A TIMP2-ARMED CONDITIONALLY-REPLICATING ADENOVIRUS FOR THE TREATMENT OF OVARIAN CANCER

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A DISSERTATION

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Ovarian cancer remains the most lethal gynecological malignancy in the U.S. Conventional therapies have limited therapeutic value due to advanced stage of the disease at diagnosis. Conditionally-replicating adenoviruses (CRAds) are promising, novel anti-cancer agents that are designed to selectively replicate in and lyse tumor cells. In clinical trials, CRAds exhibited limited efficacy thus far. Second generation CRAds are being developed to express a therapeutic protein to further enhance antitumor efficacy. One attractive target in ovarian tumor microenvironment is the matrix metalloproteinases (MMPs) that degrade the extracellular matrix. Thus, tissue inhibitor of metalloproteinase 2 (TIMP2), an endogenous inhibitor of MMPs, is a promising candidate to arm a CRAd intended to treat ovarian cancer. We hypothesize that a CRAD armed with TIMP2 will inhibit ovarian cancer progression through two distinct mechanisms. First, specific replication in tumor cells should lead to oncolysis. Second, secretion of TIMP2 into the tumor microenvironment should inhibit excess extracellular MMPs and activate other MMP-independent signaling pathways to inhibit tumor growth, angiogenesis and metastasis.

A targeted and armed CRAd, Ad5/3-CXCR4-TIMP2 was developed using the CXCR4 promoter for enhanced replication, expressing the TIMP2 transgene. First, we confirmed the secretion and functional activity of TIMP2, as demonstrated by the inhibi-
tion of gelatin degradation by MMPs. In addition, arming with TIMP2 did not inhibit viral replication nor oncolytic potency, as the TIMP2 armed viruses showed enhanced killing of cancer cells when compared to the unarmed viruses. Examination of viral replication in primary stage III and IV ovarian cancer samples revealed a consistent high level of viral replication with Ad5/3-CXCR4-TIMP2. The therapeutic efficacy of the TIMP2-armed CRAd was then evaluated in an orthotropic model of ovarian cancer, in which athymic mice were intraperitoneally injected with human ovarian cancer cells. The results demonstrated that the Ad5/3-CXCR4-TIMP2 delayed tumor growth and significantly increased survival when compared to the unarmed CRAd. This effect was mediated through inhibition of MMPs. Collectively, the present study demonstrated the enhanced therapeutic efficacy of the dual-action, TIMP2-armed CRAd in vivo and the translational potential of using Ad5/3-CXCR4-TIMP2 for treatment in patients with advanced ovarian cancer.

Keywords: CRAds, CXCR4, TIMP2, ovarian cancer
DEDICATION

This dissertation is dedicated to my parents. Thank you for all your sacrifices, support and encouragements throughout the years. Your high hopes for me have motivated me to be the best that I could be and to always strive for more. I am grateful and feel blessed to have you both in my life.
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<tr>
<td>Ad5</td>
<td>human adenovirus serotype 5</td>
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<td>ADP</td>
<td>adenovirus death protein</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BCIP/NBT</td>
<td>5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CAR</td>
<td>coxsackievirus and adenovirus receptor</td>
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<td>CD</td>
<td>cytosine deaminase</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CRAd</td>
<td>conditionally replicating adenovirus</td>
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<tr>
<td>CXCR4</td>
<td>CXC chemokine receptor 4</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGFP</td>
<td>enhanced green fluorescent</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
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<tr>
<td>GCV</td>
<td>gancyclovir</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>H &amp; E</td>
<td>hematoxylin and eosin</td>
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<td>Abbreviation</td>
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<tr>
<td>hNIS</td>
<td>human sodium iodide symporter</td>
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<td>HSV-TK</td>
<td>herpes simplex virus thymidine kinase</td>
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<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<td>IU</td>
<td>infectious units</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>Luc</td>
<td>luciferase</td>
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<td>MLP</td>
<td>major late promoter</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MSLN</td>
<td>mesothelin</td>
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<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-buffered saline with tween</td>
</tr>
<tr>
<td>pIX</td>
<td>protein IX</td>
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<tr>
<td>Q-PCR</td>
<td>quantitative polymerase chain reaction</td>
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<td>QRT-PCR</td>
<td>quantitative reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic acid</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SLPI</td>
<td>secretory leukoprotease inhibitor</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline, 0.1% Triton X-100</td>
</tr>
<tr>
<td>Abbreviation</td>
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<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>tissue culture infectious dose 50</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
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<tr>
<td>TSP</td>
<td>tumor- or tissue-selective promoter</td>
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<tr>
<td>UAB</td>
<td>The University of Alabama at Birmingham</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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CHAPTER 1

INTRODUCTION

Impact of Ovarian Cancer

The American Cancer Society estimates that 21,880 women will be diagnosed with ovarian cancer in the U.S., and an estimated 13,850 women will die from the disease in 2010 [1]. Today, ovarian cancer ranks as the leading cause of gynecological cancer death in the U.S. In the early stages, patients with ovarian cancer present with non-specific symptoms that often mimic digestive and bladder disorders, which precludes its real diagnosis. In addition, there is a lack of an effective and accurate screening test. Thus, in majority of patients, diagnosis is made at an advanced stage (stage III), where the cancer has disseminated within the peritoneal cavity and often accompanied with malignant ascites. Consequently, surgery alone is rarely sufficient and adjuvant chemotherapy is often employed postoperatively. Despite advances in surgical techniques and chemotherapeutic agents, the overall mortality rate has remained unchanged over the last four decades. This presents an apparent need for new and effectively targeted therapies that can treat the disseminated disease.

Conditionally-Replicative Adenoviruses are a New Class of Anticancer Agents

Conditionally-replicative adenoviruses (CRAds) are appealing anticancer agents because they can be engineered to replicate selectively in the tumor cells [2] (Figure 1).
Figure 1: Viral replication cycle of a CRAd. Following infection, a CRAd will only replicate in the target tumor cells and kills the cell by lysis. Then, the released viral progeny infects and replicates in the surrounding target cells. The achievement of this lateral spread is a key event for the effectiveness of CRAd based therapy. (from ref. [2])
Following infection, these viruses will replicate only in the tumor cells, amplifying the initial viral inoculum, which leads to viral oncolysis and the release of viral progeny. Subsequently, the progeny virions can self-perpetually repeat the cycles of infection, replication, and cell lysis until potentially all the cells of the tumor are destroyed, while sparing normal cells [3, 4].

Human adenovirus serotype 5 (Ad5) has several advantages over other oncolytic viruses [5, 6]. Adenoviruses are associated with mild disease. Moreover, the Ad5 genome has been extensively studied and its protein functions are well understood, which allows for easy genetic modifications. Extensive modifications have been made to the capsid protein, fiber, for enhanced and specific infection of tumor cells. In addition, adenovirus can be purified to high titers and is stable in the bloodstream, two features that permit systemic administration and thus the possibility of treating metastases. Collectively, these characteristics have led to the extensive use of Ad5 as an oncolytic agent.

Thus far, tumor-selective replication of CRAds has been achieved via two main strategies. One strategy involves deletions in viral genes necessary for replication in the normal cells but not in the tumor cells. The first and most prominent example of this is the dl1520 virus, also known as Onyx-015 [7]. The Onyx-015 adenovirus contains a deletion of the E1B-55k gene that is responsible for p53 inactivation. Thus, it was conceived as an oncolytic virus that would selectively replicate in p53-deficient tumor cells, while lacking the ability to replicate in normal cells. Later investigations revealed that the late function of E1B-55k, nuclear export of viral mRNAs, was the key determinant of its selectivity [8]. Alternatively, selective replication can be achieved by
placing the expression of the viral E1A gene, which is essential for viral replication, under the control of a tumor or tissue-selective promoter (TSP) [9]. Several promoters have been tested and shown selectivity for the targeting of ovarian cancer, including secretory leukoprotease inhibitor (SLPI) [10], survivin [11], CXCR4 [12], human telomerase reverse transcriptase (hTERT) [13] and mesothelin (MSLN) [14] promoters.

**CRAds Exhibit Limited Efficacy in Clinical Trials**

In recognition of the oncolytic potential of CRAds, they have been rapidly translated into human clinical trials, where their safety has been demonstrated. A phase I clinical trial with the aforementioned replication selective Onyx-015 adenovirus was conducted in patients with recurrent or refractory epithelial ovarian cancer [15]. This study demonstrated that the virus is safe, as it was well tolerated and no dose-limiting vector-related toxicity was experienced. However, the results proved disappointing, indicating no clear-cut evidence of clinical or radiologic response in any patient. Similar results were observed for other cancers as well [16]. This suggests that efficacy of CRAds must be enhanced for successful clinical translation.

**Oncolytic Potency of a CRAd Also Depends on the Expression of the Primary Adenovirus Receptor on the Target Cell**

A major factor limiting the efficacy of CRAds as cancer therapeutics is due to the underexpression or deficiency of the primary receptor on the surface of cancer cells [17]. The binding of an adenovirus to a target cell is dependent upon the interaction of the knob domain of fiber with its receptor on the target cell [18]. For Ad5, the primary
receptor that the knob binds to is the coxsackievirus and adenovirus receptor (CAR) [19]. Following binding to CAR, the Arginine-Glycine-Aspartic acid (RGD) motif on the penton base interacts with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the cell surface and leads to virus internalization. Cells deficient in CAR, including many types of cancer cells, are poorly transduced by the unmodified adenoviruses and result in the low therapeutic efficacy observed [17]. This is not unexpected, as CAR has been implicated to function as a tumor suppressor protein, and is often down regulated during cellular transformation [20]. The problem with low CAR levels is augmented in CRAds, where the potential therapeutic advantage of replication by CRAds is negated by poor intratumoral spread due to low CAR levels [21].

**Adenoviruses Can be Targeted at the Level of Transduction**

To increase the transduction of cancer cells, adenoviruses have been engineered to target alternative receptors that are more prevalent on the cancer cells when compared to normal cells. These tropism alterations are broadly classified into two groups: adaptor-mediated or genetic modification. Genetic modification is further subdivided into pseudotyping and ligand incorporation. Pseudotyping is the incorporation of a fiber or knob from a different serotype, whose receptor is more abundantly expressed on target cells and allows for enhanced transduction. A prime example of this is the Ad5/3 virus, which still retains the Ad5 fiber shaft and tail, but whose knob domain has been replaced with that of the Ad3 virus. This knob modification allows CAR-independent binding to target cells expressing the Ad3 receptor [22]. Alternatively, a ligand can be incorporated into the fiber that mediates binding to an alternative receptor. An example is the
Ad5.RGD virus, in which a ligand containing RGD motif is incorporated into the HI loop of the fiber knob, which allows for direct interaction of the RGD-containing knob with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [23]. In studies evaluating the transduction of ovarian cancer cells by adenovirus with different fiber modifications, Ad5/3 consistently demonstrated enhanced transduction over Ad5.RGD vectors both in vitro and in vivo [24, 25]. Furthermore, the increased transduction seen with Ad5/3 chimeric fiber has been shown to increase the potency of CRAds [26, 27].

**CRAds Can Be Armed with Therapeutic Genes to Increase Anti-tumor Efficacy**

Another strategy to augment efficacy of CRAds is to use them for the delivery of a therapeutic transgene, also called arming, in which the input dose of the transgene is amplified by replication of the virus [28]. Transgenes employed to date include suicide genes such as cytosine deaminase (CD) and herpes simplex virus thymidine kinase (HSV-TK), the tumor suppressor p53, and the pro-inflammatory cytokine, tumor necrosis factor $\alpha$ (TNF$\alpha$) [29-32]. The inclusion of these therapeutic transgenes has been shown to enhance the oncolytic potency of CRAds in both in vitro and in vivo studies. Unfortunately, the use of cytotoxic genes also presents drawbacks. The timing of prodrug administration is crucial with adenoviruses armed with HSV-TK or CD, which work by converting the prodrug into a cytotoxic analog that inhibits DNA replication. Bad timing has been shown to limit viral replication and spread by causing premature killing [33].
Current arming strategies of CRAds for ovarian cancer focus primarily on enhanced killing of tumor cells. However, a tumor is a heterogeneous population composed not only of cancerous cells, but also fibroblasts, endothelial cells and immune cells. This heterogeneity results from the complex processes of invasion and angiogenesis, during which malignant cells recruit normal stromal cells to help support their growth. Thus, tumor growth, invasion and metastasis formation all require the active participation of the tumor-reactive stroma for the remodeling of the extracellular matrix and formation of new blood vessels [34]. It is increasingly apparent that the proliferative and invasive behavior of ovarian cancer is modified by the microenvironment. In light of this, we proposed that it is rational to arm CRAds with a transgene that modulates the microenvironment, and thus in turn, acts on the cancer cells indirectly.

**Strategies for Targeting of Ovarian Cancer Should Consider the Properties of the Microenvironment**

The importance of the interplay between the cancer cell and the microenvironment in modulating tumor progression has long been recognized. Tumor growth, angiogenesis, invasion and metastasis all require the degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are a family of endogenous proteinases capable of degrading all components of the ECM. Physiologically, MMPs play an important role in tissue remodeling, however their dysregulation has been revealed to be central to tumor progression [35] (Figure 2). Furthermore, numerous studies have shown that MMPs are upregulated in virtually all
**Figure 2: Current perspective of MMP roles in cancer.** MMPs are now known to contribute to multiple steps of tumor progression including tumor growth, angiogenesis, invasion and the establishment and growth of metastatic lesions. In addition, it is recognized that MMPs are not only synthesized by tumor cells but are frequently produced by surrounding stromal cells, including fibroblasts and infiltrating inflammatory cells. (From ref. [35])
human cancers, including ovarian cancer and their overexpression has been linked to malignant transformation [36]. In particular, MMP-2 and MMP-9 have been shown to be consistently upregulated in ovarian cancer cells as well as in the ascites fluid [37]. MMP-2 and MMP-9 are collagenases and are capable of degrading type IV collagen in the basement membrane, thus playing an important role in promoting metastasis formation. In a study using both MMP-9 wildtype and knockout (KO) mice, it was demonstrated that MMP-9 wildtype mice had consistently higher numbers of tumor incidence, ascites and blood vessel formation when compared to the MMP-9 KO mice. This suggests that host stromal MMP-9 expression plays a critical role in angiogenesis and growth of xenograft human ovarian cancer cells in the peritoneal cavity of mice [38]. This data is further supported by an immunohistochemical study on biopsy samples from patients with various stages of ovarian cancer. Kamat et al. found an increased level of stromal MMP-2, MMP-9 and MT1-MMP and these increases correlated with aggressive clinical features, such as advanced stage, high-grade ascites and positive lymph node. MT1-MMP contains a transmembrane domain and thus is attached to the cell surface; it is responsible for the activation of pro-MMP-2. Furthermore, stromal expression of MMPs was shown to be a better predictor of clinical outcome than epithelial MMP expression [39]. Collectively, these studies confirm that the tumor-host microenvironment modifies the proliferative and invasive behavior of ovarian cancer cells. Thus it is rational to arm a CRAd with a secreted protein targeting the microenvironment for the treatment of ovarian cancer.
**Tissue Inhibitors of Metalloproteinases are Key Regulator of MMPs**

Increasing data support the role of stromal MMPs in tumor growth, angiogenesis and metastasis of ovarian tumors, which validates MMPs as good therapeutic targets for ovarian cancer therapy. Activity of MMPs is tightly regulated by a group of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). To date, TIMPs 1 though 4 have been identified. TIMPs are a family of small (21-29 kDa) secreted proteins. Although any TIMP can bind and inhibit any active MMP in a 1:1 stoichiometric ratio, there are preferential pairings [40] (Figure 3). Furthermore, numerous studies conducted using replication-deficient adenovirus expressing TIMPs have proved their utility for anticancer therapy [40-43]. These viruses, armed with TIMP-1, -2 or -3, resulted in the reduction of tumor growth, angiogenesis and metastasis in various cancer models.

**TIMP2 is Unique Among Members of the TIMP Family**

Despite similarities between members of the TIMP family, it is important to recognize that each TIMP exhibits distinctive structure, biochemistry and expression profile [44, 45]. Among the members of the TIMP family, TIMP2 has been most extensively studied. In addition to its antitumor activity as an inhibitor of MMPs, TIMP2 can also inhibit tumor progression by a variety of mechanisms independent of MMP inhibition. Seo *et al.* have shown that TIMP2 can inhibit the proliferation of endothelial cells in response to angiogenic stimuli from fibroblast growth factor (FGF) 2 or vascular endothelial growth factor (VEGF) A *in vitro* and angiogenesis *in vivo*. These effects require the binding of TIMP2 to $\alpha_3\beta_1$ integrin on endothelial cells, which leads to the dissociation of HSP60 and SHP-1 from $\beta_1$ subunits. Released SHP-1, a protein tyrosine
Figure 3: Interaction between TIMP and MMP. TIMP binds active MMP in a 1:1 stoichiometric ratio and results in the inactivation of MMP. (From ref. [40])
phosphatase, associates with the FGFR-1 and VEGFR-2, cognate receptors for FGF-2 and VEGF-A respectively, and prevents receptor phosphorylation and activation [46]. Oh et al. have demonstrated that TIMP2 inhibits migration of endothelial cells through an indirect effect on MMPs that is mediated by the upregulation of the RECK protein, a membrane anchored MMP inhibitor [47]. Finally, Feldman et al. have reported on an increased activity of mitogen-activated protein kinase phosphatase 1 in tumors overexpressing TIMP2, which leads to an increase dephosphorylation of p38 mitogen-activated protein kinase that results in inhibition of tumor growth and angiogenesis [48].

Our hypothesis is that a CRAd armed with TIMP2 will inhibit the progression of ovarian cancer by two distinct mechanisms. First, viral replication should lead to direct lysis of cancer cells. Second, secretion of TIMP2 from infected tumor cells will inhibit tumor growth and angiogenesis through the direct inhibition of excess extracellular MMPs, as well as through MMP-independent pathways.

**Experimental Design**

To validate our hypothesis, we first constructed a TIMP2-armed CRAd, Ad5/3-CXCR4-TIMP2, for the treatment of disseminated ovarian cancer. The TIMP2 transgene was inserted into the CRAd backbone in place of the viral E3B genes, which are dispensable in an oncolytic adenovirus and has been reported to yield high levels of transgene expression, late in the infection cycle [49]. We decided to incorporate the Ad5/3 fiber modification into our armed CRAd based on its ability to highly transduce ovarian cancer cell lines relative to fibroblasts when compared to the other fiber modifications (Figure 4). Since the Ad5/3 modified fiber also enhances transduction of
Figure 4: Transduction of ovarian cancer cells by different fiber modifications. To determine which of these fiber-modified vectors would be most selective and efficient for the transduction of ovarian cancer cells, three human ovarian carcinoma-derived cell lines Hey, SKOV3.ip1, OV4, and a control fibroblast (VH10) cell line, were transduced at a multiplicity of infection (MOI) of 10 infectious units (IU) per cell. Forty-eight hours post-transduction, the cells were lysed and assayed for luciferase activity. The results shown are normalized to the luciferase activity of the vector with the wildtype Ad5 fiber.
non-target fibroblasts, a TSP will be used to achieve selective viral replication in the ovarian cancer cells. Therefore even if the virus infects normal fibroblasts, it should not be able to replicate and thereby preventing any harmful effects on non-target cells. As discussed above, this Ad5-based virus was rendered conditionally-replicative in cancer cells though the usage of a TSP in E1A region. Based on our preliminary studies, the TSP we choose was the CXCR4 promoter, which exhibited highest ratio of replication in ovarian cancer cells relative to control fibroblasts when compared to survivn, Flt1, MSLN, SLPI, and hTERT promoters (Figure 5).

Studies performed in this thesis work are presented as two chapters. The first chapter, entitled “Conditionally Replicating Adenovirus Expressing TIMP2 for Ovarian Cancer Therapy” contains necessary data that characterized Ad5/3-CXCR4-TIMP2 and evaluated its efficacy in vitro using human epithelial ovarian cancer cell lines and ex vivo in primary ovarian tumor samples from patients with stage III and IV epithelial ovarian cancer. The second chapter, entitled “Conditionally Replicating Adenovirus Expressing TIMP2 Increased Survival in a Disseminated Model of Ovarian Cancer” contains data that examined the efficacy of Ad5/3-CXCR4-TIMP2 in a murine model of disseminated ovarian cancer. Collectively, these studies support the use of TIMP2 in ovarian cancer therapy.
Figure 5: Different TSP activity in ovarian cancer cells. To determine which of these promoters would be the most selective for the targeting of ovarian cancer, a panel of isogenic recombinant adenoviral vectors containing the promoters driving the expression of the luciferase gene was used. Three ovarian carcinoma-derived cell lines, Hey, SKOV3.ip1, OV4, and the control fibroblast, VH10, cell line were transduced by the vectors at an MOI of 10 IU/cell. Forty-eight hours post-transduction, the cells were lysed and assayed for luciferase activity. The results are normalized to the luciferase activity in cells transduced with the vector containing the control cytomegalovirus (CMV) promoter.
CHAPTER 2

CONDITIONALLY-REPLICATING ADENOVIRUS EXPRESSING TIMP2 FOR OVARIAN CANCER THERAPY

BY

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Abstract

**Purpose:** Current treatments for ovarian cancers have limited therapeutic outcomes due to advanced stage of the disease at diagnosis. Among new therapies, conditionally-replicating adenoviruses (CRAds), designed to selectively lyse cancer cells, hold promise. In clinical trials, CRAds exhibited limited efficacy thus far. Second generation CRAds are being developed to express a therapeutic protein to enhance antitumor efficacy. One attractive target in the tumor microenvironment is the matrix metalloproteinases (MMPs) that degrade the extracellular matrix, and are upregulated in ovarian cancer. Tissue inhibitor of metalloproteinase 2 (TIMP2) is an endogenous inhibitor of MMPs. The present study developed and evaluated a novel CRAd (Ad5/3-CXCR4-TIMP2) for ovarian cancer therapy.

**Experimental Design:** A targeted CRAd, Ad5/3-CXCR4-TIMP2 was developed using the CXCR4 promoter for enhanced replication, expressing the TIMP2 transgene. The efficacy of this armed CRAd was determined in both established human ovarian cancer cell lines and in primary ovarian tumor samples.

**Results:** Ad5/3-CXCR4-TIMP2 mediated expression of functional TIMP2, as demonstrated by the inhibition of MMP activity. In addition, arming with TIMP2 did not inhibit viral replication nor oncolytic potency, as the TIMP2 armed viruses showed enhanced killing of cancer cells when compared to the unarmed viruses. We also examined viral replication in primary ovarian cancer tissues obtained from patients with stage III and IV ovarian cancer. In four of the tissue samples, Ad5/3-CXCR4-TIMP2 revealed a 4- to 30- fold increase in replication when compared to the Ad5/3 virus.
**Conclusion:** Results support the translational potential of Ad5/3-CXCR4-TIMP2 for treatment of patients with advanced ovarian cancer.
**Translational Relevance:**

Ovarian cancer remains the leading cause of gynecological cancer death in the U.S. due to advanced stage of cancer at diagnosis along with the lack of effective therapy for disseminated disease. Clinical trials using adenoviral based gene therapy have exhibited safety but suggest the need to increase vector efficacy for successful clinical translation. Towards this goal, we designed a CRAd which combines the property of tumor selective replication with the delivery of a therapeutic transgene, TIMP2, which targets the tumor microenvironment to inhibit growth and dissemination. Our article is the first study of a CRAd armed with a non-cytotoxic transgene for ovarian cancer therapy. Moreover, we have employed an *ex vivo* model system using primary ovarian tumors slices to examine efficacy of replication of these viruses. These promising preclinical results provide the basis for clinical translation.
**Introduction**

Ovarian cancer is the leading cause of gynecological cancer death in the U.S., accounting for approximately 14,600 deaths annually. In most cases, diagnosis is made at an advanced stage, where the cancer has disseminated, thus significantly lowering survival. Despite advancements in surgical techniques and chemotherapeutic agents, the overall mortality rate has remained unchanged over the last four decades (1). Therefore, new and effectively targeted therapies are urgently needed to limit ovarian cancer progression.

Among new therapies based on the approaches of molecular medicine, the use of conditionally replicative adenoviruses (CRAds) are highly promising for the treatment of ovarian cancer. Due to their oncolytic potential, CRAds have been rapidly translated into human clinical trials, where their safety has been demonstrated, but with only modest clinical results (2), indicating the need for further improvement of CRAds for successful clinical translation. Towards this goal, we designed a CRAd which combines the property of tumor selective replication with delivery of a therapeutic transgene to target ovarian cancer survival and dissemination.

Many cancer cells, including ovarian cancer cells, exhibit a paucity of the adenovirus serotype 5 (Ad5) receptor, the coxsackie and adenovirus receptor (CAR), thereby limiting CRAds infection and thus therapeutic potential (3, 4). To increase the transduction of ovarian cancer cells, adenoviruses have been genetically manipulated allowing retargeting to an alternative receptor that is more abundantly expressed on target cells. The Ad5/3 virus, which still retains the shaft and tail of Ad5 fiber, but has had the Ad5 knob replaced with the Ad3 fiber knob, exhibits enhanced transduction both *in vitro* and
in vivo as compared to the Ad5 wildtype vector (5, 6). This fiber knob modification was included in the CRAd used in this study. For targeted replication and to minimize deleterious effects on non-target tissues, a tumor selective promoter (TSP) was also used to limit viral replication to cancer cells. The CXCR4 promoter exhibits a “tumor on, liver off” profile, thus making it ideal for the targeting of ovarian cancer cells (7).

As with most solid tumors, ovarian cancer growth, angiogenesis, invasion and metastasis all require the degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are capable of degrading all components of the ECM. Physiologically, MMPs play an important role in tissue remodeling. However, their dysregulation has been shown to be central to tumor progression (8, 9). In particular, MMP-2 and MMP-9 have been shown to be consistently upregulated in ovarian cancer and are associated with poor prognosis (10, 11). Activity of MMPs is tightly regulated by a group of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). To date, four mammalian TIMPs have been identified; with each TIMP exhibiting differences in structures, biochemical properties and expression profiles (12, 13). Among the members of the TIMP family, TIMP2 has been most extensively studied. In addition to its antitumor activity as an inhibitor of MMPs, TIMP2 can also inhibit tumor growth and angiogenesis by a variety of mechanisms independent of MMP inhibition (14-16).

The present study developed and determined the therapeutic potential of an armed CRAd (Ad5/3-CXCR4-TIMP2) for ovarian cancer therapy. Transductional selectivity of the CRAd was achieved by genetically replacing the Ad5 knob with the Ad3 knob. The CXCR4 promoter was used to mediate tumor selective replication of the vector. The TIMP2 gene was incorporated to increase the antitumor effects. We hypothesized that the
production of TIMP2 from virally infected cells should bind and inhibit the excess extracellular MMPs and thereby inhibit tumor progression through both MMP-dependent and MMP-independent pathways. In addition to validating the virus with ovarian cancer cell lines, the present study also evaluated the effects of the armed-CRAd in tumor tissues obtained from stage III and IV ovarian cancer patients, identifying the superior potential of this vector for patients presenting with an advanced stage of ovarian cancer.
Materials and Methods

Cell lines. The A549 human lung carcinoma cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Ovarian adenocarcinoma cell lines SKOV3.ip1 and OV-4 were kind gifts from Drs. Janet Price and Judy Wolf, respectively (MD Anderson Cancer Center, Houston, TX). Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Ham’s F-12 medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). All cell lines were cultured at 37 °C in a humidified atmosphere and at 5% CO₂. Media and supplements were purchased from Mediatech (Herndon, VA).

Human ovarian cancer tissue and culture. Human primary ovarian tumor samples were obtained from the Division of Gynecologic Oncology, Department of Obstetrics & Gynecology, University of Alabama at Birmingham, with institutional review board approval at the time of initial debulking from patients with histologically confirmed ovarian adenocarcinoma. Samples were kept on ice in University of Wisconsin solution (Barr Laboratories, Inc. Pomona, NY) until slicing. Time from harvest to slicing was kept at an absolute minimum (≤ 2 hours). The precision tissue cut technique was performed using the Krumdieck Tissue Slicer as previously described (17). Briefly, tissue slices (250 μm -thick) were placed into 24-well plates (1 slice per well) containing 1 mL of complete culture media (RPMI with 1% antibiotics, 1% L-glutamine, and 10% FCS). The plates were then incubated at 37°C and 5% CO₂ in a humidified environment.
under normal oxygen concentrations for up to 4 days. A plate rocker set at 60 rpm was used to agitate slices to ensure adequate oxygenation and viability (18).

**Construction and production of the viruses.** The wild-type human adenovirus serotype 5, Adwt300, was purchased from ATCC. The tropism-modified control virus, Ad5/3, containing the Ad5 fiber shaft and tail has had the knob domain replaced with that of the Ad3 virus (19). The control, Ad5/3-CXCR4, is a CRAd with the CXCR4 promoter inserted into the E1A position (20).

The TIMP2 armed viruses were constructed as follows: A full-length human TIMP2 cDNA was isolated from Ad-TIMP2, an E1- and E3-deleted replication deficient Ad5 vector, which expresses TIMP2 under the control of the CMV promoter (21). The TIMP2 gene was subcloned in place of the E3B region into pZErO-2 E3 6.9, a transfer vector containing a 6.9 kb fragment of the Ad5 genome including the E3 region (kind gift of Dr. Nik Korokhov, VectorLogics, Inc., Birmingham, AL). The resultant plasmid was linearized with *Bam*HI and cotransformed into *E. coli* BJ5183 (Stratagene, La Jolla, CA) with a *Swa*I-linearized plasmid, pVK500CΔE3, containing the Ad5 genome deleted for both the E3 region and the fiber gene (22). The resulting plasmid was cotransformed into *E. coli* BJ5183 with an *EcoRI*-linearized plasmid, pKAN.F5/3, containing the Ad5 fiber shaft and tail and an Ad3 fiber knob (23). The resultant plasmid, pVK500C-TIMP2-F5/3 was linearized with *Pac*I and used to transfect A549 cells to generate the tropism-modified armed replicating adenovirus, Ad5/3-TIMP2. To produce the tropism-modified armed CRAd, Ad5/3-CXCR4-TIMP2, pVK500C-TIMP2-F5/3, was subjected to a final round of recombination by linearization with *Swa*I and cotransformation into *E. coli*
BJ5183 with a PmeI-linearized pCXCR4, a plasmid containing the CXCR4 promoter (7). This final plasmid, pVK500C-CXCR4-TIMP2-F5/3, was linearized with PacI and used to transfect A549 cells to generate Ad5/3-CXCR4-TIMP2. Viruses were amplified in the A549 cell line and purified by two rounds of cesium chloride density centrifugation. The titers of viral particles and infectious units were determined (24).

**Quantitative reverse-transcriptase polymerase chain reaction (QRT-PCR) for expression of TIMP2 and viral genes.** SKOV3.ip1 and OV-4 cells were infected with Ad5/3-CXCR4 or Ad5/3-CXCR4-TIMP2 at a multiplicity of infection (MOI) of 10 infectious units (IU) per cell. At 12, 24, and 36 h post-infection, total RNA was isolated from cell lysates using the RNeasy Mini Kit (Qiagen, Valencia, CA) and subjected to QRT-PCR analysis (LightCycler 480 system, Roche Diagnostics, Indianapolis, IN). RNA from cells infected with the armed CRAd was assayed for expression of TIMP2, whereas samples from cells infected with Ad5/3-CXCR4 were assayed for expression of the E3B gene RIDα. All samples were analyzed for expression of ADP and fiber. Expression of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Results were expressed as copy number/ng of total RNA. Primer sequences used in the study are as follows:

**TIMP2:**
forward primer: 5’- TCTGGATGGACTGGGTACCA-3’
reverse primer: 5’-CTTGATGCAGGCGAAGAACTT-3’
probe: 5’-6FAM -AGAAGAACATCAACGGGCACCAGGC- TAMRA-3’
RIDα:
forward primer: 5’-GCTGGAAACGAATAGATGCCA-3’
reverse primer: 5’-GTTGCAGTGAAGCATAGCG-3’
probe: 5’-6FAM-ACCACCCAACCTTTCCCGGC-TAMRA-3’

ADP:
forward primer: 5’-TCTGCTGCCTAAAGCGCAA-3’
reverse primer: 5’-TTTGGGTGTAGCACAATGATGG-3’
probe: 5’-6FAM-CGCGCCCGACCACCATCCTATAGT-TAMRA-3’

Fiber:
forward primer 5’-TGATGTTTGACGCTACAGCCATA-3’
reverse primer: 5’-GATTGTTGTTGGTGCCATTAGGTG-3’
probe: 5’-6FAM-ACCAAATTCAAGCCCATCTCCTGCATTAATG-TAMRA-3’

GAPDH:
forward primer: 5’-GGTTTACATGTTCCAATATGATTCCA-3’
reverse primer: 5’-ATGGGATTTCCATTGATGACAAG-3’
probe: 5’-CGTTTCGCTAGCCTTGAGCGGTGCCAT-3’

Expression and bioactivity of the TIMP2 protein from armed viruses. SKOV3.ip1 cells were infected with Ad5/3-CXCR4, Ad5/3-TIMP2 or Ad5/3-CXCR4-TIMP2 at an MOI of 10 IU per cell and overlaid with 1 ml serum-free medium. At 24, 48, and 72 hours post-infection, 1 ml samples of medium were concentrated to 100 µl using a Microcon centrifugal filter device (Millipore, Bedford, MA). The presence of TIMP2 was detected by SDS-PAGE followed by immunoblotting using a mouse anti-human
TIMP2 primary antibody (R&D Systems Inc., Minneapolis, MN; diluted 1:250) and an anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham Biotech, Arlington Heights, IL; diluted 1:5000). Blots were developed with enhanced chemiluminescence Western blotting detection reagents (Amersham Biotech). Recombinant human TIMP2 was used as the positive control (R & D Systems Inc.).

To determine functional activity of TIMP2, reverse zymography was performed as previously described (25). Briefly, the samples were electrophoresed in 0.1% SDS, 12% polyacrylamide gels containing 1 mg/ml gelatin and MMP-2 (Reverse Zymography Kit, School of Biological Sciences University of East Anglia, UK). After electrophoresis the gels were washed overnight with 2.5% Triton X-100, incubated in 50mM Tris/HCl, pH 7.5 and 5mM CaCl₂ for 16–18 h at 37°C, then stained and destained to visualize TIMP activity. Dark bands on the gel indicated the inhibition of gelatin degradation by MMP due to the presence of TIMP.

Viral DNA replication in human ovarian cancer cell lines. SKOV3.ip1 and OV-4 cells were infected with Ad5/3-CXCR4-TIMP2 or each of the control viruses at an MOI of 0.1 IU per cell and overlaid with 1 ml medium. Two and 4 days post-infection, DNA was extracted from 200 µl samples of medium (QIAamp DNA Blood Mini Kit, Qiagen) and analyzed by Q-PCR for the Ad5 E4 gene as an indicator of viral replication, using previously described primers and probe (26). Results were expressed as copy number/ng of total DNA.
**Viral DNA replication in human ovarian tumor tissues.** Tumors were sliced as described above. These slices were approximately 15-cell layers thick and contained about 1 x 10^6 cells per slice. Tumor slices were placed in 24-well plates and infected with 5 x 10^6 IU of Ad5/3-CXCR4-TIMP2 or each of the control viruses, diluted in 1 ml RPMI medium with 2% (v/v) FBS. The following day, infection mixtures were replaced with 1 ml RPMI medium containing 10% (v/v) FBS and all supplements. At 1 to 4 days post-infection, 200 μl samples of medium were harvested, DNA was purified as described above, and samples were analyzed for the presence of the Ad5 E4 gene by Q-PCR, using previously described primers and probe (26). Results were expressed as copy number/ng of total DNA.

**Oncolytic potency of CRAbs.** To determine the oncolytic potency of the viruses, cell viability was measured quantitatively with a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, SKOV3.ip1 and OV-4 cells were plated in 96 well plates and infected in triplicate with the replicating viruses at a range of MOIs: 1 – 10^-5 IU per cell. Eight days post-infection, 20 μl of the MTS reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium] was added to each well that contained 100 μl of medium. Cells were then incubated for 3 h at 37°C and the resultant absorbance was record at 490 nm. All cell viability results were expressed as a percentage of viable cells compared to uninfected control.
Immunohistochemistry. Briefly, paraffin sections of human primary ovarian tumors were deparaffinized in xylene and hydrated through graded alcohol. Antigen retrieval was performed in citrate buffer (pH 6.0), under steam for 20 min. Sections were cooled to room temperature, and endogenous peroxidase was removed using 0.3% H$_2$O$_2$ in methanol for 30 min and blocked with 5% BSA for 30 min. Tissue sections were then incubated with primary antibodies overnight at 4°C. Sections were washed in Phosphate-buffered saline (PBST) containing 0.05% Tween-20 and again incubated at room temperature with biotin-conjugated goat anti-goat/anti-mouse secondary antibody for 2 h. After washing, sections were incubated with streptavidin-conjugated horseradish peroxidase for 1 h at room temperature. After another wash with PBST, immunodetection was performed using 3,3′-diaminobenzidine-H$_2$O$_2$ (Vector Labs) and counterstained with hematoxylin.

Statistical analysis. Data were analyzed by one-way ANOVA. A Tukey test was also applied for multiple comparisons wherever applicable. Values provided are the mean ± SE, and the differences were considered significant if $P < 0.05$. 
Results:

Construction and characterization of a tropism-modified, TIMP2 armed CRAd. A tropism-modified CRAd, armed with TIMP2, was constructed for the treatment of ovarian cancer. The genomes of this CRAd (Ad5/3-CXCR4-TIMP2), along with control viruses are shown schematically in Fig. 1. The TIMP2 transgene was engineered into the E3B region of the genome under the control of the native promoter, splicing and polyadenylation signals. The gene encoding the adenovirus death protein (ADP) was retained to mediate efficient lysis and viral progeny release from infected cells. This armed CRAd was validated first for transgene expression in the human ovarian cancer cell line, SKOV3.ip1. Quantitative RT-PCR confirmed the expression of TIMP2 from the E3B region of Ad5/3-CXCR4-TIMP2. More importantly, TIMP2 expression in the armed CRAd correlated with the expression profile of the native E3B gene, RIdα, from the unarmed CRAd, Ad5/3-CXCR4 (Fig. 2A). In addition, expression of TIMP2 by the armed CRAd, did not inhibit upstream ADP (Fig. 2B) or downstream fiber gene expressions (Fig. 2C). Secreted TIMP2 was detectable in the medium at 24 hours postinfection from ovarian cancer cells infected with the armed viruses (Fig. 2D). Further, TIMP2 produced by the armed viruses was biologically active, which was indicated by the inhibition of gelatin cleavage by the MMPs in the reverse zymography analysis (Fig. 2E). Similar results were obtained when OV-4 ovarian cancer cells were infected (not shown).

Expression of TIMP2 does not inhibit the oncolytic potency of a replicating adenovirus in vitro. Monolayers of SKOV3.ip1 and OV-4 cells were infected with
either Ad5/3-CXCR4-TIMP2 or each of the control viruses at an MOI of 0.1 infectious units per cell (IU/cell). On days 2 and 4 postinfection, DNA was extracted from the media and subjected to quantitative PCR (Q-PCR) analysis to determine the copy number of adenoviral E4 region as a measure of viral DNA replication. The tropism modified TIMP2 armed virus, Ad5/3-TIMP2 and the armed CRAd, Ad5/3-CXCR4-TIMP2, replicated on day 4 at levels similar or higher to their respective unarmed controls in both SKOV3.ip1 (Fig. 3A) and OV-4 cells (Fig. 3B). This indicated that arming with TIMP2 does not inhibit viral replication. Moreover, selectivity of viral replication was examined in control fibroblast cells. As shown in Fig. 3C, Ad5/3-CXCR4-TIMP2 replicated less efficiently than Ad5/3-TIMP2 in control fibroblasts, suggesting that replication was selective with the CXCR4 promoter in ovarian cancer cells. Next, oncolytic potency of the viruses was examined quantitatively using the MTS assay on both SKOV3.ip1(Fig. 3D) and OV-4 cells (Fig. 3E). Results of this assay further demonstrated the oncolytic potency of the TIMP2 armed viruses, which exhibited enhanced killing of ovarian cancer cells when compared to the unarmed viruses in both SKOV3.ip1 and OV-4 cells.

**CRAd armed with TIMP2 replicates efficiently in primary ovarian cancer tissues.** Following successful testing of the armed CRAd (Ad5/3-CXCR4-TIMP2) in established human ovarian cancer cell lines that indicated both its therapeutic transgene expression and oncolytic potential, we sought to determine if these effects would be similar in ovarian cancer tissues as a prelude to clinical translation of this virus. To this end, we employed an *ex vivo* model system to further examine replication efficacy of Ad5/3-CXCR4-TIMP2 and the control viruses. Five primary epithelial ovarian tumors were
collected and examined for viral replication from patients with advanced disease. The median patient age was 60 years old (range 49 – 69) and all patients were Caucasian. Four of the five patients were diagnosed with stage IIIC papillary serous adenocarcinoma of the ovary, where tumors larger than 2 cm were present in the peritoneal cavity. The fifth patient was diagnosed with stage IV metastatic ovarian carcinoma. The tumors were obtained during debulking surgeries and maintained in culture immediately following surgery. Tumor slices of 250 μm -thick were made, and were infected at MOI of $5 \times 10^6$ IU/slice with Ad5/3-CXCR4-TIMP2 or each of the control viruses. Viral replication was accessed through determination of E4 copy number. Consistently, Ad5/3-CXCR4-TIMP2 exhibit high levels of viral replication (Fig. 4A-E). Moreover, in four of the five patients, Ad5/3-CXCR4-TIMP2 exhibited 4- to 30- fold increase in viral replication when compared to the Ad5/3 virus, suggesting the potential of this vector for ovarian cancer therapy. Furthermore, we examined by immunohistochemistry, the expression level of the CXCR4 receptor as an indicator of the CXCR4 promoter activity, which we have used to achieve selective replication. Shown in Fig. 5A, there is a strong CXCR4 staining in the four of the five primary ovarian tumors examined, indicating that CXCR4 promoter is a good promoter for targeted replication in ovarian cancer cells. Examination of primary tumor sample from patient 4 reveled low level of endogenous TIMP2 expression. However, 48 hours after infection with the TIMP2 armed viruses, the level of TIMP2 present in the tissue increased significantly (Fig. 5B).
Discussion:

In recognition of the oncolytic potential of replicating adenoviruses, these microbiologica lls have been rapidly translated into human clinical trials for patients with advanced cancer. These studies have demonstrated safety, but suggest the need for strategies to improve their efficacy for successful clinical translation (27). One strategy used to improve the efficacy of adenoviruses is to engineer the virus to deliver a therapeutic transgene (28). Most of these “armed” oncolytic adenoviruses have been designed to carry suicide genes, such as cytosine deaminase or herpes simplex virus thymidine kinase, that augment virus mediated killing of the infected tumor cells (29, 30). However, the timing of the prodrug administration is extremely crucial, as bad timing could potentially inhibit viral spread due to premature killing of the virus prior to the completion of the viral replication cycle. Instead of focusing solely on enhanced killing of the infected cancer cells, we hypothesized that it would be rational to arm the replicating adenovirus with a secreted protein that can also modulate the tumor microenvironment and thereby limit or prevent tumor progression.

Toward this goal, we sought to enhance the efficacy of a replicating adenovirus by arming it with TIMP2. TIMP2 possesses a number of attractive features that favor its use in this therapeutic strategy. TIMP2 is a small, 21 kDa unglycosylated protein that is naturally secreted. TIMP2 binds in a 1:1 stoichiometric ratio to the active forms of MMPs, including MMP-2 and MMP-9, thereby inhibiting the MMP activity associated with tumor growth, angiogenesis and invasion (9). Thus, overexpressing TIMP2 in the context of a tumor microenvironment should directly block tumor growth. A distinct advantage of TIMP2 over other members of the TIMP family is that it has also been
shown to inhibit tumor growth and angiogenesis by a variety of mechanisms independent of direct MMP-inhibition (14-16). TIMP2 can inhibit the proliferation of endothelial cells in response to angiogenic stimuli such as fibroblast growth factor 2 or vascular endothelial growth factor A by binding to $\alpha_3\beta_1$ integrin and cause the induction of protein tyrosine phosphatase activity (16). TIMP2 also inhibits migration of endothelial cells through an indirect MMP inhibitor effect that is mediated by the upregulation of the RECK protein, a membrane anchored MMP inhibitor (15). Finally, tumors overexpressing TIMP2 have reported to have an increased activity of mitogen-activated protein kinase phosphatase 1, which leads to an increase dephosphorylation of p38 mitogen-activated protein kinase that, in turn, results in inhibition of tumor growth and angiogenesis (14). Studies with non-replicative adenoviral vectors expressing TIMP2 have proved their utility for cancer therapy, resulting in the reduction of tumor growth, angiogenesis and metastasis in various cancer models (31-33). Collectively, these studies provide a clear rationale for a therapeutic strategy exploiting the localized overexpression of TIMP2 in the tumor microenvironment.

We hypothesized that Ad5/3-CXCR4-TIMP2, a CRAd armed with TIMP2, should directly kill the cancer cells through viral oncolysis, while secretion of TIMP2 by the infected cells into the tumor microenvironment would augment the therapeutic effect by preventing tumor growth and angiogenesis via both MMP-dependent and MMP-independent mechanisms. We have validated \textit{in vitro} that our TIMP2 armed viruses are secreting functional TIMP2, as indicated by the inhibition of the enzymatic degradation of gelatin by active MMPs. For the TIMP2 armed CRAd to be efficacious, it is important that the expression of TIMP2 does not impair viral replication nor its oncolytic potency.
We have demonstrated that both viral replication and oncolytic potency were not compromised in both SKOV3.ip1 and OV-4 human ovarian cancer cells by arming with TIMP2. Moreover, we showed that with the CXCR4 promoter, there is selectivity in replication, as indicated by higher level of replication in the ovarian cancer cells, when compared to the control fibroblasts. In the hopes of translating this virus into the clinic, we have employed the usage of an \textit{ex vivo} model system to further examine viral replication. Tumors samples examined from five patients with stage III and IV ovarian cancer revealed a consistent high level of replication with Ad5/3-CXCR4-TIMP2 when compared to the other control viruses. This data is very encouraging, suggesting that Ad5/3-CXCR4-TIMP2 might be effective for the treatment of advanced ovarian cancer. Studies are underway to examine the biodistribution and toxicity of Ad5/3-CXCR4-TIMP2 in a murine model of orthotopic disseminated ovarian cancer.
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33. Rigg AS, Lemoine NR. Adenoviral delivery of TIMP1 or TIMP2 can modify the invasive behavior of pancreatic cancer and can have a significant antitumor effect in vivo. *Cancer Gene Ther* 2001;8: 869-78.
Figure Legends

Figure 1. Genomic organization of armed CRAd and control viruses. Adwt300, is the wild-type Ad5 virus. Ad5/3 is a replicating Ad5 virus with a modified fiber that contains the shaft and tail of Ad5 fiber and the Ad3 fiber knob. Ad5/3-CXCR4 is a CRAd with a CXCR4 promoter in E1 to limit viral replication to the cancer cells in conjunction with the aforementioned fiber modification. Ad5/3-TIMP2 is a replicating virus armed with TIMP2 in E3B region and with the F5/3 modified fiber. Ad5/3-CXCR4-TIMP2 is a CRAd with the CXCR4 promoter in E1, armed with TIMP2 in the E3B region and the F5/3 modified fiber.

Figure 2. Characterization of armed CRAd. SKOV3.ip1 ovarian cancer cells were infected with Ad5/3-CXCR4-TIMP2 (■), or Ad5/3-CXCR4 (♦) at a MOI of 10 IU per cell. At the indicated times postinfection, cellular RNA was extracted and subjected to QRT-PCR to detect expression of: A, the TIMP2 gene in cells infected with Ad5/3-CXCR4-TIMP2 or the RIDα gene in cells infected with Ad5/3-CXCR4. B, the ADP gene. C, the fiber gene. All samples were tested in triplicate and graphed as the mean ± SD. D, Secretion of TIMP2 by infected SKOV3.ip1 cells. At the indicated times postinfection, conditioned medium was harvested and subjected to immunoblot analysis using an anti-TIMP2 antibody. E, Secreted TIMP2 binds active MMPs and inhibits the degradation of gelatin. Conditioned medium was harvested from SKOV3.ip1 cells at indicated times postinfection and subjected to reverse zymography.
Figure 3. Viral replication and oncolytic potency of the armed CRAds. SKOV3.ip1 (A), OV-4 (B) ovarian cancer cells and control fibrolasts (C) were infected with the indicated viruses. The conditioned culture medium was harvested on 2 and 4 days postinfection. DNA was extracted and subjected to Q-PCR to detect the E4 gene copy number as a measure of viral DNA replication. SKOV3.ip1 (D) and OV-4 (E) ovarian cancer cell lines were infected at indicated MOIs. Eight days postinfection, cell viability was assessed quantitatively using the MTS assay. Results were normalized to the uninfected cells and plotted as relative viable cell fraction. All samples were performed in triplicate. *p< 0.05.

Figure 4. TIMP2 armed CRAd exhibit enhanced viral replication in primary epithelial ovarian tumor samples. (A-E) Five human primary ovarian tumor tissues were obtained from debulking surgeries of patients with either stage III or stage IV disease. Slices from the tissues were made, then infected with the indicated viruses and maintained in culture for up to four days. DNA was extracted from the media at the indicated times and subjected to Q-PCR to detect the E4 gene copy number as a measure of viral DNA replication. Data indicate mean ± SD from triplicate assays for each patient tissue. *p< 0.05.

Figure 5. Immunohistochemistry in primary epithelial ovarian tumor slices. (A) Four human primary ovarian tumor tissues from patients with advanced cancer, were examined for expression of CXCR4. Primary ovarian tumor from patient 4 was examined for TIMP2 (B) expression with the indicated viruses at 48 hrs postinfection.
Figure 1
Figure 2A
Figure 2B
Figure 2C
Figure 2D
Figure 2E
Figure 3A
Figure 3B

The figure shows a bar graph with the x-axis labeled "Time post-infection (days)" and the y-axis labeled "E4 copy number." The graph compares five different conditions labeled as follows:

- Adwt 300
- Ad 5/3
- Ad5/3-CXCR4
- Ad5/3-TIMP2
- Ad5/3-CXCR4-TIMP2

The bars indicate the copy number of E4 at different time points (2 and 4 days) for each condition, with error bars showing variability. The graph includes a marker (*) indicating a statistically significant difference.
Figure 3C
Figure 3D
Figure 3E
Figure 4A
Figure 4B
Figure 4C
Figure 4D
Figure 4E
CHAPTER 3

CONDITIONALLY-REPLICATING ADENOVIRUS EXPRESSING TIMP2
INCREASED SURVIVAL IN A DISSEMINATED MODEL OF OVARIAN CANCER

BY

SHERRY W. YANG, DIPTIMAN CHANDA, JAMES J CODY, ANGEL A. RIVERA,
REINHARD WAЕHLER, GENE P. SIEGAL, JOANNE T. DOUGLAS, AND
SELVARANGAN PONNAZHAGAN
Abstract

**Purpose:** Ovarian cancer remains the most lethal gynecological malignancy in the U.S. Conventional therapies have limited therapeutic value due to advanced stage of the disease at diagnosis. Conditionally-replicating adenoviruses (CRAds) are promising novel anticancer agents that are designed to selectively infect and lyse tumor cells. Antitumor efficacy of CRAds can be further enhanced by delivering a therapeutic protein. Matrix metalloproteinases (MMPs) is a potential therapeutic target as they are upregulated in ovarian cancer and have been shown to play many roles in tumorigenesis. Tissue inhibitor of metalloproteinase 2 (TIMP2) is an endogenous inhibitor of MMPs. The present study evaluated the efficacy of a novel CRA (Ad5/3-CXCR4-TIMP2) with the CXCR4 promoter for selective viral replication in cancer cells together with TIMP2 as a therapeutic transgene in a murine orthotopic model of ovarian cancer.

**Experimental Design:** The Ad5/3-CXCR4-TIMP2 vector was further modified by replacing Ad5 fiber knob with Ad3 fiber knob to enhance infectivity of ovarian cancer cells. An orthotopic model of ovarian cancer was developed in athymic nude mice by intraperitoneal injection of the human ovarian cancer cell line, SKOV3-Luc that constitutively expresses luciferase. Eight days following tumor cell injection and upon confirmation of peritoneal dissemination of the cells by non-invasive bioluminescence imaging, mice were randomly divided into four treatment groups: PBS, Ad-TIMP2, Ad5/3-CXCR4, and Ad5/3-CXCR4-TIMP2, with \( n = 10 \) per group. All mice were imaged weekly to monitor tumor growth and were sacrificed upon reaching any of the
endpoints, including tumor burden exceeding 10% of the animal’s body weight, weight loss exceeding 15%, weight gain exceeding 5 g. Survival analysis was performed using the Log-rank (Mantel-Cox) test.

**Results:** Mice were followed for survival, the median survival for the PBS cohort was 33 days, for the Ad-TIMP2 cohort it was 39 days, for the Ad5/3-CXCR4 cohort it was 52.5 and for Ad5/3-CXCR4-TIMP2 cohort it was 63 days. The TIMP2-armed CRAd delayed tumor growth and significantly increased survival when compared to the unarmed CRAd (P<0.05). This therapeutic effect was mediated through inhibition of MMP9, as demonstrated by a decrease in active MMP-9 in the cohort treated with TIMP2-armed CRAd.

**Conclusion:** Results of the study support the translational potential of Ad5/3-CXCR4-TIMP2 for treatment of patients with advanced ovarian cancer.
Introduction

Ovarian cancer is the leading cause of gynecological cancer death in the U.S. (1). The significant morbidity and mortality in patients with ovarian cancer is attributed to the fact that patients present with nonspecific symptoms at the early stages, which precludes its diagnosis (2). Majority of the patients are diagnosed at Stage III, where the cancer has disseminated throughout the peritoneal cavity, which leads to poor prognosis. Current therapies have limitations, as the five-year survival rate has remained unchanged at 50% over the past four decades (3). Thus, novel and effectively targeted therapies for the disseminated disease are urgently needed.

Conditionally replicating adenoviruses (CRAds) are promising new class of therapeutics, as these viruses have the potential to selectively and self-perpetually replicate and lyse cancer cells to eradicate the entire tumor (4). Clinical trials with CRAds have so far demonstrated the safety of oncolytic adenoviruses. However, modest therapeutic efficacy with CRAds suggests further optimization for effective clinical translation (5). One strategy of augmenting antitumor efficacy utilizes the CRAd as a platform for the delivery of a therapeutic transgene, in addition to their oncolytic potential (6). To this end, we hypothesized that efficacy of a replicating adenovirus can be improved for the treatment of ovarian cancer by arming with a gene that acts on the microenvironment, as the interplay between the cancer cells and its microenvironments has been known to modulate tumor progression (7-9).

Matrix metalloproteinases (MMPs) is a diverse family of proteases capable of degrading all components of the extracellular matrix (ECM) (10). They are the ideal class of target, as their dysregulation has been shown to contribute to the promotion of
tumor growth, angiogenesis, invasion and metastasis (11). In particular, MMP-2 and
MMP-9 are consistently upregulated in ovarian cancer and are associated with poor
prognosis (12, 13). An important regulator of MMPs is its endogenous inhibitor, the
tissue inhibitor of metalloproteinases (TIMP), a family of small secreted proteins that act
by binding directly to active MMPs and inhibit their action. To date, four mammalian
TIMPs have been identified (14). Among the TIMP family members, TIMP2 is unique in
its ability to inhibit tumor growth and angiogenesis through pathways independent of
MMP inhibition (15-17). We hypothesized that the production of TIMP2 from virus
infected cells should bind and inhibit the excess extracellular MMPs and thereby inhibit
tumor progression through both MMP-dependent and MMP-independent pathways.

Previously, we developed and determined the therapeutic potential of a TIMP2-
armed CRAd, Ad5/3-CXCR4-TIMP2, for ovarian cancer therapy. Transductional
selectivity of the CRAd was achieved by genetically replacing the Ad5 knob with the
Ad3 knob, which circumvents the coxsackie and adenovirus receptor (CAR) and redirects
binding to the Ad3 receptor, which is more abundantly expressed on ovarian cancer cells
(18, 19). The CXCR4 promoter was used to mediate tumor selective replication of the
vector, as it exhibits a “tumor on, liver off” profile (20). The TIMP2 gene was
incorporated to inhibit tumor progression. We have validated in vitro that our Ad5/3-
CXCR4-TIMP2 is secreting functional TIMP2, as indicated by the inhibition of the
enzymatic degradation of gelatin by active MMPs. For the TIMP2-armed CRAd to be
efficacious, it is important that the expression of TIMP2 does not impair viral replication
nor its oncolytic potency. We have demonstrated that both viral replication and oncolytic
potency were not compromised in both SKOV3.ip1 and OV-4 human ovarian cancer
cells by arming with TIMP2. Moreover, we showed that there is selectivity in replication with the CXCR4 promoter, as indicated by higher level of replication in the ovarian cancer cells, when compared to the control fibroblasts. In the hopes of translating this virus into the clinic, we further employed the usage of an *ex vivo* model system to further examine viral replication. Tumors samples examined from five patients with confirmed stage III and IV ovarian cancer revealed a consistent high level of replication with Ad5/3-CXCR4-TIMP2 when compared to the other control viruses. Collectively, these data were very encouraging, suggesting that Ad5/3-CXCR4-TIMP2 might be effective for the treatment of advanced ovarian cancer. In the present study, we sought to determine the therapeutic efficacy of Ad5/3-CXCR4-TIMP2 in a murine, orthotopic model of disseminated ovarian cancer. Tumor progression was monitored through non-invasive *in vivo* imaging.
Materials and Methods

Cells. The firefly luciferase–expressing ovarian adenocarcinoma cell line SKOV3-luc was kindly provided by Dr. R. Negrin (Stanford Medical School, Stanford, CA). The A549 human lung carcinoma cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Both cell lines were cultured in a 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Ham’s F-12 medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37 °C in a humidified atmosphere and at 5% CO₂. Media and supplements were purchased from Mediatech (Herndon, VA).

Construction and production of the viruses. Ad-TIMP2 is an E1-, E3-deleted replication-deficient Ad5 vector which expresses TIMP2 under the control of the CMV promoter (21). Ad5/3-CXCR4 is a CRAd with the CXCR4 promoter inserted into the E1A position along with a tropism-modified fiber, containing the Ad5 fiber shaft and tail and has had the knob domain replaced with that of the Ad3 virus (22). The Ad5/3-CXCR4-TIMP2 was constructed as follows: A full-length human TIMP2 cDNA was isolated from Ad-TIMP2, an E1- and E3-deleted replication deficient Ad5 vector, which expresses TIMP2 under the control of the CMV promoter (21). The TIMP2 gene was subcloned in place of the E3B region into pZErO-2 E3 6.9, a transfer vector containing a 6.9 kb fragment of the Ad5 genome including the E3 region (kind gift of Dr. Nik Korokhov, VectorLogics, Inc., Birmingham, AL). The resultant plasmid was linearized with BamHI and cotransformed into E. coli BJ5183 (Stratagene, La Jolla, CA) with a
Swai-linearized plasmid, pVK500CΔE3, containing the Ad5 genome deleted for both the E3 region and the fiber gene (23). The resulting plasmid was cotransformed into *E. coli* BJ5183 with an *EcoRI*-linearized plasmid, pKAN.F5/3, containing the Ad5 fiber shaft and tail and an Ad3 fiber knob (24). The resultant plasmid, pVK500C-TIMP2-F5/3, was subjected to a final round of recombination by linearization with *Swai* and cotransformation into *E. coli* BJ5183 with a *PmeI*-linearized pCXCR4, a plasmid containing the CXCR4 promoter (25). This final plasmid, pVK500C-CXCR4-TIMP2-F5/3, was linearized with *PacI* and used to transfect A549 cells to generate Ad5/3-CXCR4-TIMP2. Viruses were amplified in the A549 cell line and purified by two rounds of cesium chloride density centrifugation. The titers of viral particles and infectious units were determined as previously described (26).

**Orthotopic ovarian cancer model and vector treatment.** Female BALB/c nu/nu mice (Harlan) were obtained at ages 6 to 8 week and quarantined for 2 weeks. Mice were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. *In vivo* optical imaging was done at the small animal imaging core facility in the Comprehensive Cancer Center of the University of Alabama at Birmingham. On day 0, mice were injected intraperitoneally (i.p.) with $5 \times 10^6$ SKOV3-luc cells in 250 µl PBS and imaged for bioluminescence to obtain baseline reading. Eight days later, mice were reimaged to ensure the implantation of the tumors and were randomly divided into four groups: (1) PBS, (2) Ad-TIMP2, (3) Ad5/3-CXCR4, (4) Ad5/3-CXCR4-TIMP2,
with n = 10 per group. Each mouse was injected i.p. with 2 x 10^8 IU of the given virus in 200 µl PBS, or PBS alone. All mice were imaged for bioluminescence weekly after tumor cell injection to monitor tumor growth. All mice were followed daily to record survival. Survival time reflects the time required for the animals to reach any of the endpoints, including tumor burden exceeding 10% of the animal’s body weight, weight loss exceeding 15%, weight gain exceeding 5 g. Survival data were plotted on a Kaplan-Meier curve, and different groups of data were compared using the Log-rank (Mantel-Cox) test (GraphPad Prism Software v.5).

**Bioluminescence imaging to detect in vivo luciferase expression.** Mice were imaged at day 0 first for bioluminescent signal after injection of SKOV3-luc cells to obtain baseline reading of tumor burden. Imaging was performed again before initial treatment on day 8 and then weekly until mice were sacrificed due to tumor burden. Briefly, 150 mg/kg D-luciferin was injected i.p., and mice were anesthetized with isoflurane gas anesthesia and placed in a light-tight chamber. The photographic (gray-scale) reference image was obtained at 10 min after D-luciferin injection, and the bioluminescent image was collected immediately thereafter. Images were obtained with a CCD cooled to -120°C, using the IVIS -100 Imaging System (Xenogen Corp., Alameda, CA), with the field of view set at 25 cm height. The bioluminescent images used exposures ranging from 1 to 10 s, 1 f/stop, 4 binning, and open filter. Data acquisition software was calibrated so that no pixels were saturated during image collection. The bioluminescent and gray-scale images were overlaid using LivingImage software (Xenogen Corp.). Living Image Software (Xenogen, Alameda, CA) was also used to obtain a pseudocolor image.
representing bioluminescence intensity (blue, least intense, and red, most intense). Regions of interest were drawn around the i.p. tumors, and the total counts (photons) were summed for the entire tumor areas. The total counts were normalized to image acquisition time (photons/sec). Statistical differences of tumor size among groups were assessed with Student’s t test. A P value of <0.05 was considered statistically significant. Data were presented as mean values ±SD.

**Immunohistochemistry.** Mice were sacrificed upon high tumor burden by predetermined parameters. At time of sacrifice, necropsies were performed on a few mice from each treatment group, in which tumors and major organs were harvested and fixed into paraffin blocks. Briefly, paraffin sections of xenografted human ovarian tumors were deparaffinized in xylene and hydrated through graded alcohol. Antigen retrieval was performed in citrate buffer (pH 6.0), under steam for 20 min. Sections were cooled to room temperature, and endogenous peroxidase was removed using 0.3% H₂O₂ in methanol for 30 min and blocked with 5% BSA for 30 min. Tissue sections were then incubated with primary antibodies overnight at 4°C. Sections were washed in Phosphate-buffered saline (PBST) containing 0.05% Tween-20 and again incubated at room temperature with biotin-conjugated goat anti-goat/anti-mouse secondary antibody for 2 h. After washing, sections were incubated with streptavidin-conjugated horseradish peroxidase for 1 h at room temperature. After another wash with PBST, immunodetection was performed using 3,3’-diaminobenzidine-H₂O₂ (Vector Labs) and counterstained with hematoxylin.
Results:

Arming with TIMP2 delayed tumor growth in vivo. To evaluate the therapeutic efficacy of Ad5/3-CXCR4-TIMP2 in a clinically relevant in vivo model, we generated orthotopically disseminated ovarian cancer by injecting female nude mice with human SKOV3-luc cells intraperitoneally (i.p.). Luciferase expressing stable cell line was used to monitor the therapeutic efficacy of our treatments by noninvasive imaging of the tumor burden in vivo. Eight days following tumor cell administration, mice were reimaged to ensure the implantation of the tumors and divided into four treatment groups, with 10 mice per group. The control groups received PBS, or either replication deficient vector expressing TIMP2 (Ad-TIMP2), or CXCR4 promoter based CRAd with a chimeric Ad5/3 fiber (Ad5/3-CXCR4). The therapeutic group received TIMP2-armed CXCR4 promoter based CRAd with a chimeric Ad5/3 fiber (Ad5/3-CXCR4-TIMP2) (Fig. 1). All mice were imaged after i.p. injections of D-luciferin on a weekly basis. A panel of representative images is shown in Fig. 2A, while quantitative determination of bioluminescent signals is shown in Fig 2B. Low bioluminescent signals of SKOV3-luc cells were detected as early as 8 days following tumor cell injection, and were relatively consistent between the four groups. By day 30, there was significant tumor growth in the PBS group, as mice exhibited considerable increase in abdominal girth and were beginning to show signs of distress. In contrast, tumor growth was significantly inhibited in all groups treated with viruses (p < 0.001). At day 36, majority of the mice in the PBS group have died from heavy tumor burden. At this time point, mice treated with Ad-TIMP2, began to display considerable tumor burden. However, both groups treated with CRAds had significantly less bioluminescent signal (p < 0.001), indicating a slower rate
of tumor development. Furthermore, tumor burden in the cohort of mice treated with Ad5/3-CXCR4-TIMP2, the armed CRAd, was significantly less when compared to the tumor burden in mice treated with Ad5/3-CXCR4, the unarmed CRAd (P < 0.01; Fig. 5B). Collectively, these data suggest that both viral replication and arming with TIMP2 contributed to delaying the onset of tumor growth.

**Arming with TIMP2 increased survival.** All mice were then followed for survival as shown in Fig. 3A. The median survival time increased from 33 days, for the PBS cohort, to 39 days in the cohort injected with the nonreplicating vector expressing TIMP2 (Ad-TIMP2), this difference was statistically significant (P < 0.03). The cohort treated with the unarm ed CRAd (Ad5/3-CXCR4) had median survival time of 52.5 days, whereas cohort treated with the TIMP2-armed CRAd (Ad5/3-CXCR4-TIMP2) had a median survival time of 63 days (Fig. 3B), this difference with also statistically significant, with a p value of < 0.002. In summary, arming with TIMP2 is useful for the treatment of disseminated ovarian cancer, as it increased survival in vivo.

**TIMP2 is expressed in the tumors of armed viruses.** To determine if the increased survival seen in the TIMP2-armed CRAd when compared to the unarm ed CRAd was due to modulation of the microenvironment by TIMP2, expression of TIMP2 was first examined in tumors excised from each treatment group at autopsy. As seen in Fig. 4, tumors excised from groups treated with PBS and Ad5/3-CXCR4 were negative for TIMP2 expression. Result of the immunohistochemistry indicated that in tumors excised from cohort treated with the Ad-TIMP2, TIMP2 expression was limited to the periphery
of the tumor. This was not unexpected, as Ad-TIMP2 is a nonreplicating vector and was not expected to disseminate within the tumor effectively. In contrast, tumors from mice treated with Ad5/3-CXCR4-TIMP2 exhibited homogenous TIMP2 staining throughout the entire cross-section of the tumor, indicating that this oncolytic virus was effective in infecting and replicating throughout the entire tumor.

**TIMP2-armed CRAd decreased level of active MMP9.** TIMP2 is a well known inhibitor of MMP9. Hence, staining for active MMP9 was performed to determine if its level was affected. As shown in Fig. 5, tumors from groups treated with PBS, Ad5/3-CXCR4, and Ad-TIMP2, all exhibited high levels of active MMP9. In contrast, tumors from group treated with Ad5/3-CXCR4-TIMP2 had very low levels of active MMP9 expression. This suggests that TIMP2 secreted by the armed CRAd was effective in inhibiting activity of MMP9.
Discussion:

The importance of the interplay between the tumor cells and its microenvironment in modulating tumor progression has long been recognized (27). In the pathogenesis of ovarian cancer, MMP-2 and MMP-9 are consistently upregulated (12). Furthermore, increased levels of these MMPs correlated negatively with survival as it is associated with advanced stage, high-grade ascites and positive lymph node (13). These data suggest that MMPs are valid therapeutic targets for the treatment of ovarian cancer. A key regulator of MMPs is its endogenous inhibitors, TIMPs. Four mammalian TIMPs have been identified thus far, among them TIMP2 has been most extensively studied due to its ability to inhibit tumor growth and angiogenesis by a variety of mechanisms independent of direct MMP-inhibition (15-17). TIMP2 can inhibit endothelial cell proliferation through induction of protein tyrosine phosphatase activity (17). TIMP2 also inhibits tumor growth and angiogenesis by increasing activity of mitogen-activated protein kinase phosphatase 1 (15). In an effort to develop a successful CRAd geared toward the treatment of disseminated ovarian cancer, we proposed that arming with TIMP2 will augment the therapeutic efficacy of a CRAd by inhibiting tumor progression through both MMP-dependent and MMP-independent pathways.

In our previous study, we characterized and evaluated the efficacy of Ad5/3-CXCR4-TIMP2 in vitro. We confirmed that TIMP2 expression did not impair viral replication or oncolytic potency of the virus. Furthermore, selective replication was achieved with the CXCR4 promoter. Validation of Ad5/3-CXCR4-TIMP2 on primary tumor samples from five patients with confirmed stage III and IV ovarian cancer, revealed consistent high level of replication when compared to the other control viruses.
Collectively, these data suggested the potential of Ad5/3-CXCR4-TIMP2 for the treatment of advanced ovarian cancer.

In the current study, we sought to determine the efficacy of Ad5/3-CXCR4-TIMP2 in vivo. Noninvasive bioluminescent imaging provides a powerful tool that permits longitudinal monitoring of tumor growth in mice. Moreover, it allows us to use a model system that is clinically relevant. In majority of cases, ovarian cancer is diagnosed at an advanced stage, where the cancer has disseminated throughout the peritoneal cavity; using subcutaneous animal model to evaluate efficacy of treatment, while convenient, does not reflect the natural clinical progression of the disease. Thus, establishing disseminated tumors with i.p. injection of SKOV3-luc cells along with imaging enables us to use a clinically valid model system that can more accurately and effectively predict the translational value of Ad5/3-CXCR4-TIMP2.

Eight days following inoculation with tumor cells, mice were randomly divided into four treatment groups: PBS, replication deficient vector expressing TIMP2 (Ad-TIMP2), unarmed CXCR4 promoter based CRAd with a chimeric Ad5/3 fiber (Ad5/3-CXCR4), and TIMP2-armed CXCR4 promoter based CRAd with a chimeric Ad5/3 fiber (Ad5/3-CXCR4-TIMP2). When the mice were followed for survival, the TIMP2-armed CRAd significantly increased survival (p = 0.002) when compared to the unarmed CRAd, however the increase was modest. One explanation is that in our animal model, we treated with a single, low dose of virus, in contrast, many in vivo experiments examining efficacy used viral doses that were at least one to two logs higher, and gave multiple administrations of the virus (28-31). It is conceivable that with a higher dose or multiple dosing, therapeutic efficacy could be further enhanced. In addition, augmentation of viral
efficacy could also be achieved by using radiation and chemotherapy as adjuvant therapies (32-34).

Another potential explanation for the modest increase might be attributed to the tumor biology of ovarian cancer, as well as highlighting the paradoxical roles of MMPs and TIMPs in cancer progression. It is well established that increased MMP-2 and MMP-9 expression in ovarian cancer correlates negatively with prognosis and survival (12, 13, 35-38). However, the specific roles of MMPs in ovarian cancer progression are not well defined. There is literature suggesting that MMP-2 is predominately important in early tumorigenesis by initiating metastasis through enhancing adhesion of ovarian cancer cells to the peritoneum (39). Thus, therapies to block it once metastasis is established are ineffective. However, other reports support the role of MMP-9 in tumor growth and angiogenesis (40). The roles of MMPs and TIMPS in ovarian cancer are further confounded by immunohistochemical studies looking at expression of TIMP2 in ovarian cancer tissues. While some groups have reported low expression of TIMP2 in primary ovarian tumors (35), others have seen increased TIMP2 expression in ovarian cancer tissues, suggesting that TIMP2 contributes to tumor progression, in part through the activation of pro-MMP2 and inhibition of anti-tumor activities of MMPs (36, 38). In contrast, several animal studies utilizing viral delivery of TIMP2 have shown its utility to suppress tumor growth, migration and metastasis in various cancer models (41-45). Collectively these studies indicate that perhaps effects of TIMP2 on tumor promotion or suppression are dependent on timing and the specific type of tumor. Rather than nonspecific inhibitions of MMPs, it is perhaps more useful to specifically inhibit different MMPs during stages of tumor progression.
Emerging data from clinical, pathological, and molecular genetic studies suggest a new model for ovarian carcinogenesis, where ovarian cancer is classified into two distinct groups, type I and II (46). Type I tumors are clinically indolent, as they are slow growing and generally confined to the ovary at diagnosis. In contrast, type II tumors are clinically aggressive, present at an advanced stage and believed to arise de novo, as precursor lesions have not been identified. The type II tumors accounts for 70% of ovarian cancer and are characterized by mutation of TP53 in 80% of the cases. This new data suggest that p53 could potentially be a good therapeutic target to arm the virus for ovarian cancer therapy. CRAd armed with p53 have been shown in vitro to enhance oncolyosis in cervical cancer (47) and a variety of other cancer cell lines (48). Another potential therapeutic target is CXCR4, the only chemokine receptor found to be expressed on ovarian cancer (49). The ligand for CXCR4 is CXCL12, which can stimulate ovarian cancer cells migration in vitro. CXCL12 is present in ascites and is expressed by peritoneal mesothelial cells, thus explaining that in part, migration and attachment of ovarian cancer cells to the peritoneum are facilitated by CXCL12–CXCR4 interactions. Ablating this interaction with a CXCR4 antagonist, AMD3100, has been shown to inhibit migration in vitro (50).

While there some literature on CRAAds for ovarian cancer therapy (22, 28-32, 51-53), there is very limited research on the usage of an armed CRAd. We report here for the first time, evaluation of CRAd delivering a noncytotoxic gene for treatment of disseminated ovarian cancer. Data suggest that Ad5/3-CXCR4-TIMP2 is promising for clinical translation of the virus for treatment of patients with disseminated ovarian cancer. It is conceivable that with further refinements in terms of therapeutic dose, multiple
dosing schedule and usage with other adjutants such as chemotherapy and radiation, therapeutic efficacy can be further enhanced.
References:


43. Rigg AS, Lemoine NR. Adenoviral delivery of TIMP1 or TIMP2 can modify the invasive behavior of pancreatic cancer and can have a significant antitumor effect in vivo. Cancer Gene Ther 2001;8: 869-78.


Figure Legends

Figure 1. Genomic organization of armed CRAd and control viruses. Adwt300, is the wild-type Ad5 virus. Ad-TIMP2 is an E1-, E3-deleted replication-deficient Ad5 vector which expresses TIMP2 under the control of the CMV promoter in E1. Ad5/3-CXCR4 is a CRAd with a CXCR4 promoter in E1 to limit viral replication to the cancer cells in conjunction with Ad5/3 modified fiber that contains the shaft and tail of Ad5 fiber and the Ad3 fiber knob. Ad5/3-CXCR4-TIMP2 is a CRAd with the CXCR4 promoter in E1, armed with TIMP2 in the E3B region and the aforementioned F5/3 modified fiber.

Figure 2. Bioluminescent imaging of mice before and after treatment. Disseminated tumors were established by injecting 5 x 10^6 SKOV3-luc cells i.p. into female athymic nude mice on day 0. On day 8, mice were treated with either i.p. injection of PBS, or 2 x 10^8 IU of Ad-TIMP2, Ad5/3-CXCR4, and Ad5/3-CXCR4-TIMP2. Bioluminescence levels were measured weekly. (A), pseudocolor image representing bioluminescence intensity on days 8, 22, 30 and 36. (B), bioluminescence intensity from abdominal region was quantitated and normalized, and the mean photon counts per second for each group are shown for the given days. Data indicate mean ± SD for each treatment group. *, P < 0.05.

Figure 3. Survival analysis of treatment groups. Mice were monitored for survival. Survival data are plotted on a Kaplan-Meier curve (A). Treatment with Ad5/3-CXCR4-
TIMP2 significantly enhanced survival when compared to treatment with Ad-TIMP2, Ad5/3-CXCR4, or PBS (P < 0.05). (B), median survival in days for each treatment group.

**Figure 4. TIMP2 expression in excised epithelial ovarian tumor.** At death, tumors were collected from each treatment group and examined for expression of TIMP2.

**Figure 5. MMP-9 expression in excised epithelial ovarian tumor.** At death, tumors were collected from each treatment group and examined for expression of active MMP9.
Figure 1
Figure 2A

- Day 8
- Day 22
- Day 30
- Day 36

**PBS**

**Ad-TIMP2 (ΔE1)**

**Ad5/3-CXCR4**

**Ad5/3-CXCR4-TIMP2**

Color Scale
Min = 1400
Max = 47000
Figure 2B
<table>
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<th>Median Survival (days)</th>
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<tr>
<td>PBS</td>
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</tr>
<tr>
<td>Ad-TIMP2</td>
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</tr>
<tr>
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Figure 3B
Figure 4
Figure 5
CHAPTER 4

DISCUSSION

Rationale

Ovarian cancer continues to be a significant clinical problem, with the majority of patients diagnosed at an advanced stage and suffering from high disease burden. Current therapies can slow the progression of the disease, but do not significantly alter survival, which underscores the need for new and effective treatment options. Armed CRAds represent a new class of anticancer agents with great therapeutic potential, as they combine the lytic effect of viral replication with the antitumor activity of a therapeutic transgene. Furthermore, usage of a replication-competent virus allows the amplification of the transgene [28]. A number of armed CRAds have been described, carrying transgenes ranging from ones with direct cytotoxic activity to others that modulate the tumor microenvironment. To date, animal studies employing armed CRAds have shown that a rationally chosen therapeutic transgene can enhance antitumor efficacy over that of an unarmed CRAd.

In the selection of a therapeutic gene intended to treat ovarian cancer, we need to first understand the development and modulation of the tumor microenvironment. MMPs are a diverse family of proteases capable of degrading virtually all components of the
ECM. During the early stages of tumorigenesis, MMPs mediate ECM and basement-membrane degradation, which contributes to the formation of a microenvironment that favors tumor growth [36, 63]. In the later stages of tumorigenesis, MMPs promote metastasis and sustain the growth of tumors. Studies in ovarian cancer have shown that MMP-2 and MMP-9 are consistently upregulated [37]. Furthermore, increased levels of these MMPs correlated negatively with survival as they are associated with advanced stage, high-grade ascites and positive lymph node [39]. These data suggest that MMPs are the ideal therapeutic target, as their inhibition could potentially block different steps in tumorigenesis. A key regulator of MMPs is its endogenous inhibitors, TIMPs, which binds to the active forms of MMPs, thereby abrogating MMP activity associated with tumor growth, angiogenesis and invasion [36]. Four mammalian TIMPs have been identified thus far, among them TIMP2 has been most extensively studied for its ability to inhibit tumor growth and angiogenesis by a variety of mechanisms independent of direct MMP-inhibition [46-48]. TIMP2 can inhibit the proliferation of endothelial cells in response to angiogenic stimuli such as FGF-2 or VEGF-A by binding to $\alpha_3\beta_1$ integrin and cause the induction of protein tyrosine phosphatase activity [46]. TIMP2 also inhibits migration of endothelial cells through an indirect MMP inhibitor effect that is mediated by the upregulation of the RECK protein, a membrane anchored MMP inhibitor [47]. Finally, tumors overexpressing TIMP2 have reported to have an increased activity of mitogen-activated protein kinase phosphatase 1, which leads to an increase dephosphorylation of p38 mitogen-activated protein kinase that, in turn, results in inhibition of tumor growth and angiogenesis [48]. Studies with non-replicative adenoviral vectors expressing TIMP2 have proved their utility for cancer therapy,
resulting in the reduction of tumor growth, angiogenesis and metastasis in various cancer models [40-42]. Collectively, these studies provide a clear rationale for a therapeutic strategy exploiting the localized overexpression of TIMP2 in the tumor microenvironment. We hypothesize that a CRAd armed with TIMP2 will inhibit ovarian cancer progression through two distinct mechanisms. First, the direct lyses of tumor cells through viral replication. Second, secretion of TIMP2 into the tumor microenvironment should inhibit excess extracellular MMPs and activate other MMP-independent signaling pathways to inhibit tumor growth, angiogenesis and metastasis.

**Experimental Observations**

We constructed a CRAd which expresses TIMP2 from the E3B region of the genome, under the control of endogenous transcription machinery. We first confirmed that the armed CRAd, Ad5/3-CXCR4-TIMP2, expresses TIMP2 with similar kinetics as do the native E3B genes from the unarmed CRAd, Ad5/3-CXCR4, and that expression depends on viral DNA replication. We also showed that the expression of TIMP2 did not affect expression of the surrounding genes in the adenoviral genome. Moreover, it was confirmed that cells infected with Ad5/3-CXCR4-TIMP2 secrete functional protein capable of inhibiting MMP activity. We showed that the expression of TIMP2 inhibits neither the replication nor the oncolytic potency of a CRAd and that it does not alter selectivity, an important feature of an agent intended for clinical use. Having characterized this new armed CRAd, we then proceeded toward the validation of our hypothesis. First, we employed the usage of an *ex vivo* model system to further examine viral replication. Tumors samples examined from five patients with stage III and IV
ovarian cancer revealed a consistent high level of replication with Ad5/3-CXCR4-TIMP2 when compared to the other control viruses. The therapeutic efficacy of our TIMP2-armed CRAd was then evaluated in an orthotopic model of ovarian cancer. Our *in vivo* results demonstrated that expression of TIMP2 by a CRAd delayed tumor growth and significantly increased survival when compared to the unarmed CRAd. This effect was mediated through inhibition of MMPs. These observations supported our hypothesis that a CRAd armed with TIMP2 have enhanced potency against tumor progression, by both direct and indirect means.

**Advantages and Limitations of the *in vivo* Model**

In the murine model employed in our studies, cells were injected directly into the peritoneal cavity of nude mice and allowed to grow for one week before treatment was administered. We used this orthotopic disseminated model because it is clinically relevant. In majority of cases, ovarian cancer is diagnosed at an advanced stage, where the cancer has disseminated throughout the peritoneal cavity; using subcutaneous animal model to evaluate efficacy of treatment, while convenient, does not reflect the natural clinical progression of the disease. Thus, establishing disseminated tumors with i.p. injection of SKOV3-luc cells along with imaging enabled us to use a clinically valid model system that can more accurately and effectively predict the translational value of Ad5/3-CXCR4-TIMP2.

Bioluminescent imaging is a powerful tool that permits longitudinal monitoring of tumor growth in mice with same level of simplicity as taking measurements with calipers in the s.c. model [64]. Moreover, it is sensitive, allowing detection of low tumor load
shortly after inoculation. In addition, the imaging is rapid, easy to perform, safe, and cost effective. Collectively, these characteristics have led to its extensive use in preclinical evaluation.

While bioluminescent imaging offers insights into tumor growth kinetics and aid in the monitoring of therapy efficacy, it also presents limitations when compared to other imaging modalities, such as positron emission tomography, magnetic resonance imaging, or ultrasound [64, 65]. Bioluminescence imaging does not allow the determination of absolute tumor mass in an animal due to quenching of bioluminescence by tissue components. In addition, it’s unsuited for the determinations of the exact location of tumors because it’s limited spatial resolution. The lack of precision is reflected in significant fluctuations in signal when a given mice is reimaged. Also, in the later stages of the experiment, we experienced bioluminescent signals exceeding the camera’s limit of detection when bulky tumors were present in the peritoneal cavity of mice. Collectively, these characteristics resulted in large error bars seen with our imaging data.

**Other Studies Employing TIMP2**

In the work described here, we sought to treat established disseminated tumor with Ad5/3-CXCR4-TIMP2. Since these tumors had already progressed from micrometastases at the time of treatment, this more closely reflects the clinical situation than other studies involving TIMP2. In most studies, recombinant TIMP2 has been administered in a preventative protocol, meaning that treatment was initiated either before or on the same day as injection of the tumor cells. In those situations, TIMP2 is highly effective at reducing tumor burden, and has been shown to inhibit tumor growth,
angiogenesis and prevent establishment of tumor metastases [41, 42]. Model employing TIMP2 as a treatment for established subcutaneous tumor has also been described, where TIMP2 has been shown to reduce tumor burden and decreased angiogenesis, but there were no discussions on survival [66, 67]. This reflects the difficulty of treating disseminated disease and is not surprising, given that the ability of TIMP2 to reduce tumor burden is the product of its MMP-inhibition and potentially activation of other anti-tumor signaling cascade rather than direct cytotoxicity. The effectiveness of TIMP2 gene therapy has been demonstrated in models of pancreatic cancer, employing an adenoviral vector [41], colorectal cancer [42], squamous cell carcinoma [67] and breast cancer [66]. However, this work is the first report of efficacy in a disseminated model of ovarian cancer with an oncolytic virus that expresses TIMP2.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

From our in vitro studies we have demonstrated that Ad5/3-CXCR4-TIMP2 secretes functional TIMP2. Moreover the usage of the CXCR4 promoter is selective, as we see increases in viral replication in ovarian cancer cells, but no in non-target normal cells, such as fibroblasts. In addition, our ex vivo studies revealed that Ad5/3-CXCR4-TIMP2 exhibited enhanced replication in primary ovarian tumors from stage III and IV ovarian cancer patients. Finally, our in vivo studies have demonstrated the suitability of TIMP2 as a transgene in a replicating adenovirus intended to treat disseminated ovarian cancer. We have confirmed the ability of TIMP2-armed CRAd to inhibit MMP activity, and in turn slowdown tumor growth and enhance survival relative to the unarmed control. We have shown in this proof-of-principle study that a CRAd armed with TIMP2 more effectively controls ovarian cancer progression than an unarmed CRAd in vivo, in a preclinical murine model of disseminated disease.

Future Directions

While the results shown here are promising, we believe that even greater efficacy can be achieved with this armed CRAd through several refinements of the approach. For
instance, the antitumor effects observed in our murine model were the result of a single administration of virus, using a relatively modest dose. Other studies employing armed virus have more frequently relied on repeated administrations, with doses that are several orders of magnitude higher than what is reported here [26, 68-70]. Another possible refinement of the current model would be the treatment of smaller metastases, either by injecting fewer cells initially or by earlier administration of the armed CRAd. This would allow us to determine the effectiveness of this therapy for the 30% of patients that are diagnosed at an earlier stage. In addition, literature shows that augmentation of viral efficacy could be achieved by using adjuvant therapies that are cytotoxic, such as radiation and chemotherapy [67, 71, 72]. Regardless of which model system is used, it would be desirable to able to monitor the levels of circulating TIMP2 following administration of the armed CRAd. This information is crucial if this armed CRAd is to be used clinically.

The ability to non-invasively monitor CRAds in vivo is an active area of research that will facilitate their translation into the clinic. Toward this goal, three categories of reporters have been developed. The first category consists of secreted reporter proteins that are not typically found in serum, thereby allowing a non-invasive means of monitoring the persistence and efficacy of a CRAd. Examples include CRAds armed with human carcinoembryonic antigen [73], human chorionic gonadotropin β chain [74], and secreted placental alkaline phosphatase [75]. While serum levels of these proteins correlate to viral replication, and allow determination of persistence and efficacy, they do not reveal the biodistribution of the armed CRAds in vivo. To do so, CRAds must be armed with imaging moieties. The second category includes reporters that allow the
infected cells to be visualized. A prominent example is the luciferase enzyme, which activates a bioluminescent substrate and thereby allowing visualization and quantification. Luciferase activity has been used extensively to evaluate the transgene expression, tumor targeting and efficacy of replicating adenoviruses [59, 76-79]. Alternatively, the human sodium iodide symporter (hNIS) gene allows quantification of gene expression following the administration of a radiological substrate [80-83]. In contrast, visualization of infected cells with the production of a fluorescent protein has the distinct advantage of not requiring the administration of a substrate. CRAds have been constructed to carry genes for green fluorescent protein (GFP) [84-86], enhanced GFP (EGFP) [87-89] and red fluorescent protein (RFP) [75, 90]. Multifunctional reporters, such as TK fused to GFP, have also been used to arm CRAds [91, 92]. The final category of reporters used to monitor replicating adenoviruses in vivo have imaging moieties directly incorporated into the viral capsid. Thus, gene transcription by the infected cell is not necessary for monitoring infection because the viral particle itself can be visualized. This has been accomplished by fusing imaging motifs to protein IX (pIX), a minor adenoviral capsid protein. Motifs used for this purpose includes the fluorescent proteins EGFP [93-95] and RFP [96], and TK [97]. Incorporation of TK was shown to be both functional with respect to its conversion of the prodrug gancyclovir as well as for imaging with positron emission tomography. As work continues in this field, other imaging modalities may also be discovered.

In this study, we utilized luciferase-expressing cells to non-invasively confirm the establishment of metastases. Viral replication and efficacy was assessed indirectly by monitoring the rate of tumor growth over time. By combining the use of a reporter-
expressing cell line with one of the CRAd-based imaging systems detailed above would permit non-invasive imaging of viral replication and distribution, with independent determination of tumor burden, allowing for a detailed understanding of efficacy of the treatment. More importantly, CRAd-based imaging would facilitate clinical translation, as it would allow us to directly monitor the virus in patients.

Finally, although the armed CRAd described here was used to treat disseminated ovarian cancer, this virus may be useful against a variety of other cancers that have well documented increases in MMPs, such as breast, melanoma and lung cancer [35, 36, 63, 98]. The ultimate goal is to translate this virus into the clinic upon further refinements. Future studies that further demonstrate the efficacy of this CRAd in enhancing survival will help to realize this goal.
REFERENCES


41. Rigg AS, Lemoine NR. Adenoviral delivery of TIMP1 or TIMP2 can modify the invasive behavior of pancreatic cancer and can have a significant antitumor effect in vivo. Cancer Gene Ther 2001; 8: 869-78.


APPENDIX

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE APPROVAL FORM
UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on October 26, 2010. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines.

Principal Investigator: YANG, SHERRY W.
Co-Investigator(s):
Protocol Number: X100222010
Protocol Title: A Dual-Action, Armed Replicating Adenovirus for the Treatment of Ovarian Cancer

The IRB reviewed and approved the above named project on 4/2/10. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.
IRB Approval Date: 4-2-10
Date IRB Approval Issued: 4/2/10

HiPAA Waiver Approved?: Yes

Marilyn Doss, M.A.
Vice Chair of the Institutional Review Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.
APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL WITH STIPULATIONS

DATE: January 13, 2010
TO: Ponnazhagan, Selvarangan
    LHRB-513 0007
    934-5731

FROM:
    Judith A. Kapp, Ph.D., Chair
    Institutional Animal Care and Use Committee

SUBJECT: Title: A TIMP2-Armed Conditionally Replicating Adenovirus for the Treatment of Ovarian Cancer (Dr. Sherry Yang)
    Sponsor: Internal
    Animal Project Number: 100109013

On January 13, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approved above referenced application for use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>C</td>
<td>240</td>
</tr>
</tbody>
</table>

Approval is granted with the following stipulation(s):

Animal procurement and initiation of studies may not commence until the Animal Use Safety Information Sheet is authorized by OHS. Once the AUSI is authorized, you will be contacted by Earle Durboraw (934-3536) to discuss specific safety precautions which may be necessary for the ARP care staff. Animal procurement and use of potentially hazardous agents in live animals may not occur until Mr. Durboraw has informed the IACUC Office that a satisfactory discussion has occurred.

This protocol is scheduled for review one year from January 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100109013 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.