplicate.

In vitro analysis of cytocidal effect by crystal violet staining

$1x10^5$ cells were plated in 24 well plates then infected with virus at 0.1 or 1vp/cell in 1ml growth medium with 5% FBS. At serial time points, cells were fixed with 10% buffered formalin for 10 minutes then stained with 1% crystal violet in 70% ethanol for 20 minutes, then washed with water and dried.

In vitro IFN production by ELISA

S2O13 cells were plated in 24 well plates at $5x10^4$ cells/well then infected with virus at 1vp/cell in 1ml growth medium with 5% FBS. At serial time points, cell culture supernatant was collected and centrifuged to remove cell debris. Samples were analyzed for IFN concentration using a commercial human IFN ELISA kit (PBL Interferonsource, Piscataway, NJ) according to the manufacturer's instructions.

In vivo antitumor effect in a PDAc xenograft model

MiaPaCa-2 or S2O13 cells ($1x10^6$ cells in 100 µl PBS) were injected in each flank of female athymic nude mice. Groups were comprised of 5 animals each with 10

tumors/group. When the nodules reached a maximum diameter of 8-10 mm, each tumor was injected once with $1x10^{10}$ vp of virus or controls per 50 µl of PBS. Tumor size was measured with calipers, and tumor volume was calculated with the formula volume=width^2 x length/2. Animals were euthanized in accordance with the approved institutional protocol.

Statistical methods

Statistical analysis of viral effect *in vitro* and *in vivo* was carried out with Excel (Microsoft, Redmond WA). Student's *t* test of means was used with a two-tailed *p* value of less than 0.05 taken to be statistically significant. Data are expressed as mean ± standard deviation of at least three results except where indicated.

Chapter 3

The Structurally Analogous Vector RGDCox2CRAdΔE3ADP-IFN Demonstrates Similarly Potent In Vivo Activity in Hamsters

Introduction

An absolute requirement for optimizing CRAd design and function is a valid experimental system. For *in vivo* usage, there are few small animal models which are permissive to human adenovirus replication. Although nude mice are critically useful for studying the *in vivo* effect of human adenovirus on human tumor xenografts, this model system suffers from two serious shortcomings. First, nude mice, being deficient in the full spectrum of cell-mediated immunity, cannot serve as a valid model of the immune modulating effects of adenovirus or any immunomodulatory transgene. Secondly, nude mice, and rodents in general, do not allow human adenoviruses to replicate except in human xenograft tissue itself (12). Syrian hamsters are one of only two small animal systems which allow systemic human adenovirus replication (54), and are the easiest of the two to handle. It is not clear, however, how closely this replication conforms to replication of adenovirus in the human host.

Therefore, to study pancreatic cancer *in vivo* using human adenoviruses expressing an immunomodulatory transgene, it is necessary to have three things: one, a permissive and immunocompetent convenient model system, two, adenoviruses designed for use in this system, and three, syngeneic cancer cells as an immune-competent animal will reject a human xenograft unlike a nude mouse. With regard to the first stipulation, Syrian hamsters are one such useful system. For the second, a structurally analogous adenovirus as tested here earlier in human cells but optimized for infectivity toward hamster pancreatic cancer cell lines is described. For the third requirement, hamster

pancreatic cancer cell lines are available which approximate human pancreatic carcinogenesis.

The point of the CRAd system is that a promoter is chosen due to its differential activity in the cancer target of interest compared to normal tissue to allow for specific transcriptional targeting of cancer. For proof of concept, the identity of the promoter is not important. In the case of hamster pancreatic cancer cell lines, one needs to select a tumor-specific promoter that will approximate the differential activity only of the human situation, not necessarily the identical promoter as the human tumor. In this respect it is interesting that hamster pancreatic cancer, like human cancer, is known to be a Cox2-positive tumor (55).

Hamster pancreatic cancer, to the extent that it is studied here, is the result of experimental administration of potent carcinogens to induce pancreatic neoplasia, and the resulting cells immortalized (56). This is unlike the human situation of spontaneously arising cancer specimens being used for generating cell lines. Typically this has been done using live hamsters and harvesting of the cancerous pancreas (57) however this has also been described in vitro (58).

This laboratory has previously designed and tested adenoviruses with the RGD fiber modification (45) on hamster pancreatic cancer cell lines as well as confirmed the lack of cross-reactivity of human and hamster IFN (data not shown). An adenovirus which is fiber-modified for optimal infectivity toward pancreatic cancer, with replication controlled by the Cox2 promoter, and expressing hamster interferon α was created. It is hypothesized that the effect of this virus, and specifically the effect of its interferon

production, will show potent effects *in vivo*, and should be stronger in effect than the results of testing the analogous human IFN vector in nude mice.

Results

Confirmation of viral structure

PCR of viral DNA was used to confirm structure of the novel Cox-2 controlled virus RGDCox2CRAdΔE3ADP-IFN. Analysis was done for both the fiber region and for Cox2 promoter status as previously described (45).

IFN expression occurs after infection of hamster cell line HP1 with IFN-expressing virus

The ability of the novel virus to express hamster IFN on infection of HP1 was verified by ELISA of cell culture supernatant (Figure 3-1). This both verifies that the correct structure is present as well as ensures that IFN is expressed well upon infection, a critical requirement for eventual testing *in vivo*.

Oncolytic efficiency of IFN-expressing Ads in vitro

The novel adenovirus optimized for hamster use RGDCox2CRAdΔE3ADP-IFN was tested for cytolytic potency against two hamster cell lines, HP1 and HapT1 (Figure 3-2). Although all viral preparations were of high quality (data not shown), effective oncolysis was not seen until titers of at least 2000 vp/cell in either cell line. Compared to human PDAc cell lines (Figure 2-5A-C), a much higher titer is required for productive infection and killing.

Therapeutic efficacy of replication competent IFN-producing Ads in vivo

In vivo analysis of antitumor efficacy of replicating IFN producing viruses was performed using subcutaneous xenograft models in immunocompetent Syrian hamsters. This experiment tested the *in vivo* antitumor efficacy of RGDCox2CRAdΔE3ADP-IFN in both its direct and indirect immunomodulating effects. Established tumors were treated

with a single intratumoral injection of $3x10^{10}$ vp of virus at day 0. At day 24, the RGDCox2CRAdΔE3ADP-IFN group showed an average relative tumor volume of 1.65, compared to 4.64 with RGDCox2CRAd, 5.6 with RGDwt, and 5 with PBS. Results demonstrate a statistically significant tumor suppression of RGDCox2CRAdΔE3ADP-IFN at day 24 when compared to saline and to all other groups ($p<0.05$ for all). (Figure 3-3).

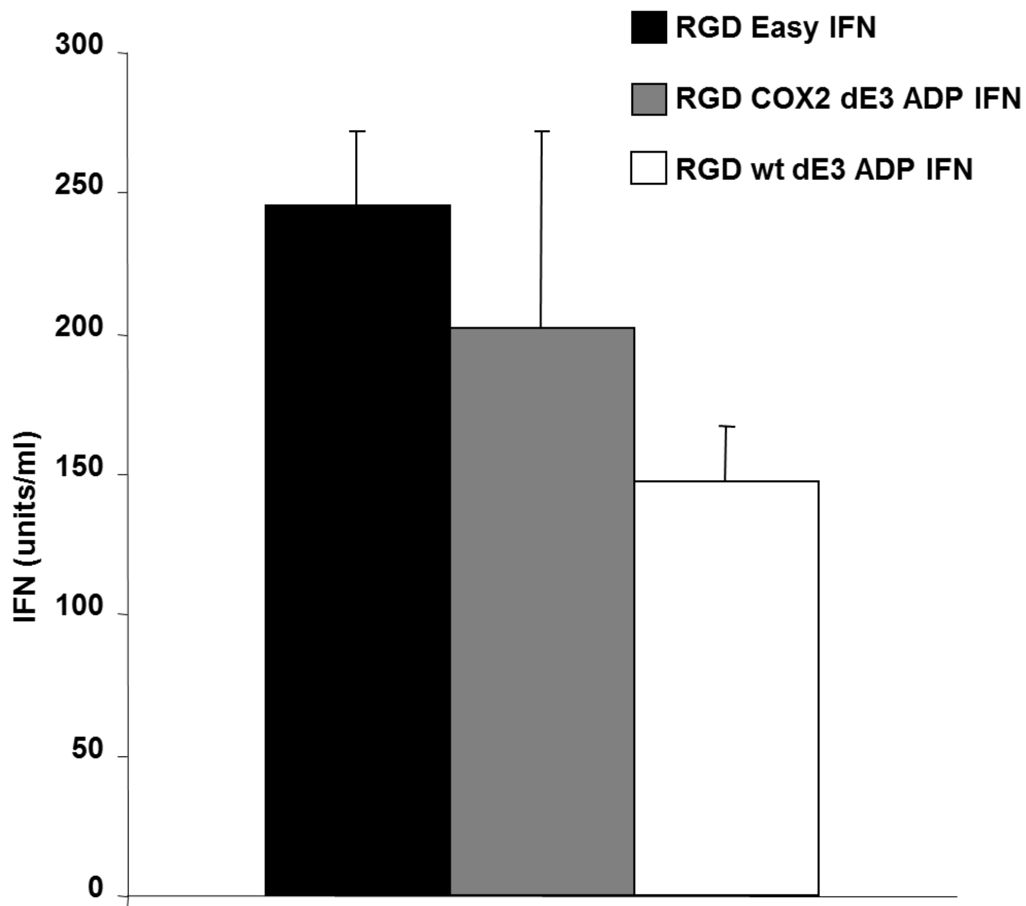


Figure 3-1. IFN production by viral infection in hamster cell line HP1. IFN levels were assayed from cell culture supernatant. This verifies the capability of all IFN viruses including Cox2-controlled IFN virus RGDCox2ΔE3DAP-IFN to produce IFN in this cell line after infection.

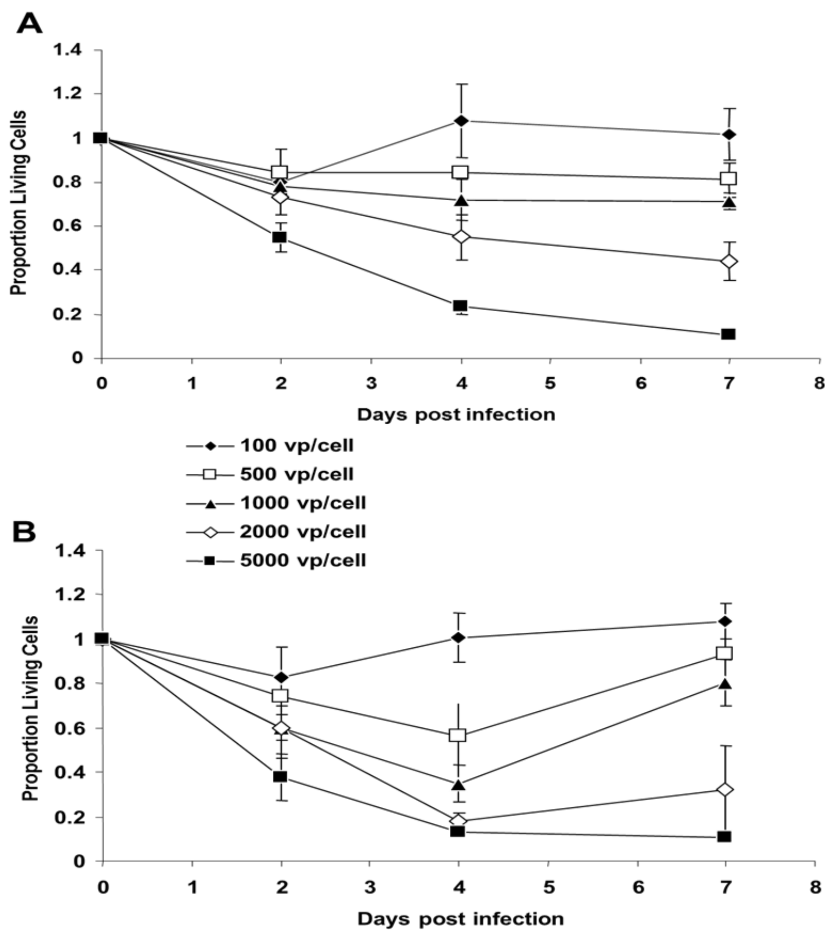


Figure 3-2. *In vitro c*ytocidal effect of differing infectious concentrations of RGDCox2CRAdΔE3ADP-IFN. Hamster pancreas cancer cell lines **(A)** HP1 and **(B)** HapT1 respectively were infected at day 0. Cell viability was determined with a colorimetric cell proliferation assay. The results are shown as proportion of living cells remaining relative to unifected cells. Killing either cell line requires titers of at least 2000 vp/cell in this assay, with cell line HapT1 appearing slightly more sensitive to higher viral titers.

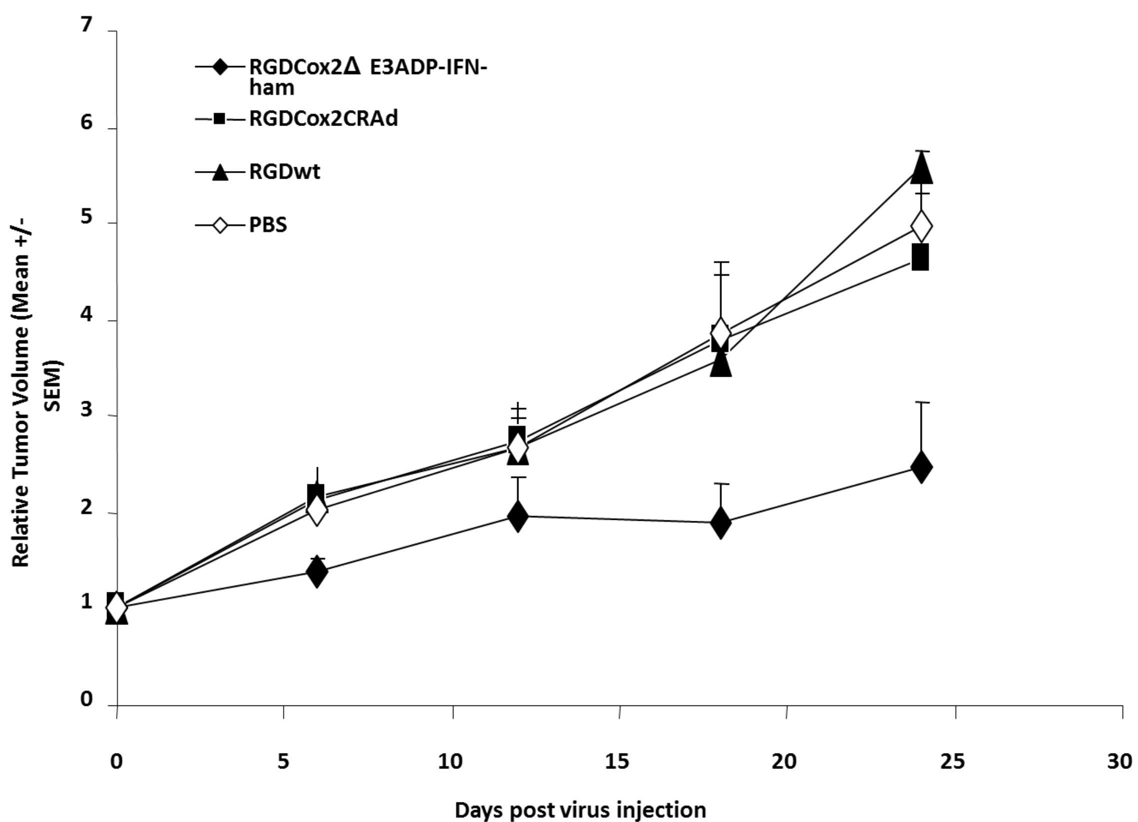


Figure 3-3. Superiority of replication-competent, ADP- and IFN-expressing adenovirus in an *in vivo* immunocompetent hamster PDAc model. HP1 syngeneic allograft hamsters were treated with a single intratumoral injection of PBS or vector. Tumor size is shown as relative tumor volume compared to day 0. Error bars indicate standard error of the mean (SEM). On day 24 the RGDCox2CRAdΔE3ADPIFN showed significantly stronger antitumor effect than RGDCox2CRAd, RGDwt, or PBS. (p<0.05 for all).

Materials and Methods

Cell lines and animals

The hamster pancreatic cancer cell lines HP1 and HapT1 were obtained from the American Type Culture Collection (Manassas, VA). Both were maintained in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Herndon, VA) with 5% fetal bovine serum (FBS). 911 cells (a kind gift of Dr. Van Der Eb, Leiden University, Netherlands (50)) were maintained in DMEM supplemented with 5% FBS. All media were supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were grown in a humidified incubator at 37ºC with a 5% CO_2 atmosphere.

Female Syrian hamsters (Charles River Laboratories, Wilmington, MA) at 7-8 weeks of age were used for *in vivo* studies. All animals received humane care based on the guidelines set by the American Veterinary Association. All experimental protocols involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Adenoviral vectors

Replication-deficient Ad vectors (AdCMVLuc, AdCox2Luc) encoding the firefly luciferase reporter gene (Luc) were generated as described previously (44). The RGD fiber modification was incorporated into the adenoviral structure as we previously reported (45). To generate IFN-expressing vectors, an expression cassette containing adenoviral death protein (ADP), an enhancer of apoptosis and viral spread, and the gene for IFN were cloned into an E3 shuttle plasmid and introduced into the E3 region of the viral genome by homologous recombination in *E. coli* (51, 52). Cox2 promoter-

controlled Ad vectors were generated using homologous recombination in *E. coli* as described previously (42, 52). All viruses were propagated in the 911 cell line and purified by double CsCl density gradient ultracentrifugation, followed by dialysis against phosphate-buffered saline (PBS) with 10% glycerol. The vectors were titrated by plaque assay, and viral particle (vp) number was measured spectrophotomectrically with absorbance at 260 nm (53). Vectors were stored at -80 ºC until ready for use. Viral structure was confirmed by PCR for Cox2 and RGD fiber structure as described previously (45).

In vitro quantitative analysis of cancer cell killing ability

Cells were seeded in 96-well plates at 2000vp/cell then infected with RGDCox2CRAdΔE3ADP-IFN at 0, 100, 500, 1000, 2000, or 5000 vp/cell in 100 µl of DMEM 2.5% medium. The cells were incubated under standard conditions and the number of living cells was measured colorimetrically at serial time points using the Cell Titer Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. The proportion of living cells at each time point was normalized to the number of living uninfected cells. All experiments were done in triplicate.

In vitro IFN production by ELISA

HP1 cells were plated in 24 well plates at $5x10^4$ cells/well then infected with virus at 100vp/cell in 1ml growth medium with 5% FBS. At day 3, cell culture supernatant was collected and centrifuged to remove cell debris. Samples were analyzed for IFN concentration using a commercial mouse IFN ELISA kit (PBL Interferonsource,

Piscataway, NJ) according to the manufacturer's instructions. According to documentation, hamster IFN is detectable with this kit.

In vivo antitumor effect in a PDAc xenograft model

HP1 cells (4×10^6 cells in 100 μl PBS) were injected in each flank of female Syrian hamsters. Groups were comprised of 5 animals each with 10 tumors/group. When the nodules reached a maximum diameter of 10 mm, each tumor was injected once with 3×10^{10} vp of virus or controls per 50 μl of PBS. Tumor size was measured with calipers, and tumor volume was calculated with the formula volume=width^2 x length/2. Animals were euthanized in accordance with the approved institutional protocol.

Statistical methods

Statistical analysis of viral effect *in vivo* was carried out with Excel (Microsoft, Redmond WA). Student's *t* test of means was used with a two-tailed *p* value of less than 0.05 taken to be statistically significant. Data are expressed as mean ± standard deviation of at least three results except where indicated.

Chapter 4

Discussion

Discussion

Currently, only surgery offers a possibility for the cure of pancreatic cancer. However, among the mere 10% or so in whom complete tumor resection is possible, many will ultimately succumb to local and distant recurrence (29, 32). Thus, a highly active targeted therapy which may be used alone or as an adjunct to multimodality therapy for local, advanced, and metastatic disease is acutely needed.

Adenoviral therapy offers this potential, but faces problems of low infectivity of cancer cells, inefficient viral spread within tumors, and off-target effects (21). Through rational design we have mitigated these problems to construct a novel IFN-expressing virus. Additionally, this virus offers the potential for localized, high-level IFN expression in the tumor itself. This may contribute to improved tolerability of an IFN-based multimodality strategy, which offers dramatic improvement in outcome of pancreatic cancer but is constrained by treatment-limiting toxicity (34-37). IFN-expressing viruses have previously been used to good effect by other investigators(48, 49), however the design of these vectors has been a nonreplicating one, which requires higher viral doses and lacks the benefit of viral persistence by replication.

The significance of our design strategy was demonstrated through analysis of infectivity and tumor specificity of our novel virus. The improved infectivity of the 5/3 fiber-knob chimera was demonstrated by using different Luc expression vectors to compare infectivity with the wild type capsid proteins to that of the 5/3 chimera (Figure 2-2). Significantly increased reporter gene activity was found in all cell lines with 5/3, indicating its superiority for tumor cell infection, and corroborating our earlier report (21).

Improved cancer cell infectivity, however, does not indicate cancer specificity. This element is achieved by using the Cox2 promoter (21, 22, 44) to drive viral replication as well as detargeting Cox2-negative normal hepatocytes. As the majority of systemically administered adenovirus is sequestered in the liver and as the E1 gene product of early adenoviral replication is toxic to hepatocytes, restricted replication is a crucial requirement for clinical use. Again using two reporter vectors, with one driven by Cox2 and the other by a nonselective promoter, Cox2 function is shown to be high in all PDAc cell lines tested and almost completely absent in BT474, a Cox2-negative control cell line (Figure 2-3). This indicates that the desired "tumor ON/liver OFF" profile was achieved. Additional demonstration of specificity by Cox2 status, using this cell line in an assay for cytopathic effect, showed no oncolysis with Cox2-controlled vectors including our novel virus. This was in contrast to near complete clearing of infected cells by viruses with an intact E1 region and unrestricted replication (Figure 2-4E).

We previously reported a strategy to massively express a transgene upon Ad replication in tumor cells, which uses the gene placed into the Ad E3 region. This structure has been proven to overexpress ADP, which facilitates viral spread and leads to a more efficient intratumoral spread of the virus (51, 52). Based on this system, we constructed an IFN- and ADP-expressing virus, incorporating the 5/3 capsid modification, and with an intact E1 region for unrestricted replication as a proof of concept (5/3wtΔE3ADP-IFN). Owing to lack of control of replication as outlined above, this virus is not suitable for human clinical use. *In vitro* testing of this virus (Figure 2-4A) shows a superior cytopathic effect to a similar complex construct lacking IFN

expression as well as 5/3Ad wt, the infectivity-enhanced control virus without ADP overexpression. These data indicate that genetic modification of the adenoviral capsid and ADP overexpression can greatly enhance the low efficacy of conventional Ads in pancreatic cancer.

When similar *in vitro* testing was applied to our novel 5/3Cox2CRAdΔE3ADP-IFN (Figure 2-4B-D), equal or improved potency compared with Ad5wt, the gold standard control virus without selectivity, is noted across all cell lines tested, in particular in both S2O13 and S2VP10, which are derived from metastatic tumors and thought to represent a more aggressive tumor phenotype. This would again indicate no detrimental loss of replicating ability or killing effect imposed by Cox2 replication control and the complex viral structure in our design.

To additionally characterize the oncolytic potency of 5/3Cox2CRAdΔE3ADP-IFN, we performed quantitative cell survival assays across multiple PDAc cell lines (Figure 2-5A-C). When compared to 5/3wtΔE3ADP-IFN as a positive control, a "lag phase" of cell death with 5/3Cox2CRAdΔE3ADP-IFN was repeatedly seen, which is potentially due to the artificial replication control imposed by the Cox2 promoter and resulting disruption of replication timing of viral genes (42). However, in all cell lines the Cox2-controlled virus trended toward eventual equivalence in oncolytic effect with the uncontrolled IFN virus, reaching statistical equivalence in two out of three experiments. Virtually no effect was seen upon low titer infection with 5/3EasyIFN indicating the increased dependence of *in vitro* cytopathic effect on replication-dependent IFN production and ADP overexpression.

In the case of hamster cell infection with RGDCox2CRAdΔE3ADP-IFN, a dramatically higher titer is required to cause cell death or even suppression of cell growth over time (Figure 3-2). This is undoubtedly a multifactorial phenomenon, likely contributed to by the intermediate level of replication of human adenoviruses in hamster cell lines (12) as well as the known low level of Cox2 activity in the cell line used. Additional unpublished work from this laboratory corroborates low Cox2 activity in HP1 cells and across several other hamster pancreatic cancer cell lines. Notably, this is not a phenomenon of poor cell targeting and inefficient expression, as the RGD modification has previously been shown to optimally target hamster pancreatic cancer cell lines as compared to wild-type adenovirus and Ad 5/3, the most effective for human pancreatic cancer (data not shown).

With regard to the separate issue of IFN expression, we analyzed levels seen after infection of a representative PDAc cell line with time (Figure 2-6). As expected, 5/3wtΔE3ADP-IFN produced large and increasing amounts of IFN, with a similar lagging increase produced by 5/3Cox2CRAdΔE3ADPIFN. Additionally, 5/3Cox2CRAdΔE3ADP-IFN produced a significantly higher IFN concentration than 5/3EasyIFN, in amounts that were continuing to increase at the final time point. This virus may be expected to have both a longer-lasting and more substantial IFN production after infection of cancer cells.

To demonstrate *in vivo* antitumor effect, subcutaneous PDAc xenografts in nude mice were treated with the IFN vectors. In an earlier experiment using 5/3wtΔE3ADP-IFN as the experimental vector, superior treatment effect compared to both ADP

expression alone and IFN expression alone was demonstrated. 5/3wtΔE3ADP-IFN virtually abrogated tumor growth until over one month post treatment (data not shown). Similarly, in a second experiment with the more aggressive S2O13 cell line which was chosen in order to highlight treatment effect, 5/3Cox2CRAdΔE3ADP-IFN showed superiority over the nonreplicating 5/3EasyIFN by day 21 post treatment (Figure 2-7). Although the result is significant, the immunodeficient nature of the experimental animals limits demonstration of the IFN component's systemic immune-stimulatory antitumor effect, which also may affect a difference seen in this system using an IFN-expressing virus.

Even more powerful results were evident in the hamster *in vivo* model (Figure 3-3). Somewhat surprisingly, despite the much lower *in vitro* effect of the hamster IFN virus as compared to the effect of the novel human virus on human cells, RGDCox2CRAdΔE3ADP-IFN strongly and persistently suppressed tumor growth in the hamster model. Compared to the human cell-nude mouse experiment (Figure 2-7), the hamster virus suppressed tumor growth over 3 times as potently when compared timepoint to timepoint at the end of the experiment. The cell line used is only weakly Cox2-positive. Had a stronger promoter in hamster pancreas cancer been selected, the results would have, undoubtedly, been even more profound. One interesting future experiment would be to treat only one of the bilateral flank tumors with adenovirus and observe for tumor regression on the opposite side. Such an effect has previously been reported in the hamster system with an IFN-expressing adenovirus, however this was with an earlier-generation virus of the nonreplicating type (48). A replicating IFN vector

as used here would be expected to not only be stronger in effect but also to be longer-acting owing to the replicating nature, higher transgene expression, and longer persistence.

It is not difficult to imagine a clinical scenario using intratumoral viral delivery (e.g., for locally advanced unresectable disease with administration by endoscopic ultrasound) and indeed at this institution investigators in the department of surgery have proposed such a phase I study using our viruses, and at least 1 study with this methodology is currently accruing patients (clinicaltrials.gov # NCT00415454). Systemic viral delivery as a route of administration offers the advantage of convenience, and we have previously shown in an orthotopic model that such a strategy also has an antitumor effect with limited toxicity (21).

Interferon alpha is a cytokine with pleiotropic effects. It has well-described biological properties including inhibition of cellular proliferation by cell cycle arrest, induction of apoptosis, anti-angiogenic effects, as well as a diverse immunostimulatory role. It is known to stimulate CD_8+ T cells, as well as NK cells and monocytes, and additionally to upregulate MHC expression for enhanced effector cell targeting (33). In terms of direct cellular effects, IFN acts through binding of its common receptor (composed of IFNAR-1 and -2 chains) to induce downstream tyrosine kinase signaling. However, the complexities of these processes present multiple avenues for escape mechanisms.

Differing susceptibility to alpha interferon has been well-described, with some cancer cell types, including some melanoma, ovarian carcinoma, and multiple myeloma,

responding poorly or not at all. However, these cell types do respond to the related cytokine interferon beta (33). The development of resistance to interferon, moreover, has been a widely reported phenomenon. Cells which adapt to downregulate pro-apoptotic IFN target genes such as TRAIL or which overexpress anti-apoptotic genes display two such avenues of escape. A third means of IFN resistance is increased expression of EGFR and its downstream targets, which actually appears to be directly stimulated by IFN as a stress response (59). It is likely that within PDAc itself, there exists differing susceptibility to IFN treatment, which may explain the differing strengths of 5/3Cox2CRAdΔE3ADP-IFN seen *in vitro* (Figs. 2-4B-D). Additionally, IFN is thought of as a cytostatic agent, best used with agents which either cause a separate tumoricidal effect such as an oncolytic virus or chemotherapy, or with complementary targeted agents such as EGFR inhibitors (59).

In summary, we have established a novel tumor-specific conditionally replicative adenovirus which expresses IFN and demonstrated proof of its *in vitro* and *in vivo* effect. Owing to its nature as a targeted tumor therapy with restricted replication, this offers the potential of local delivery of IFN in massive quantities without the need for systemic IFN administration and its attendant complications. By mitigating the toxicity of this element of IFN-based chemoradiotherapy, this strategy may find clinical use to expand the application of this robust and promising multimodality therapy to meet the pressing and continued need.

References

1. Kelly E, Russell SJ. History of oncolytic viruses: Genesis to genetic engineering. Mol Ther. 2007 Apr;15(4):651-9.

2. Cross D, Burmester JK. Gene therapy for cancer treatment: Past, present and future. Clin Med Res. 2006 Sep;4(3):218-27.

3. Hammill AM, Conner J, Cripe TP. Oncolytic virotherapy reaches adolescence. Pediatr Blood Cancer. 2010 Dec 15;55(7):1253-63.

4. Dock G. The influence of complicating diseases upon leukemia. Am J Med Sci. 1904;127:563-92.

5. Verma IM, Weitzman MD. Gene therapy: Twenty-first century medicine. Annu Rev Biochem. 2005;74:711-38.

6. Alemany R. Cancer selective adenoviruses. Mol Aspects Med. 2007 Feb;28(1):42-58.

7. Shirakawa T. The current status of adenovirus-based cancer gene therapy. Mol Cells. 2008 Jun 30;25(4):462-6.

8. Pesonen S, Kangasniemi L, Hemminki A. Oncolytic adenoviruses for the treatment of human cancer: Focus on translational and clinical data. Mol Pharm. 2011 Feb 7;8(1):12-28.

9. Russell WC. Update on adenovirus and its vectors. J Gen Virol. 2000 Nov;81(Pt 11):2573-604.

10. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab. 2003 Sep-Oct;80(1-2):148-58.

11. Garber K. China approves world's first oncolytic virus therapy for cancer treatment. J Natl Cancer Inst. 2006 Mar 1;98(5):298-300.

12. Yamamoto M, Curiel DT. Current issues and future directions of oncolytic adenoviruses. Mol Ther. 2010 Feb;18(2):243-50.

13. Dobbelstein M. Viruses in therapy--royal road or dead end? Virus Res. 2003 Apr;92(2):219-21.

14. Tripathy SK, Black HB, Goldwasser E, Leiden JM. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. Nat Med. 1996 May;2(5):545-50.

15. Mathis JM, Stoff-Khalili MA, Curiel DT. Oncolytic adenoviruses - selective retargeting to tumor cells. Oncogene. 2005 Nov 21;24(52):7775-91.

16. Chuah MK, Collen D, VandenDriessche T. Biosafety of adenoviral vectors. Curr Gene Ther. 2003 Dec;3(6):527-43.

17. Fukazawa T, Matsuoka J, Yamatsuji T, Maeda Y, Durbin ML, Naomoto Y. Adenovirus-mediated cancer gene therapy and virotherapy (review). Int J Mol Med. 2010 Jan;25(1):3-10.

18. Hardcastle J, Kurozumi K, Chiocca EA, Kaur B. Oncolytic viruses driven by tumor-specific promoters. Curr Cancer Drug Targets. 2007 Mar;7(2):181-9.

19. Curiel DT. The development of conditionally replicative adenoviruses for cancer therapy. Clin Cancer Res. 2000 Sep;6(9):3395-9.

20. Krasnykh VN, Mikheeva GV, Douglas JT, Curiel DT. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. J Virol. 1996 Oct;70(10):6839-46.

21. Ramirez PJ, Vickers SM, Ono HA, Davydova J, Takayama K, Thompson TC, et al. Optimization of conditionally replicative adenovirus for pancreatic cancer and its evaluation in an orthotopic murine xenograft model. Am J Surg. 2008 Apr;195(4):481-90.

22. Wesseling JG, Yamamoto M, Adachi Y, Bosma PJ, van Wijland M, Blackwell JL, et al. Midkine and cyclooxygenase-2 promoters are promising for adenoviral vector gene delivery of pancreatic carcinoma. Cancer Gene Ther. 2001 Dec;8(12):990-6.

23. Wesseling JG, Bosma PJ, Krasnykh V, Kashentseva EA, Blackwell JL, Reynolds PN, et al. Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors. Gene Ther. 2001 Jul;8(13):969-76.

24. Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ, Wold WS. 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. 1996 Apr;70(4):2296-306.

25. Hamid O, Varterasian ML, Wadler S, Hecht JR, Benson A,3rd, Galanis E, et al. Phase II trial of intravenous CI-1042 in patients with metastatic colorectal cancer. J Clin Oncol. 2003 Apr 15;21(8):1498-504.

26. Seth P, Zhang Z, Krimmel J, Zhang Z, Hu Z. Systemic delivery of a novel liver-detargeted oncolytic adenovirus causes reduced liver toxicity but maintains the anti-tumor response in a breast cancer bone metastasis model. Hum Gene Ther. 2011 Apr 12.

27. Cancer facts & figures 2010 [Internet]. Atlanta: American Cancer Society; 2010. Available from: http://www.cancer.org/Research/CancerFactsFigures/CancerFactsFigures/cancer-facts-figures-2010-rev.

28. Nakeeb A, Lillemoe KD, Yeo CJ, Cameron JL. Neoplasms of the exocrine pancreas. In: Greenfield's Surgery. 4th ed. Lippincott Williams & Wilkins; 2006.

29. Merchant NB, Parikh AA, Liu EH. Adjuvant chemoradiation therapy for pancreas cancer: Who really benefits? Adv Surg. 2010;44:149-64.

30. Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: A randomized controlled trial. JAMA. 2007 Jan 17;297(3):267-77.

31. NCCN clinical practice guidelines in oncology: Pancreatic adenocarcinoma [Internet].: National Comprehensive Cancer Network. Available from: http://www.nccn.org/professionals/physician_gls/PDF/pancreatic.pdf.

32. Katz MH, Wang H, Fleming JB, Sun CC, Hwang RF, Wolff RA, et al. Long-term survival after multidisciplinary management of resected pancreatic adenocarcinoma. Ann Surg Oncol. 2009 Apr;16(4):836-47.

33. Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, et al. Apoptosis and interferons: Role of interferon-stimulated genes as mediators of apoptosis. Apoptosis. 2003 Jun;8(3):237-49.

34. Nukui Y, Picozzi VJ, Traverso LW. Interferon-based adjuvant chemoradiation therapy improves survival after pancreaticoduodenectomy for pancreatic adenocarcinoma. Am J Surg. 2000 May;179(5):367-71.

35. Linehan DC, Tan MC, Strasberg SM, Drebin JA, Hawkins WG, Picus J, et al. Adjuvant interferon-based chemoradiation followed by gemcitabine for resected pancreatic adenocarcinoma: A single-institution phase II study. Ann Surg. 2008 Aug;248(2):145-51.

36. Picozzi VJ, Abrams RA, Decker PA, Traverso W, O'Reilly EM, Greeno E, et al. Multicenter phase II trial of adjuvant therapy for resected pancreatic cancer using cisplatin, 5-fluorouracil, and interferon-alfa-2b-based chemoradiation: ACOSOG trial Z05031. Ann Oncol. 2010 Aug 9.

37. Picozzi VJ, Kozarek RA, Traverso LW. Interferon-based adjuvant chemoradiation therapy after pancreaticoduodenectomy for pancreatic adenocarcinoma. Am J Surg. 2003 May;185(5):476-80.

38. Tucker ON, Dannenberg AJ, Yang EK, Zhang F, Teng L, Daly JM, et al. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res. 1999 Mar 1;59(5):987-90.

39. Cerella C, Sobolewski C, Dicato M, Diederich M. Targeting COX-2 expression by natural compounds: A promising alternative strategy to synthetic COX-2 inhibitors for cancer chemoprevention and therapy. Biochem Pharmacol. 2010 Dec 15;80(12):1801-15.

40. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, et al. Cyclooxygenase in biology and disease. FASEB J. 1998 Sep;12(12):1063-73.

41. Martin-Sanz P, Mayoral R, Casado M, Bosca L. COX-2 in liver, from regeneration to hepatocarcinogenesis: What we have learned from animal models? World J Gastroenterol. 2010 Mar 28;16(12):1430-5.

42. Yamamoto M, Davydova J, Wang M, Siegal GP, Krasnykh V, Vickers SM, et al. Infectivity enhanced, cyclooxygenase-2 promoter-based conditionally replicative adenovirus for pancreatic cancer. Gastroenterology. 2003 Oct;125(4):1203-18.

43. Yip-Schneider MT, Barnard DS, Billings SD, Cheng L, Heilman DK, Lin A, et al. Cyclooxygenase-2 expression in human pancreatic adenocarcinomas. Carcinogenesis. 2000 Feb;21(2):139-46.

44. Yamamoto M, Alemany R, Adachi Y, Grizzle WE, Curiel DT. Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers. Mol Ther. 2001 Mar;3(3):385-94.

45. Davydova J, Le LP, Gavrikova T, Wang M, Krasnykh V, Yamamoto M. Infectivity-enhanced cyclooxygenase-2-based conditionally replicative adenoviruses for esophageal adenocarcinoma treatment. Cancer Res. 2004 Jun 15;64(12):4319-27.

46. Hatanaka K, Suzuki K, Miura Y, Yoshida K, Ohnami S, Kitade Y, et al. Interferon-alpha and antisense K-ras RNA combination gene therapy against pancreatic cancer. J Gene Med. 2004 Oct;6(10):1139-48.

47. Ohashi M, Yoshida K, Kushida M, Miura Y, Ohnami S, Ikarashi Y, et al. Adenovirus-mediated interferon alpha gene transfer induces regional direct cytotoxicity and possible systemic immunity against pancreatic cancer. Br J Cancer. 2005 Aug 22;93(4):441-9.

48. Hara H, Kobayashi A, Yoshida K, Ohashi M, Ohnami S, Uchida E, et al. Local interferon-alpha gene therapy elicits systemic immunity in a syngeneic pancreatic cancer model in hamster. Cancer Sci. 2007 Mar;98(3):455-63.

49. Narumi K, Kondoh A, Udagawa T, Hara H, Goto N, Ikarashi Y, et al. Administration route-dependent induction of antitumor immunity by interferon-alpha gene transfer. Cancer Sci. 2010 Jul;101(7):1686-94.

50. Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoeben RC, et al. Characterization of 911: A new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum Gene Ther. 1996 Jan 20;7(2):215-22.

51. Ono HA, Le LP, Davydova JG, Gavrikova T, Yamamoto M. Noninvasive visualization of adenovirus replication with a fluorescent reporter in the E3 region. Cancer Res. 2005 Nov 15;65(22):10154-8.

52. Davydova J, Gavrikova T, Brown EJ, Luo X, Curiel DT, Vickers SM, et al. In vivo bioimaging tracks conditionally replicative adenoviral replication and provides an early indication of viral antitumor efficacy. Cancer Sci. 2010 Feb;101(2):474-81.

53. Maizel JV,Jr, White DO, Scharff MD. The polypeptides of adenovirus. I. evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology. 1968 Sep;36(1):115-25.

54. Thomas MA, Spencer JF, La Regina MC, Dhar D, Tollefson AE, Toth K, et al. Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. Cancer Res. 2006 Feb 1;66(3):1270-6.

55. Crowell PL, Schmidt CM, Yip-Schneider MT, Savage JJ, Hertzler DA,2nd, Cummings WO. Cyclooxygenase-2 expression in hamster and human pancreatic neoplasia. Neoplasia. 2006 Jun;8(6):437-45.

56. Ulrich AB, Schmied BM, Standop J, Schneider MB, Pour PM. Pancreatic cell lines: A review. Pancreas. 2002 Mar;24(2):111-20.

57. Townsend CM,Jr, Franklin RB, Gelder FB, Glass E, Thompson JC. Development of a transplantable model of pancreatic duct adenocarcinoma. Surgery. 1982 Jul;92(1):72-8.

58. Ikematsu Y, Liu G, Fienhold MA, Cano M, Adrian TE, Hollingsworth MA, et al. In vitro pancreatic ductal cell carcinogenesis. Int J Cancer. 1997 Sep 17;72(6):1095-103.

59. Caraglia M, Marra M, Tagliaferri P, Lamberts SW, Zappavigna S, Misso G, et al. Emerging strategies to strengthen the anti-tumour activity of type I interferons: Overcoming survival pathways. Curr Cancer Drug Targets. 2009 Aug;9(5):690-704.