RICE UNIVERSITY

Enhancing Transduction of Breast and Ovarian Cancer Using EGF and Herceptin Complexed Adenoviral Vectors

by

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ABSTRACT

Enhancing Transduction of Breast and Ovarian Cancer Using EGF and Herceptin Complexed Adenoviral Vectors

By

Kristen E. Adams

Successful gene therapy for breast and ovarian cancer will likely require that anti-cancer genes be delivered specifically to primary and metastatic tumor sites while avoiding normal tissues. Adenoviral vectors are attractive for cancer gene therapy, since they can deliver transgenes to many different tumors. While adenovirus is quite potent at gene delivery, it is also non-specific and delivers genes into tumor and non-tumor cells in vivo. For effective gene therapy, the natural tropism of adenovirus must be removed and the virus re-targeted to tumor cells using cancer-specific ligands.

To identify new cell binding ligand, peptide presenting phage libraries were selected against human breast cancer cell lines. Displayed on phage, these peptides bound specifically to their selection target, cross-reacted to varying degrees on other breast cancer cell lines, and did not bind to normal breast epithelial cells. The binding properties of these peptides were compared with those of commercially available antibodies such as Herceptin and binding proteins such as EGF to determine viable candidates for vector targeting.
Viral targeting methods developed in our laboratory show promise in both ablating the natural tropism of adenovirus and retargeting the virus. The targeting ligands were complexed to biotinylated adenovirus through avidin bridges and chemically cross-linked to adenovirus using bifunctional PEG molecules. The viral complexes were tested \textit{in vitro} before delivery was evaluated in the \textit{in vivo} xenograft tumor models. Fluorescent and luminescent reporter genes were used to determine the location of vector delivery through gene expression \textit{in vivo}. Targeted adenovirus had reduced background transduction and somewhat increased breast and ovarian cancer transduction.

Finally to better evaluate ligand performance, real time, dynamic imaging was used to track ligand distribution and kinetics \textit{in vivo} in tumor models. Fluorescent conditions were first evaluated in mouse models, demonstrating that imaging in the near infrared had superior signal to noise profiles over fluorescence in the visual range. Therefore ligands were labeled with the near infrared dye IR800 and their distribution was tracked in real time. To evaluate the feasibility of tracking virus (not transgene products), adenovirus was labeled with IR800, given to mice and virion trafficking was successfully imaged.
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A Ph.D. knows absolutely everything about absolutely nothing.

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# TABLE OF CONTENTS

ABSTRACT.............................................................................................................ii

ACKNOWLEDGEMENTS.........................................................................................iv

TABLE OF CONTENTS.............................................................................................vi

LIST OF FIGURES....................................................................................................viii

LIST OF ABBREVIATIONS.........................................................................................xii

CHAPTER 1 Background............................................................................................1

1.1 Breast Cancer.....................................................................................................1

1.2 Ovarian Cancer..................................................................................................2

1.3 The EGFR Family..............................................................................................2

1.4 Breast and Ovarian Cancer Gene Therapy.........................................................3

1.5 Adenoviral Gene Therapy for Breast and Ovarian Cancer...............................4

1.6 Specific Targeting of Adenovirus......................................................................5

1.7 Identifying Ligands for Vector Targeting.........................................................6

1.8 Combining Targeting Ligands with Adenovirus..............................................7

1.9 Cancer Imaging.................................................................................................9

1.10 Near Infrared Imaging.....................................................................................10

CHAPTER 2 Specific Aims.........................................................................................11

CHAPTER 3 Discovery and testing of novel breast cancer targeting ligands..............13

3.1 Abstract............................................................................................................13
CHAPTER 4 Complexing and purification methods for retargeting adenoviral vectors
4.1 Abstract
4.2 Introduction
4.3 Materials and Methods
4.4 Results and Discussion
4.5 Conclusions

CHAPTER 5 In vivo evaluation of targeted adenoviral vectors
5.1 Abstract
5.2 Introduction
5.3 Materials and Methods
5.4 Results and Discussion
5.5 Conclusions

CHAPTER 6 In vivo near infrared optical imaging
6.1 Abstract
6.2 Introduction
6.3 Materials and Methods.............................................................91
6.4 Results and Discussion..........................................................95
6.5 Conclusions.............................................................................113

CHAPTER 7 Conclusions and Future Directions...............................114
7.1 Ligand Discovery....................................................................114
7.2 Viral Complexing....................................................................114
7.3 In Vivo Targeting.................................................................115
7.4 Ligand Tracking......................................................................115
7.5 Future Directions.................................................................116

Bibliography..................................................................................119
LIST OF FIGURES

Chapter 3

3-1 Cell Binding by Selected Phage................................................................. 19
3-2 FITC-Labeled Peptides on Different Cell Types......................................... 21
3-3 Comparison of Fluorophores for Detection of Ligand Binding on K562 Cells....... 24
3-4 Peptide Specificity...................................................................................... 25
3-5 Biacore Antibody-Cell Binding Interactions............................................... 28
3-6 Biacore Peptide-Cell Binding Interactions................................................ 29
3-7 MDA 231 and SKBr3 EGFR and Her-2 Receptor Expression Levels............... 32
3-8 MDA 435 and MDA 468 EGFR and Her-2 Expression Levels....................... 33
3-9 SKOV-3 EGFR and Her-2 Expression Levels............................................. 35

Chapter 4

4-1 Biotinylated Cell Targeting of Biotinylated Virus......................................... 44
4-2 Ad-BAP Transduction.................................................................................. 45
4-3 Streptavidin 488 Added to Ad-Fiber-BAP, Purified in Cesium Gradient........... 48
4-4 Antibody Targeting of Avidin-added, Biotinylated Virus.............................. 49
4-5 Viral Complex Transduced K562 Cells....................................................... 52
4-6 Viral Complexes on HeLa Cells................................................................ 54
4-7 Viral Complexes on K562 Cells................................................................ 55
4-8 Herceptin Blocking of Herceptin Complexed Virus..................................... 57
4-9 Viral Layering Compared to Viral Complexes............................................. 59
4-10 Viral Transduction of MDA 435 and 468 Cells..................................................59
4-11 Viral Transduction of SKOV-3 Cells.................................................................61
4-12 Ad-PEG Conjugated to Peptides Transduction.................................................63

Chapter 5
5-1 Transduction Levels After SKBr3 Xenograft It Injection of Adenoviral Vectors…..72
5-2 Transduction Levels After SKBr3 Tumor It Injection of Adenoviral Vectors……..73
5-3 Transduction Levels After MDA 468 Xenograft Intratumoral Injection of Targeted
Adenovirus..............................................................................................................75
5-4 In Vivo and In Vitro Luciferase Imaging Using the Night Owl System...............77
5-5 In Vivo CD59 Targeting Adenoviral Complexes..............................................79
5-6 Light Intensity From Luciferase/Luciferin in SKOV-3 Tumors and Livers After Ip
Injection of Virus..............................................................................................82
5-7 Lumens Measured After Adenovirus Injection.................................................83
5-8 Light Intensity From Expressed Luciferase After Ip Injection of Adenovirus……..85
5-9 Lumens Measured in SKOV-3 Tumors and Livers After Adenovirus Injection…..86

Chapter 6
6-1 Biological Compounds and the Associated Absorbance at Different Wavelengths..97
6-2 Autofluorescence of a Mouse in 520 and 710 nm Wavelengths.........................98
6-3 Mouse Autofluorescence at 830 nm Wavelength..............................................99
6-4 Real Time Imaging of IR800, EGF-IR800, and C225 Blocked Tumors With EGF-
IR800 in Nude Mice.........................................................................................101
6-5 Tumor and Background Uptake of IR800 and EGF-IR800.........................105
6-6 Tumor to Background (TBR) Ratios of IR800, EGF-IR800, and C225 Pre-blocked EGF-IR800..................................................................................106
6-7 Relative Clearing Times of IR-800 and EGF-IR800.................................108
6-8 Measurement of Virion Fluorescence......................................................110
6-9 Imaging of Ad-WT-IR800 in Nude Mice..................................................110
6-10 Imaging of Orally Delivered Ad-WT-IR800............................................112
6-11 Imaging of Mouse Feces.........................................................................112
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>Ad-BAP</td>
<td>Biotinylated adenovirus (fiber modified with biotin acceptor peptide)</td>
</tr>
<tr>
<td>Ad-BAP-NA</td>
<td>Avidin added to biotinylated adenovirus</td>
</tr>
<tr>
<td>Ad-WT</td>
<td>Adenovirus with wild type fiber</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Her-2</td>
<td>Human receptor tyrosine kinase erbB2</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>it</td>
<td>Intratumoral</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>sc</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>TBR</td>
<td>Tumor to background</td>
</tr>
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</table>
CHAPTER 1:

Background

1.1 Breast Cancer

Breast cancer is the second most common cancer in women, with approximately 180,000 new cases every year in the United States (1). Despite accounting for approximately 30% of total cancers in women, only 16% of estimated cancer deaths are due to breast cancer (1). Recent improvements in breast cancer survival have been attributed to the use of the anti-estrogen tamoxifen against primary and metastatic estrogen-receptor (ER) positive tumors. Tamoxifen generates responses in about 50% of ER-positive tumors (2), only a fraction of breast cancers, between 30 and 50%, are ER-positive and are therefore susceptible to this therapy (3). As the cancerous cells continue to mutate (4) and divide, continued tamoxifen therapy can result in a selection of ER-negative tumors or tumors otherwise resistant to the therapy (5). As tumors have become tamoxifen resistant, new therapies are being developed targeting breast cancer through the overexpression of other receptors such as the members of the human epidermal growth factor receptor family such as EGFR and Her-2. While these therapies hold great promise for ER-positive, EGFR-positive, and Her-2-positive breast cancers, these approaches cannot address the substantial fraction of tumors that are ER-negative, EGFR-negative, and HER2-negative. Treatment of breast cancer has been difficult because the heterogeneity of breast tissue and the increased rate of mutation present in carcinoma cells lead to diverse tumor development. This combined with the metastatic nature of cancer makes local delivery ineffective. New therapeutics must be developed to target gene and chemotherapeutic
agents to these refractory breast cancers. A number of approaches including gene therapy are being developed to treat metastatatic breast cancer that is resistant to more conventional therapies.

1.2 Ovarian Cancer

Accounting for 5% of all cancer deaths, ovarian cancer is the fifth highest cancer death level in women (1). Ovarian cancer is generally diagnosed in late stages due to the lack of symptoms in the early stages (6). The EGFR and Her-2 are overexpressed in some ovarian cancers and are generally found to be poor prognostic indicators (6). There is evidence that Her-2 is also a predictor for poor chemotherapeutic effect (7). Therapies such as E1a are used to sensitize ovarian cancer cells to chemotherapy agents (8). Although 60-80% of the cancers initially respond to treatment, on average, the 5-year survival rate is 20-30% (9). The high mortality rate combined with the lack of a good therapeutic option make ovarian cancer an important target for new treatments such as targeted gene therapy.

1.3 The EGFR Family

The epidermal growth factor receptor family is commonly overexpressed in solid tumors. This set of receptor tyrosine kinases (RTK) includes EGFR (Her-1), erbB2 (Her-2), erbB3, and erbB4 is activated through ligand binding which causes homo- and heterodimerization, leading to phosphorylation and the activation of a complex signaling pathway involved in cell proliferation (10). Specifically, both EGFR and Her-2 overexpression have been implicated with more aggressive, metastatic tumors and poor
prognosis (11). This discovery has led to the development of targeting agents which
inhibit EGFR and Her-2 for anti-cancer therapy. Cetuximab (C225) has been developed
against EGFR which blocks the proliferation of many cancer types including breast and
ovarian (reviewed in (12)). Herceptin has been developed to target breast cancer via the
HER2 receptor, which is amplified in approximately 30% of tumors (reviewed in (13)).

1.4 Breast and Ovarian Cancer Gene Therapy
Gene therapy is an alternate approach for cancer treatment, where a viral or non-viral
vector delivers a transgene encoding a therapeutic protein, which is expressed by the
transduced cells to mediate the therapeutic effect. Early preclinical and phase I cancer
gene therapy trial results have shown some success, but these studies have been
performed by application of vectors into physically confined or constrained tumor targets
(e.g. in a tissue culture dish or by direct injection into tumor). In metastatic disease,
tumor deposits are often distributed throughout the body in a manner that prevents
targeting by direct injection to a local tumor site. To reach disseminated tumors in
metastatic breast cancer, vectors will need to be systemically injected. Unfortunately,
intravenous (i.v.) injection allows the vector to circulate throughout the body and deliver
genes to multiple non-tumor organ sites with poor gene delivery to tumor sites (14).
Promiscuous gene delivery can be a significant problem for some of these anticancer
approaches, particularly since the objective is to kill the cancerous cells through delivery
of toxic or immune activating transgenes, neither of which are desired in normal tissues.
1.5 Adenoviral Gene Therapy for Breast and Ovarian Cancer

Recombinant adenoviruses are versatile gene delivery vectors with diverse therapeutic applications in the areas of gene correction and cancer therapy. Adenovirus is easily amplified and purified in high viral titer, efficiently transduces many different cell lines and tissues, and is capable of delivering transgenes to both dividing and non-dividing cells (15-17). Transgenes are expressed off episomal DNA, avoiding complications seen with genomic integration into undesirable locations such as proto-oncogenes or tumor suppressor genes (18). These characteristics make adenovirus a good candidate for applications where transient transgene expression is therapeutically effective. For cancer gene therapy two different types of adenovirus are clinically relevant, conditionally replication competent adenovirus, where the virus has been engineered to selectively replicate and kill tumor cells, and replication incompetent adenovirus, where the genome is deleted for both E1 and E3.

Conditionally replication competent adenoviral vectors can to some degree selectively replicate in tumor, but not in normal cells. While this achieves some tumor-specificity, transcriptional control for tumor specificity can be leaky and probably will not be effective if only a small fraction of the virus circulates and physically reaches distant tumor sites to mediate these effects. Given that one cannot physically locate and target metastatic breast carcinomas throughout the body, safe and effective gene therapy for this disease will likely require the development of gene therapy vectors that can deliver genes specifically to breast cancer cells while avoiding delivery into non-tumor cells. Unfortunately, no current gene therapy vector has specificity to only breast cancer cells,
so one must be constructed. Adenovirus type 5 (Ad5) is a versatile gene therapy vector, easily produced and capable of delivering large transgenes to many different tissues including breast and ovarian cancer. As such, adenovirus can be used to deliver a variety of genes to breast cancer including immunostimulatory genes and cytotoxic genes like thymidine kinase.

While it is generally useful that Ad5 can transduce many cancer cells, its ability to deliver genes into most other tissues makes it less effective and sometimes even dangerous in vivo. To develop adenovirus for breast cancer therapy, the natural tropism of the virus must be ablated and the virus re-targeted with new ligands, which allow the vector to deliver its transgene to primary and metastatic tumors and block binding to non-tumor cells.

1.6 Specific Targeting of Adenovirus

Ad5 attaches to cells through its fiber and penton base proteins. The fibers are displayed as 12 trimers extending from the vertices of the icosahedral virus (19-21). The knob domain of fiber binds to cells by interactions with the coxsackie and adenovirus receptor (CAR) (22). The penton base sits on the bottom of the fiber trimer where it meets the viral vertices. The penton base mediates Ad5 internalization through interactions with cell surface integrins (23-27). Ablating CAR binding generally reduces transduction by at least 10-fold on most CAR positive cells (28). If CAR binding is ablated, residual transduction can still be mediated by the interaction of the adenoviral penton through its solvent-exposed RGD motif and the cell surface $\alpha v \beta 3$ or $\alpha v \beta 5$ integrins (21). However,
RGD can also be deleted to generate double-ablated vectors with drastically reduced abilities to transduce cells. Such a doubly-ablated vector would be an ideal platform on which to add new cell targeting ligands to mediate true targeting to tumor cells.

1.7 Identifying Ligands for Vector Targeting

The current use of targeting molecules such as monoclonal antibodies in breast cancer therapy is hindered by the percentage of breast cancers expressing the target receptor. Even if an antibody specific for each breast cancer type existed, antibodies are bulky and when linked to a gene therapy vector, could sterically hinder the binding and internalization steps. Given the lack of useful ligands for cell targeting, our laboratory developed peptide-presenting phage library technology to identify these needed cell-specific ligands (29). Peptides were chosen rather than antibodies to produce small targeting ligands that should permeate more readily into tissues and can be more readily genetically engineered into the proteins of viral vectors. This technology was designed to function without prior knowledge of the target cell’s biology or receptors to accelerate the rate of ligand identification. The original work involved the use of phage libraries representing ~10^9 different 12 or 20 random amino acids on the amino-terminus of the phage pIII protein to select a cell-binding peptide against cells in vitro (29) and in vivo (30). In the experiments, phage were selected directly on the target cells without attempting to deplete or clear the library of peptides that can bind other cell types and these peptides cross-react with several other cell types (29). Previous work has shown that peptide selection without clearing selects promiscuous ligands (29). When clearing cell lines were used, the selected peptide showed specificity to the target cells when in the
context of phage ((31) and data not shown). Using a clearing strategy and phage libraries, targeting ligands can be identified for different human breast cancer cells types. In the future, the selection could be done on a patient-by-patient level for cancers not responsive to the cell line generated peptides.

1.8 Combining Targeting Ligands with Adenovirus

Previous viral targeting efforts have included genetically inserting a targeting ligand into the viral capsid and conjugating the targeting ligand to the viral particle. Many methods for viral re-targeting intend to ablate or at least block the native tropism of the virus while adding function through a novel, cell-specific targeting molecule. Conjugation of the ligands to the virus can be done by covalent or non-covalent methods. Bifunctional antibodies can non-covalently link ligands and the virus (32). Chemical conjugation can cross-link the virus and the ligand (33) or it can add a motif for non-covalent interaction such as biotin/avidin complex formation (34). Insertion of ligands into the viral capsid requires genetic engineering of the virus to produce the capsid proteins with the inserted targeting ligand instead of the wild type capsid proteins (15, 17, 35) as well as using the ligand to block or ablating the native tropism of the virus (28, 36). Genetic changes are made through recombinant plasmid systems and work to have the virus produced displaying the desired targeting ligand. The fiber protein of adenovirus is a popular site for inserting ligands since this protein normally mediates binding to cells. Some ligands can effectively re-target adenovirus when genetically inserted into capsid proteins, while the genetic insertion of other ligands disrupts the trimerization of the adenoviral fiber, particularly when inserted at the fiber c-terminus (15). In other cases, insertion of some
ligands caused these to lose their binding specificity when displayed on the virus. Either of the last two cases effectively destroy the function of the viral targeting complex.

There are two virus modifications used in our laboratory to circumvent these problems in adding ligands to adenovirus: metabolic biotinylation and chemical coupling with polyethylene glycol (PEG). In the first approach, a biotin acceptor peptide (BAP) was genetically engineered onto the C-terminus of the fiber gene. When this BAP-modified vector is produced in 293 helper cells, the BAP and the virus are covalently biotinylated by the endogenous biotin ligase enzyme. Once produced, one simply needs to conjugate the biotinylated form of the ligand to the vector using tetrameric avidin as a bridge (34, 37). For the second approach, bifunctional NHS-PEG-maleimide cross-linkers are used to first conjugate PEG to the virion by the NHS group and then capture a cysteine-labeled ligand by conjugation to the maleimide (33). Addition of PEG to Ad5 at different PEG/virus ratios is able to reduce immunogenicity without loss of viral function. In our laboratory, metabolically and chemically biotinylated and PEGylated viruses have shown promise at re-targeting. Having successfully engineered the biotinylated virus once, any biotinylated ligand should be able to be attached to the virus using the biotin/avidin interaction without losing viral function or ligand targeting ability. This viral construct could theoretically be systemically delivered, then during circulation encounter both primary and metastatic tumors. Using the existing peptide discovery technology and our ability re-targetable virus, this project will develop breast cancer-targeting adenoviral vectors. Once developed these vectors could be applied to deliver therapeutic transgenes
to primary and metastatic tumor sites or be combined with tumor-specific replication-competent adenoviral vectors to increase their specificity and safety.

1.9 Cancer Imaging

Cancer diagnosis, therapy, and prognosis are all dependent upon cancer imaging. The early detection of abnormalities within tissues is increasing the overall cancer survival rates. Common imaging modalities include magnetic resonance imaging (MRI), molecular imaging, nuclear imaging (gamma imaging, positron emission tomography (PET), and single-photon emission computed tomography (SPECT)), and x-ray computed tomography (CT). Millimolar amounts of contrast agent are typically required for magnetic resonance and x-ray imaging, although recent advances are decreasing this amount (38). In contrast, nanomolar amounts of the imaging agent are necessary for nuclear and optical molecular imaging (38).

In our laboratory the imaging system of choice is fluorescence for two main reasons. First, radioactive probes require long imaging times and include exposure to radioactivity for the mouse and the researcher. Second, fluorescence is renewable, because it is less energetic, so the tag can be re-energized in situ in the body. Each photon event occurs on the order of a nanosecond, meaning that there are \(10^9\) imagable events per second, making this a very powerful technique capable of sensing extremely low amounts of dye (39). One could theoretically use any wavelength of light to image small animals, however, biological tissues have their own wavelengths of absorbance, which are minimized around 1000 nm (39).
1.10 Near Infrared Optical Imaging

Near infrared (NIR) light includes wavelengths between 700 and 1000 nm and penetrates several centimeters into tissue. Optical imaging in the NIR wavelengths is very sensitive and versatile, capable of real time, dynamic imaging (40, 41). It is an attractive set of imaging wavelengths partially due to lower background auto-fluorescence than other fluorescent molecules such as fluorescein which emits around 520 and has high skin auto-fluorescence. NIR dyes such as Cy5.5 (710 nm) and IR800 (830 nm) are available with reactive groups for conjugation to cancer targeting ligands or molecules. The real time biodistribution of antibodies and small targeting molecules can be imaged using NIR fluorescent dyes (42-45). This approach would be valuable for screening cell-targeting peptides and other ligands in vivo to determine which may useful before conjugating them to adenovirus. If antibodies can be labeled with NIR dyes and tracked, larger entities such as virions could also be labeled and their in vivo biodistribution determined in real time. A method for virus tracking would provide the ability to measure not only where transgene expression happens, but also the circulation path of the targeted and untargeted viruses.
CHAPTER 2

Specific Aims

This thesis project focuses on the development of breast cancer targeting adenoviral vectors. First ligands must be found to specifically target breast cancer cells. Once targeting ligands exist, it is necessary to develop a method for re-targeting the virus using these ligands that will retain both the functionality of the virus and that of the ligands. The resulting retargeted viruses then need to be tested for their ability to specifically transduce breast cancer xenograft tumors in mice. This work will be addressed through the following specific aims.

Specific Aim 1: To develop a novel method to identify breast cancer targeting ligands using bioengineering principles. This aim will test new methods of selecting peptide ligands from phage display libraries by applying shear forces during binding using flow cell technology.

Specific Aim 2: To examine the binding characteristics of the selected peptides and commercially available antibodies. This aim will determine the force with which each ligand binds to breast cancer cells and the optimal ligand for viral re-targeting.

Specific Aim 3: To attach peptide and antibody ligands to adenoviral vectors by covalent and non-covalent chemical coupling strategies. This aim will develop breast cancer targeting adenoviral vectors using viral modification techniques developed in our laboratory including biotinylated and PEGylated adenovirus.
Specific Aim 4: To perform in vivo studies with retargeted adenovirus. For this aim breast cancer targeting vectors will be tested in vivo in a mouse xenograft model of human breast cancer.
CHAPTER 3

Discovery and testing of novel breast cancer targeting ligands

3.1 Abstract
For breast cancer gene therapy, ligands must be identified that bind specifically to human breast cancer cells. Ideally, these ligands could be used to mediate gain of function transduction \textit{in vitro} or for the direct targeting \textit{in vivo}. To test this application, we have selected peptide-presenting phage libraries against breast cancer cell lines with clearing on other non-related human and mouse cell lines. After selection, these peptides were tested for their \textit{in vitro} binding characteristics using cell binding assays. The binding affinity of the selected peptides for the cells was determined to be too low for gene therapy applications.

3.2 Introduction
Previous work with random phage libraries demonstrated that cell binding peptide ligands can be selected from phage libraries by binding directly to cells in culture (29) or by binding to vascular cells \textit{in vivo} (30). In this work, libraries with large peptides have always out-competed libraries with smaller peptides and the build up of consensus peptide motifs is unusual. Selection against normal and tumor cells demonstrates that cell-binding peptides are relatively easy to select, however, \textit{in vitro} affinity selection alone usually generates promiscuous ligands that bind common receptors on a variety of cells. These promiscuous peptides do not appear to bind common cellular receptors like heparin sulfate. Nor do they appear to bind common \(\alpha_v\) integrins, as evidenced by the selection of only two RGD peptides in eight years of selection against diverse cell targets.
While simple affinity selection *in vitro* usually (but not always) generates promiscuous peptides, application of effective clearing strategies and target cell purification approaches appear to be robust methods to generate quite specific tumor or patient-specific targeting peptides.

### 3.3 Materials and Methods

#### Materials

Cell lines for this work were purchased from American Type Culture Collection (ATCC) in Manassas, VA. Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12), Fetal Bovine Serum (FBS), l-glutamine (l-glut), Penicillin-Streptomycin (P/S), Cell Dissociation Buffer (CDB), and Hanks Based Salt Solution (HBSS) were purchased from Invitrogen in Carlsbad, CA. The phage library ON543 was provided by Affymax in Palo Alto, CA. The peptides selected through phage display were synthetically generated by Biosynthesis Incorporated in Lewisville, TX, either biotinylated or covalently tagged with fluorescein isothiocyanate (FITC). The FACScan and the data analyzing CellQuest software were purchased from Becton-Dickinson in Franklin Lakes, NJ. Primary antibodies were ordered from BDPharmingen in San Diego, CA, except Herceptin which was a gift from Dr. Malcom Brenner. FITC labeled secondary antibodies or targeting molecules and propidium iodide (PI) were ordered from Vector Laboratories in Burlingame, CA. Biotinylated epidermal growth factor (EGF), alexafluor 488 and phycoerythrin labeled secondary antibodies or ligands were purchased from Molecular Probes in Eugene, OR. The Biacore and related software were purchased
from Biacore, Piscataway, NJ, by the Biacore Facility at University of Texas at Houston. General laboratory supplies were purchased through Fisher Scientific and VWR.

Cell Culture

The breast cancer cell lines in use are MCF-7, MDA 231, MDA 435, MDA 468, and SKBr3. A primary breast cell line HMEC was used as normal tissue. The non-breast cell lines used are Chinese Hamster Ovarian cells (CHO), muscle cells (C_{2}C_{12}), prostate cancer cells (DU 145), hepatocytes (Hepa 1-6), human pancreatic cancer cells (HPAC), mouse monocyte-macrophage cells (Raw 264.7), and human ovarian cancer cells (SKOV-3). Cells were cultured in DMEM/F12 with 10% FBS, 1% l-glut, and 1% P/S. All cells were cultured in incubators at 37°C with 5% CO₂ and humid conditions.

Phage Panning

Phage display techniques developed previously in our laboratory, attempt to determine cell-specific small targeting ligands which will re-target gene therapy vectors. The phage library ON543 displays approximately 10^9 20 amino acid peptides. To identify ligands that bind directly to human breast cancer cells, we have selected ON543 against the human breast cancer cell lines (SKBr3 and MDA 231). To reduce selection of nonspecific peptides, these selections directed at the breast cancer cells were combined with clearing strategies in which promiscuous peptides are removed from the population by depletion on CHO cells, DU 145 cells, Hepa 1-6 cells, and Raw 264.7 cells. First the library is allowed to bind the breast cancer cells, either MDA 231 or SKBr 3, and the peptides that do not bind are removed. The peptides that bind the cells are divided into
two categories: binding and internalizing peptides. The focus of this selection was the peptides displayed on the internalized phage. The cell binding phage were washed off the cells with an acid solution, and saved for possible future work. The breast cancer cells were lysed, the internalized phage harvested, and amplified in bacteria. In subsequent binding rounds the amplified phage is first allowed to bind to the clearing cells. The phage that did not bind and remained in solution are collected and allowed to bind to the cell of interest, the cells are lysed and the internalized phage harvested and amplified. Selections were done in sets of five rounds after which the resulting phage was amplified in bacterial cells, ten colonies picked and the phage DNA sequenced to produce the selected peptide. Five rounds of selection on the SKBr3 cells collapsed the $10^8$ population of the library down to essentially one peptide, SKBr5C1. After ten rounds of selection on the MDA 231 cells, peptide MDA10.2 was selected out of the $10^8$ population. Selection against the two different breast cancer cell types produced two different peptides. The peptides were tested for specificity in the context of phage. The XS 5.2 peptide was selected against dendritic cells and is the negative control peptide in this experiment.

**Cell Staining**

Adherent cell lines were treated with cell-dissociation buffer (CDB) to detach them from their culture flasks. All binding assays were done in HBSS with 1% BSA (HBSS-BSA). Cells were lifted from culture flasks with CDB, divided into tubes of $1 \times 10^6$ cells, and washed 3 times with HBSS-BSA.
For peptide binding, the FITC-labeled peptides are allowed to attach to the cells at 37°C for 60 minutes in two different concentrations, 0.5μM and 5.0μM, after three washes, the cells were resuspended in 500μL Phosphate Buffered Saline (PBS) for analysis. Biotinylated peptides at a concentration 500μM were allowed to bind to the cells for 60 minutes at 37°C, in HBSS-BSA, the cells were washed, and incubated with 1/100 SA-488 for 60 minutes at 37°C and were resuspended in 500μL Phosphate Buffered Saline (PBS) for analysis. For antibody binding, the cells were resuspended in 100 μl of HBSS-BSA containing antibody or nothing (control) and incubated for 30 minutes at 4°C. The cells were washed 3 times with 1 ml of HBSS with 1% BSA (HBSS-BSA). Secondary antibodies conjugated to FITC, Alexafluor 488, PE, or Streptavidin-alexafluor 488 (SA 488) diluted to 0.01 mg per ml in HBSS-BSA was added and the cells were incubated for 30 minutes at 4°C. The cells were then wasted 3 times with 1 ml Phosphaste Buffered Saline (PBS) and then resuspended in 500 μl PBS with PI. Labeling was quantified by measuring 10,000 events per sample on the FACScan. Positive cells were defined on the negative control sample with no antibody, setting approximately 99% of the cells as negative and the other approximately 1% of the cells as positive.

**Fluorescence Microscopy**

Cells were fluorescently labeled using the same procedure as for cell staining. Cells were then resuspended in 100 μl PBS (without PI), and 20 μl of the cell solution was pipetted into a chamber slide.
**BiaCore**

Biotinylated ligands were allowed to attach to SA chips at concentrations of 5 mg/ml at flow rates of 5 μl/min for 70 μl (or 14 min). Cells were then circulated over the chip for 10 minutes at 10 μl/min. The Biacore and its software were then used to analyse the relative binding amounts of the cell types to the peptidies.

**3.4 Results and Discussion**

**Random Peptide Selection.**

The ON543 20-mer peptide library was selected for five rounds on SKBr3 cells with clearing. This selection collapsed the $10^8$ population of peptides in the library down to essentially one peptide, SKBr5C1, with the amino acid sequence GQIPITEPELCCVPWTEAFY. After ten rounds of selection with clearing on the MDA 231 cells, peptide MDA10.2, with the amino acid sequence, PQPPNSTAHPNPHKAPPNTT, was selected out of the library. Selection against each cell type produced a different peptide, and in additional testing of the peptides in phage context, both the SKBr5C1 and the MDA10.2 peptide bind SKBr3, MDA-231, MCF-7 breast cancer cells, but do not bind to normal breast epithelial cells, nor to most non-target cell types, with MDA10.2 appearing to bind both SKBr3 and MDA 231 cells better than SKBr5C1 (Figure 3-1).
Figure 3-1 Cell Binding by Selected Phage. The indicated peptide-presenting phage were bound to the indicated cells and the number of phage bound were quantitated by titration in bacteria. XS5.2 was selected against mouse dendritic cells and was used as a negative control. The peptide selected against MDA 231 cells was amplified from two different colonies and there is very little difference between the two peptides in the context of phage.
Both the SKBr5C1 and the MDA10.2 peptides bind SKBr3, MDA-231, MCF-7 breast cancer cells, better than they bind to the representative non-target cell types. The MDA10.2 peptide appears to bind both SKBr3 and MDA 231 cells better than SKBr5C1 peptide. It is possible that the SKBr peptide displaying amplifies more quickly in bacteria that the one displaying the 231 peptide. This could affect the results of these and future selections.

Cell staining

Synthetically generated peptides may interact differently with the cells than the peptides displayed on phage. To test the behavior of the selected peptides MDA10.2 and SKBr5C1, each was synthetically generated with a biotin or a FITC tag. A liver specific peptide (HepaCD8) and peptide L10.5F are both negative controls for peptide binding. There were only enough cells for analysis in the SKBr 3 and C2C12 cell samples, so they were analyzed without the other cell types. The FITC-labeled peptide data (Figure 3-2) do not show the same targeting specificity as was observed on phage in Figure 3-1. This suggests, that the peptides may have lost specificity out of context of the phage proteins, or that their affinity as single peptides is insufficient to mediate detectable binding. In contrast, when they are displayed on phage, they can bind by avidity, since up to 5 peptides are displayed per phage.
**Figure 3-2 FITC-Labeled Peptides on Different Cell Types.** FITC labeled peptides were allowed to bind to negative control mouse skeletal muscle cells (C2C12) and breast cancer cells (SKBr3). Breast cancer specific peptide (SKBr3R5.1) and negative control peptides (HepaCD8 and L10.5F) were bound to the cells at 0.5µM and 5µM concentrations and the fluorescent intensity was measured on the FACScan, N=1 in this experiment.
For the SKBr3 peptide on SKBr3 cells, the mean fluorescence intensity does not change much between the two peptide concentrations. The SKBr3 peptide bound better to the C_2C_{12} cells than to the SKBr3 cells. These MFIs are relatively low, despite high percent positive numbers (not shown). The low fluorescence intensity combined with the high percent positive reading could mean that each cell was bound by a peptide or a few peptides, but not enough to produce high levels of fluorescence. To test this, after flow analysis, 40 μl of cells were inoculated into six well chambers on microscope slides for fluorescence microscopy. The resulting images also showed very low levels of fluorescence (data not presented). There was not enough fluorescence to determine if the cells do have very few peptides bound or if there is just background fluorescence which could be due to unbound peptide in the solution, dead cells non-specifically binding peptides and/or auto-fluorescing or there might be problems with the assay.

To test the methodology used to measure peptide binding, a very strongly binding antibody (α-CD59) was chosen along with a cell type (K562) known to display a high number of CD59 molecules. Due to the low signals detected from the fluorescence of FITC labeled peptides, streptavidin labeled with three common fluorescent tags FITC, Alexafluor 488, and PE were compared to determine which gives the strongest signal. The antibody concentration was titrated to determine the detection limit of each fluorophore, and the fluorescent tag determined to have the lowest detection limit and give the largest difference in signal with each change in antibody concentration was chosen to be the fluorophore for the future experimentation. To remove possible fluorescence contamination from dead cells, cells were incubated in propidium iodide
(PI) to detect dead cells, for 5 minutes on ice and then PI-positive (dead) cells were excluded from analysis. The results of the fluorophore comparison are shown in Figure 3-3.

Although PE had the highest measured fluorescence, it has spectral overlap with PI. For this reason, Alexafluor 488 was chosen as the best fluorophore. This allows binding experiments to be done with PI used to mark the dead cells, so they can be excluded from the experimental analysis. The biotinylated peptides were then tested using Streptavidin-Alexafluor 488 (SA-488) as a secondary fluorescent molecule to amplify the signal from the peptide binding. Using SA-488 as a secondary does not seem to increase the signal with the peptides bound to cells, as shown in Figure 3-4.
Figure 3-3 Comparison of Fluorophores for Detection of Ligand Binding on K562 Cells. K562 cells were bound with primary antibody (α-CD71) shown in mg at the bottom of the graph. The streptavidin (SA) secondary was conjugated to FITC, shown with blue bars, Alexafluor 488 represented by red bars, and PE in the yellow bars, N=1 in this experiment.
Figure 3-4 Peptide Specificity. Negative control cells, CHO, DU145, and HELA and breast cancer cells MDA 231 were bound with biotinylated peptides or antibodies, then with streptavidin alexafluor 488 and measured for fluorescence. The negative control antibody IT (isotype) and negative control peptide cmyc were compared with the breast cancer peptides SKBr and 231 in addition to the positive control antibody CD 71, N=1 in this experiment.
Using Alexafluor 488 as a fluorescent marker instead of FITC did not significantly increase the signal of the peptides. Micromolar concentrations of the peptides were required to measure fluorescence by the peptides. These concentrations are too high to be biologically relevant, so methods must be developed to increase the binding while decreasing the concentration of peptide put on cells. These flow cytometry methods were unable to measure the affinity of the two breast cancer specific peptides as free peptides in solution. Although the affinity of the peptides cannot be changed without chemically or physically altering the peptides, the avidity of the peptide/cell complex could possibly be increased.

**Biacore**

The Biacore system is set up to measure specificity, concentration, kinetics, and affinity of interactions between proteins, nucleic acids, lipids, carbohydrates and even whole cells. Binding interactions can be quantitatively measured by binding a target molecule to the Biacore sensor chip surface and allowing the binding molecule to be captured from a solution as it is passed over the chip. The sensor chip consists of a glass surface, coated with a thin layer of gold enabling the use of Surface Plasmon Resonance (SPR). SPR measures changes in refractive index to determine changes in mass in the aqueous layer close to the sensor chip surface where the target molecule is held. When molecules from the test solution bind to the target molecule the mass increases, when they dissociate the mass decreases. These mass changes are measured as a function of time and from the collected data, the characteristics of the molecular interactions can be determined.
The Biacore streptavidin (SA) chips have SA attached to the slide surface through short dextran linkers. This allows biotinylated ligands to be bound to the chip and these flexible linkers allow for peptide movement and possibly the dimerization or multimerization of the peptides. To test if Biacore could provide a more sensitive and more quantitative measure of ligand interactions with cells, biotinylated antibodies against CD59 and CD71 were bound to the SA chip with a no antibody control and an isotype (IT) antibody control. CD 59 and CD 71 positive cells (K562) and CD 59 and CD 71 negative cells (CHO) were allowed to bind to each of the different surfaces and the amount of cell binding is represented in Figure 3-5.

The CD 59 and CD 71 antibodies captured the CHO cells comparably with the negative controls. The CD 59 and CD 71 antibodies captured the K562 cells better than the negative controls. The antibodies bound the CD 59 and CD 71 positive cells but not the CD 59 and CD 71 negative cells, so the Biacore was used to test the selected peptides. To expand the Biacore system for this, each peptide was synthesized with a biotin tag, bound to the SA chip, and cells were flowed across the surface of the chip in the Biacore in an attempt to measure ligand-cell interactions. It should be noted that this is an unconventional use of the Biacore technology, since SPR is typically used to detect interactions between two proteins, rather than a protein and a cell. Biotinylated peptides were bound to the SA chip and solutions containing different types of cells flowed over the surface to determine cell binding to the peptides (Figure 3-6).
Figure 3-5 Biacore Antibody-Cell Binding Interactions. Biotinylated negative control isotype (IT) antibody or no antibody and human specific CD59 and CD71 antibodies were immobilized onto SA chips and negative control cells, CHO (A) and positive control cells, K562 (B), were allowed to bind to the antibodies under flow conditions. The binding was measured through changes in SPR and graphed as response units, notice the different scales for CHO (max 150) and K562 (max 500).
Figure 3-6 Biacore Peptide-Cell Binding Interactions. Biotinylated negative control peptides selected against chronic lymphocytic leukemia cells (CLL 1-1 and CLL 1-6) and breast cancer selected peptides (213R10.1 and SKBr3R5.1) were immobilized onto SA chips and negative control cells, Hepa 1-6 (A), MDA 231 (B), and SKBr3 (C) cells were allowed to bind to the peptides under flow conditions. The binding was measured through changes in SPR and graphed as response units.
Hepa 1-6 cells bound to 231R10.1 and CLL 1-1 the best, then SKBrR5.1 followed by CLL 1-6. Breast cancer cells, MDA 231, attached the most to the CLL 1-1 peptide, followed by the 231R10.1 peptide, then the SKBrR5.1 and finally the CLL 1-6 peptide. The other breast cancer cell line, SKBr3, bound best to the 231R10.1 and CLL 1-1 peptides, then the CLL 1-6 peptide and worst to the SKBrR5.1 peptide. When this assay, only marginal changes in SPR (as shown as “response”) were observed by each of the selected peptides and the peptides did not show the same specificity that was observed when tested on phage. These data suggest that the peptides fail to function correctly out of the context of phage or that this unconventional use of SPR is ineffective. In addition to the aforementioned methods, optical tweezers, cells binding to coated slides, and dextran complexing the peptides were all tried to measure the binding of the selected peptides. None of these methods were able to detect a reproducible binding that measured higher than a negative control peptide. Although, work continued to test these peptides in the context of virus, focus for the project was shifted towards using them more robust larger ligands epidermal growth factor (EGF targeting EGFR) and Herceptin (targeting Her-2) were examined as possible gene therapy targeting molecules.

**Antibody binding.**

Antibodies are known to bind their receptors with high affinity, making them a good option for gene therapy vector targeting, with the caveat that they are likely impossible to genetically engineer into the virus. As such, alternate, non-genetic means will need to be employed to add these large ligands to adenovirus for vector targeting.
Human breast cancer cell lines overexpress a variety of receptors at different levels, two such receptors that have been well characterized are EGFR and Her-2. Known antibodies exist to each of these receptors and EGFR has a naturally occurring ligand EGF. Initially, MDA 231 and SKBr3 were tested for their EGFR and Her-2 levels, the receptor staining levels are shown in Figure 3-7.

Unfortunately MDA 231 cells show low levels of expression of both EGFR and Her-2 and a low percent of positive cells (data not shown), making them poor candidate cells for targeting using Herceptin and EGF. SKBr3 cells show expression of both receptors, with Her-2 overexpressed to a higher degree than EGFR. Unfortunately, SKBr3 cells were found to up and down regulate Her-2 expression through their life in culture, and tended to lose Her-2 expression completely after stress or long periods of cell culture (data not shown).

For these reasons, alternative cell types were explored for targeting using EGFR and Her-2. The receptor expression levels were measured on human breast cancer cell lines MDA 435 and MDA 468, as shown in Figure 3-8.
Figure 3-7 MDA 231 and SKBr3 EGFR and Her-2 Receptor Expression Levels. MDA 231 (A) and SKBr3 (B) cells were bound with biotinylated antibodies against EGFR and Her-2, and negative controls with no primary antibody or isotype antibody (IT). The cells were then stained with streptavidin alexafluor 488 and their fluorescence intensity measured using the FACScan. In this figure, * indicates \( p < 0.0005 \), and ** indicates \( p < 0.05 \).
Figure 3-8 MDA 435 and MDA 468 EGFR and Her-2 Expression Levels. MDA 435 (A) and MDA 468 (B) cells were bound with biotinylated antibodies against EGFR and Her-2 and negative control no antibody and isotype (IT) antibodies. The cells were then stained with streptavidin alexafluor 488 and the fluorescence intensity measured using the FACSan. In this figure, * signifies p < 0.05, ** signifies p < 0.0005, and *** indicates an insignificant difference (p > 0.1).
MDA 435 cells did not over express either EGFR or Her-2. MDA 468 cells expressed high levels of EGFR, but did not overexpress Her-2. Due to low receptor expression levels in MDA 435 cells, these make excellent negative control cells for comparison to targeting via EGF on the MDA 468 cells which express high levels of EGFR and low levels of Her-2. EGFR targeting can be achieved through antibodies or through the naturally occurring small molecule ligand, EGF. Other cancer cells also over-express the EGFR and Her-2 receptors making the development of targeting vectors against these receptors applicable to many other types of neoplasia. For example, when tested by flow cytometry, SKOV-3 ovarian cancer cells express both EGFR and Her-2 (Figure 3-9). These cells therefore provide an alternate tumor model to test targeting. In addition, SKOV-3 cells have the advantage of allowing one to test targeting in the simple orthotopic peritoneal tumor model in mice, since ovarian cancer normally metastasizes to the peritoneum. SKOV-3 cells show low mean fluorescent intensities, but high percent positive levels for EGFR and Her-2, they were explored as a possible target cell for gene therapy through EGF and Herceptin targeted adenovirus.
Figure 3-9 SKOV-3 EGFR and Her-2 Expression Levels. SKOV-3 cells were bound with biotinylated antibodies against EGFR and Her-2 and negative control no antibody and isotype (IT) antibodies. The cells were then stained with streptavidin alexafluor 488 and the fluorescence intensity measured using the FACScan. In this figure, * signifies $p < 0.005$ and ** signifies $p < 0.0005$. 
3.5 Conclusions

The linear peptides selected from phage display library ON543 did not show high binding levels outside the context of phage. Two possible reasons for this are the display on phage proteins constrains the peptides in an unpredictable manner which is not reproduced by the synthetically generated linear peptides, that peptide binding occurs by avidity on phage and their affinity as single synthetic peptides is too low, or that their proximal display on phage may allow multimerization on the phage that does not occur in their synthetic form (29). Context-specific constrained peptide libraries are being developed in our laboratory to address the first possible problem. Although we attempted to recapitulate the avidity display provided by phage by other means, it is uncertain to what degree this was affected. Due to the lack of targeting from the selected peptides off of the phage, and the high binding affinities shown by antibodies against EGFR and Her-2 as well as the small molecule ligand EGF, these ligands were selected for pursuit of our targeting goals in the rest of the project.
CHAPTER 4

Complexing and purification methods for re-targeting adenoviral vectors

4.1 Abstract

Adenovirus is a versatile gene therapy vector, capable of transducing many different cell types, both dividing and non-dividing. For adenovirus to be a useful breast cancer gene therapy vector, the virus must minimally be given the ability to deliver transgene products to the cells where expression is desired. Given that breast and ovarian cancer cells can be refractory to normal adenovirus infection, this chapter describes work to enhance transduction of EGFR and Her-2 expressing breast and ovarian cancer cells utilizing the ligands EGF and Herceptin.

4.2 Introduction

As described in Chapter 1, recombinant adenoviruses are versatile gene delivery vectors with diverse therapeutic applications in the areas of gene correction and cancer therapy(46-49). The genetic introduction of even small peptide targeting ligands into adenoviral capsid proteins does not always produce functional constructs. The ligand can disrupt viral function, removing the ability of the virus assemble and grow. Conversely, display of the new ligand in the different structure of the virus protein can also change the conformation of the peptide thus affecting its ability to bind specifically to the target cells. While these issues are a difficulty for small 2 kDa peptides, genetic engineering of large complex proteins like EGF or an antibody are essentially impossible due size and
protein structure issues. Finally, genetically engineering a ligand into the virus for each target cell population is time consuming and is not feasible for every peptide.

Metabolically biotinylated adenovirus was developed in our laboratory as one approach to circumvent many of the problems described above (37, 50). As described in Chapter 1, to create a metabolically biotinylated adenovirus, a biotin acceptor peptide (BAP) was genetically engineered into the C-terminus of the viral fiber protein creating a virus known as Ad-BAP. Wild-type virus is designated Ad-WT. This strategy uses the exceptional affinity between biotin and tetrameric avidin, which is the strongest biologically occurring interaction with a $K_D$ of $10^{-15}$. Most antibodies or targeting ligands can be purchased already biotinylated or can be easily biotinylated, making this system extremely useful. It is theoretically possible to re-target the biotinylated virus using any biotinylated ligand, without requiring any further genetic or chemical modification of the virus, just the covalent interaction between biotin and avidin. This chapter describes testing the feasibility of this approach to target adenovirus to breast and ovarian cancers cells in vitro.

4.3 Materials and Methods

Materials

Cell lines for this work were purchased from American Type Culture Collection (ATCC) in Manassas, VA. Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12), Dulbecco’s Modified Eagle Medium (DMEM), Minimum Essential
Medium with Earle’s Balanced Salt Solution (EBSS), Fetal Bovine Serum (FBS), l-glutamine (l-glut), Penicillin-Streptomycin (P/S), Cell Dissociation Buffer (CDB), and Hanks Based Salt Solution (HBSS) were purchased from Invitrogen in Carlsbad, CA. The FACScan and the data analyzing CellQuest software were purchased from Becton-Dickinson in Franklin Lakes, NJ. Primary antibodies were ordered from BDPharmingen in San Diego, CA, except Herceptin which was a gift from Dr. Malcom Brenner. Biotin-NHS was ordered from Vector Laboratories in Burlingame, CA. Neutravidin (NA) was purchased from Sigma Aldrich, St. Louis, MO. Biotinylated and unlabeled epidermal growth factor (EGF) and streptavidin alexafluor 488 were purchased from Molecular Probes in Eugene, OR. Bi-functional polyethylene glycol (NHS-PEG-Mal) was purchased from Nektar Therapeutics, Birmingham, AL. DG-10 desalting columns came from Biorad in Hercules, CA. Dialysis cassettes with 300 kDa molecular weight cut-off membranes were purchased from Spectrum Laboratories, Rancho Dominguez, CA. The luminometer was purchased from Turner Biosystems in Sunnyvale, CA. General laboratory supplies were purchased through Fisher Scientific and VWR.

Cell Culture
The breast cancer cell lines MDA 231, MDA 435, MDA 468, and SKBr3 and ovarian cancer cell line SKOV-3 were cultured in DMEM/F12 with 10% FBS, 1% l-glut, and 1% P/S. The human embryonic kidney cell line (HEK 293) which expresses adenoviral genes E1 and E3 allowing for the propagation of replication incompetent adenovirus and chronic myelogenous leukemia cell line (K562) were grown in DMEM with 10% FBS, 1% l-glut, and 1% P/S. N3S cells, a variant of HEK 293 cells which are able to grow in
suspension, were maintained in EBSS with 10% FBS, 1% l-glut, and 1% P/S. All cells were cultured in incubators at 37°C with 5% CO₂ and humid conditions.

**Viral Amplification**

Ad-Fiber-BAP was amplified in HEK 293 cells grown in DMEM with 5% FBS, 1% l-glut and 1% P/S with 100μM d-biotin. Ad-Fiber-WT was amplified in HEK 293 cells grown in DMEM with 5% FBS, 1% l-glut and 1% P/S. To remove the virus from the cells after culture, cells were resuspended in 50mM Tris pH 8.0 and treated to three freeze/thaw cycles. The cell debris was spun out of solution and the viral particles were purified from the supernatant.

**Viral Purification**

The cell lysate from the viral amplification was layered on a cesium chloride gradient and spun for 3 hours at 20000 rpm in the ultra centrifuge. After the spin, the bottom viral band was removed from the tube, diluted to 4 ml with 50mM Tris pH 8.0 and layered onto another cesium chloride gradient and spun for 20 hours at 20000 rpm in the ultracentrifuge. Again the bottom viral band is removed and the virus is desalted and buffer exchanged into 500mM sucrose Tris buffer in a DG-10 column.

**Viral Layering**

K562 cells were incubated with biotinylated antibodies α-CD5, α-CD59, and α-CD71 antibodies for 30 minutes on ice. The unbound antibody was removed and then the cells incubated with neutravidin for 30 minutes on ice. The unbound neutravidin was removed
and the cells were incubated with virus for 30 minutes on ice. The cells were washed to remove excess virus, and media added to the cells. The cells were plated and incubated for 48 hours and then transgene expression measured.

Adherent cell lines were treated with CDB to detach them from their culture flasks. All binding assays were done in HBSS with 1% BSA (HBSS-BSA). Cells were lifted from culture flasks with CDB, divided into tubes of $1 \times 10^6$ cells, and washed 3 times with HBSS-BSA. The cells were resuspended in 100 μl of HBSS-BSA containing 1nmol of antibody, EGF, or nothing and incubated for 30 minutes at 4°C. The cells were washed 3 times with 1 ml of HBSS with 1% BSA (HBSS-BSA). NA was added at 0.01ng/cell or no NA in 100 μl HBSS-BSA and the cells were incubated for 30 minutes at 4°C. The cells were washed 3 times with 1 ml of HBSS with 1% BSA (HBSS-BSA). Biotinylated adenovirus (Ad-BAP) was added at 5000 vp/cell or no viral particles in 100 μl HBSS-BSA and the cells were incubated for 30 minutes at 4°C. The cells were then wasted 3 times with 1 ml culture medium, resuspended in media, and plated in dishes. The cells were cultured for 48 hours then the transgene expression was measured through GFP or luciferase transgene expression on the FACScan and Luminometer respectively.

**Viral Complexing**

The cell lysate from the viral amplification was layered on a cesium chloride gradient and spun for 3 hours at 20000 rpm in the ultra centrifuge. After the spin, the bottom viral band was removed from the tube, diluted to 4 ml with 50mM Tris pH 8.0, NA was added in 50 fold excess to the number of biotins per capsid, typically about 2mg, and incubated
for 30 min on ice. Then the virus was layered onto another cesium chloride gradient and spun for 20 hours at 20000 rpm in the ultracentrifuge. Again the bottom viral band is removed and the virus is desalted and buffer exchanged into 500mM sucrose Tris buffer in a DG-10 column. Biotinylated ligand was then added to the virus at 100 fold excess to the number of NA per virion. After a 30 min incubation on ice, the ligands were purified away by size exclusion on 400kDa beads.

**PEGylation of Virus**

After CsCl purification, the virus was stored in potassium phosphate buffer (10mM K₂PO₄, 150mM NaCl, 1mM MgCl₂, 5% w/v sucrose, pH 7.8). Bifunctional PEG was added to 1x10¹² viral particles at 1 mg/mL in 0.1mM potassium phosphate buffer at pH 8.5 to generate Ad-PEG-Mal. The reactions were carried out for an hour at room temperature with constant mixing. Excess free lysine was added to quench the reaction after an hour, followed by 300 kDa molecular weight cut off dialysis to remove unreacted PEG molecules and lysines. The last dialysis step, the virus was dialysed into 10mM TRIS, 150mM NaCl, 1mM EDTA, 5% sucrose, pH 7.0, and 1µM peptide was conjugated to 5x10¹¹ Ad-PEG-Mal particles at 4°C overnight. Excess peptides were removed by DG-10 desalting column. Viral concentrations were then determined by measuring absorbance at 260nm and real time PCR.

**4.4 Results and Discussion**

Ad-BAP was tested for biotin-dependent targeting by first chemically biotinylating K562 cells and then adding avidin to the cells and then the biotinylated virus. K562 cells were
chosen to show Ad-BAP gain of function because they are CAR-negative and thus not well transduced by wild type adenovirus. The cells were incubated with Neutravidin or plain HBSS-BSA-AbAm for 30 minutes on ice, washed, incubated with virus from 30 minutes on ice, washed and plated in 24 well dishes for 48 hours in media. Transgene (EGFP) expression was measured after the 48 hour incubation and the results are graphed in Figure 4-1.

Increasing transduction was observed with increasing amounts of avidin on the biotinylated cells only in the presence of avidin. This work demonstrated that the vector cold transduce cells in a biotin and avidin-dependent fashion. In contrast, transduction was generally at background levels in the absence of biotin, except only at the highest levels of added virus. This and other work has shown some higher level of background can be observed due to non-specific interactions of avidin with adenovirus and with cells. To test if the vector could be re-targeted to specific cell surface receptors, K562 cells were again used. K562s express CD59 on their surface. In this experiment, K562 cells were first exposed with a biotinylated antibody against CD59, and then they were treated with neutravidin and finally Ad-BAP was added, with washes at each step to remove free molecules. After incubation with the virus, the cells were cultured for 48 hours and then transduction was assessed by measurement of GFP expression by flow cytometry (Figure 4-2).
Figure 4-1 Biotinylated Cell Targeting of Biotinylated Virus. The biotinylated and control cells were incubated with neuravidin then Ad-BAP. After 48 hours, the cells were harvested and the percentage of cells expressing EGFP was measured using the FACScan, in this experiment N=1.
**Figure 4-2 Ad-BAP Transduction.** Cells were layered with antibody, then neuravidin, and finally Ad-BAP. After 48 hours the cells were harvested and the percentage of cells expressing EGFP was measured using the FACScan. Neuravidin has some slight background binding to the cells. The NA background is greater than the other control samples, but the background is probably insignificant compared to the targeted transduction. Other experiments using isotype control antibodies and irrelevant antibodies demonstrated the specificity of the observed transduction, in this experiment N=1.
These data demonstrate that Ad-BAP is able to transduce biotinylated cells or cells labeled with a biotinylated antibody when neutravidin is added as a linker between the virus and the cells. Ad-Fiber-wt does not show the same ability to transduce the biotinylated cells or cells labeled with biotinylated ligands (data not shown).

Viral Complexing

Previous targeting was done by adding first biotinylated antibody, then avidin, and finally the biotinylated virus to the cells in a “layering” approach. This biotin-avidin targeting technology would be better suited for in vivo studies if the antibody, avidin, and virus could be combined as a targeting complex rather than by the layering approach.

Adenovirus is normally purified using by two sequential ultra-centrifugations in cesium chloride gradients. While the concentration of CsCl is quite high (~1.3 g/ml or ~7.7 M), the exceptional affinity of avidin for biotin allows one to complex avidin to the virus directly in CsCl after one or more gradient spins to co-purify the biotinylated virus and avidin on the second gradient (data not shown). After adding avidin, the biotinylated targeting ligand can be added and the excess ligand removed either through size exclusion, a cesium gradient, or a binding column.

The first step in forming the targeting complex is to add avidin to the Ad-Fiber-BAP. Five 150 mm plates of 293 cells were infected with Ad-Fiber-BAP, the cells lysed to free the virus, the cell debris pelleted, and the supernatant containing the virus banded on two cesium chloride gradients. The band from each tube was collected in approximately 1 mL
volume, the bands were combined and the total volume brought up to 8 mL, which was split evenly into two tubes of 4 mL. To one tube 100 μL of fluorescent streptavidin-AlexaFluor-488 (SA-488) was added and to the other tube the same volume of 30mM Tris buffer. The tubes were mixed gently and incubated on ice for 30 minutes. After the incubation, each tube of virus was banded on a cesium chloride gradient. The tubes were set next to a UV light box and a digital photograph taken, as seen in Figure 4-3.

Once complexed, Ad-Fiber-BAP + SA-488 (Ad-Fiber-BAP-SA) was tested for activity and targeting. The bands from each tube were collected, desalted in a column, and collected in 1 mL volumes. To each band an equal volume of glycerol was added to stabilize the virus when frozen. Then Ad-Fiber-BAP or Ad-Fiber-BAP-SA was applied to K562 cells previously incubated with antibody and neutravidin in four different conditions: no antibody and no neutravidin, α-CD59 and no neutravidin, no antibody and neutravidin, and both α-CD59 and neutravidin as detailed previously. The virus was added to the cells and incubated, then washed, and plated in media for 48 hours before analysis. Transduction, as assessed by the expression of the transgene luciferase, was measured by luciferase activity from K562 cell lysates, and the results are presented in Figure 4-4.
Figure 4-3 Streptavidin 488 Added to Ad-Fiber-BAP, Purified in Cesium Gradient. The left tube is the Ad-Fiber-BAP without SA-488 added and the right tube is the Ad-Fiber-BAP with the SA-488 added. The unbound SA-488 is in the aqueous layer near the top of the tube, while the bound SA-488 is seen in the viral bands. The upper bands are empty capsids and incomplete viral particles, while the bottom band contains the viral particles. In the picture, there is some reflection from the fluorescent tube on the right to the left tube.
Figure 4-4 Antibody Targeting of Avidin-added, Biotinylated Virus. K562 cells were layered with CD59 antibody and/or neuravidin or neither, then either Ad-BAP or Ad-BAP-SA488. After 48 hours the cells were harvested and measured for luciferase expression using the luminometer, N=1 in this experiment.
This graph shows that Ad-BAP can only transduce cells that have been incubated with both antibody and avidin, while Ad-BAP-SA can transduce cells that have been incubated with antibody but no avidin. Further, Ad-BAP-SA transduction is blocked when cells are incubated with both antibody and avidin. This experiment demonstrated that we could pre-complex Ad-BAP with avidin for retargeting, but did not demonstrate if we could form the full virus-avidin-ligand complex. To test this, the Ad-BAP-SA complex was mixed with biotinylated anti-CD71 antibody and this mixture was separated on a cesium chloride gradient. Unfortunately, unlike complexing with avidin alone, this three-way complex did not form a crisp band on CsCl, but was instead was cloudy and disperse (data not shown). This disperse blob was collected and the virus incubated with K562 cells, without any additional components. In this case, Ad-BAP-SA + α-CD71 did not show the functional targeting activity (data not shown) as the Ad-BAP-SA (Fig. 4-4). This complex was tested on cells incubated with and without blocking an excess of α-CD71 and then with virus. The transgene expression was very low and showed no targeting over Ad-BAP-SA (data not shown). From this, it appears that the three-way complex could not be purified by cesium chloride gradient. This failure appears to be specific to the use of large antibody ligands, since subsequent work by others in the laboratory using smaller ligands have been able to band three-way complexes on CsCl.

Another complexing method was developed where the virus would simply be mixed with the same ratio of avidin and antibody and then the mixture put on cells to see the transduction. Ad-Fiber-BAP was mixed with 0, 1, 3, 10, and 30 Neutravidin particles per viral particles. The same particle ratio of α-CD59 antibodies to viral particles was added.
The virus, neutravidin, and antibodies were incubated for 30 minutes on ice with gentle shaking before addition to the cells. The entire volume of viral complexes was added to 1 million cells and incubated for 30 minutes on ice. The cells were washed and plated in media for 48 hours. Transgene expression was measured and the results are presented in Figure 4-5.

Ad-BAP transduced the cells about the same amount as the different Ad-BAP/SA/Antibody mixtures. Premixing Ad-BAP with different ratios of avidin and antibody per viral particle did not improve the measured transduction when compared with Ad-BAP, suggesting that this approach would not be viable, particularly in vivo.
**Figure 4-5 Viral Complex Transduced K562 Cells.** This experiment was done in duplicate and the y-axis expanded to show slight differences in measured fluorescence. Ad-BAP was mixed with neutravidin and CD59 antibody at ratios of 1:1, 3, 10, and 30 and put on cells. The cells were incubated for 48 hours and then harvested and their fluorescent intensity measured using the FACScan, in this experiment N=2.
Since neither the cesium chloride gradient and pre-mixing strategy worked, we needed to develop an alternate approach to purify the three-way complexes targeted with antibodies. As an alternate approach, 400kDa size exclusion chromatography resin was tested to determine if this could be used to separate unconjugated 150 kDa antibody away from the viral complexes. In this approach, the antibodies would enter the 400 kDa resin and more slowly elute, whereas the complexes that exceed the pore size of the resin would simply flow through in the “void” fraction on the column. To test this, Ad-Fiber-BAP-SA was incubated with α-CD59 for 30 minutes on ice with gentle mixing at 15 minute intervals. Five mL of the 400kDa size exclusion beads were allowed to settle in a five mL Qiagen column, and then washed with 10mL 30mM Tris Buffer. The viral complex mixture was added to the column and washed out with two 2 mL washes of 30 mM Tris Buffer. The flow through was collected in fractions and these fractions tested for bioactivity (transduction) on HeLa cells as shown in Figure 4-6.

Testing the activity of each fraction showed that fractions 10 to 12 had the most luciferase expression, so they were combined to test on K562 cells. No antibody or avidin were used to block the cells, instead the gained ability to transduce the cells with no additions was used as the criterion for functional viral complex formation. The transduction results were measured through transgene production, using a luciferase assay, as show in Figure 4-7.
**Figure 4-6 Viral Complexes on HeLa Cells.** Ad-BAP-SA488 was mixed with CD59 and then the viral complexes separated from the free antibody on a size exclusion column. Each elution fraction was collected and 5μl put on HeLa cell. After 48 hours, the luciferase activity of each sample was measured, in this experiment N=1.
Figure 4-7 Viral Complexes on K562 Cells. The Ad-BAP-SA488-CD59 viral complexes combined from fractions 10-12 above were allowed to bind to K562 cells in parallel with Ad-BAP. After 48 hours the cells were harvested and the luciferase expression was measured. In this figure, * indicates $p < 0.005$. 
When the excess antibody was removed through a size exclusion column, the viral complexes retained their activity, and Ad-Fiber-BAP-SA-59 shows gain of function over Ad-Fiber-BAP. This suggests that this purification scheme could allow large preparations of Ad-Fiber-BAP to be produced and then complexed with avidin during CsCl banding. These complexes can then be stored at −80°C and then be complexed with any biotinylated targeting ligand, separated by gel filtration, and then used (the Ad-Fiber-BAP-SA used in these experiments was stored for approximately 2 months between adding the avidin and the antibody).

This work and previous work shows proof of principle for targeting adenovirus using antibodies, but now the system needed to be adapted to target breast or ovarian cancer cells. To test this, Ad-BAP was complexed via Neutravidin (NA) to the clinically-approved anti-Her-2 therapeutic monoclonal antibody Herceptin. Ad-BAP-NA-Herceptin (Ad-BAP-NA-Her) was complexed and purified and tested for its ability to target SKBr3 cells that over-express Her-2. MDA 231 breast cancer cells were used as a negative control, since they do not express Her-2. Free Herceptin or NA or both were incubated with some of the cells before to show specific gain of transduction with the Herceptin targeted virus. The viral efficacy is measured through transgene expression and the results presented in Figure 4-8.
Figure 4-8 Herceptin Blocking of Herceptin Complexed Virus. Breast cancer cells MDA 231 and SKBr3 were layered first with Herceptin, then neutravidin, and finally Ad-BAP-NA-Her complexed virus. The cells were incubated for 48 hours then the luciferase expression measured, N=1 in this experiment.
Free Herceptin shows specific blocking of the Herceptin complexed virus on SKBr3 cells which overexpress Her-2 and no affect on MDA 231 cells which do not overexpress Her-2. The ability of free Herceptin to block Ad-Fiber-BAP-NA-Herceptin transduction suggest that the virus is using the Her-2/Herceptin interaction to attach to the cells.

We next compared transduction of cells by layering the antibody and avidin on the cells versus transduction by Ad-Fiber-BAP-NA-Herceptin on SKBr3 cells. The complexed virus was added in the final step of the layering experiment when the Ad-Fiber-BAP was added to the cells layered with Herceptin and NA. The transduction was measured by transgene expression, and there was no significant difference between the layered and complexed virus as seen in Figure 4-9.

The Herceptin complexed virus transduced SKBr3 cells at least as well as the layering approach. Antibodies are large molecules, about 150 kDa in size, which could be too large for effective trafficking in vivo. It is possible that smaller complexes will be needed for intravascular injections for in vivo targeting. To test this, Ad-BAP-NA was complexed with the small 7 kDa ligand EGF and this complex was tested for EGFR specific transduction on EGFR positive MDA 468 cells and EGFR negative MDA 435 cells. The expression of the luciferase transgene was measured using the luminometer, and the results are shown in Figure 4-10.
Figure 4-9 Viral Layering Compared to Viral Complexes. SKBr3 cells were transduced with no virus, Ad-BAP, Ad-BAP with layering of NA and Herceptin, or Ad-BAP-NA-Her complexes. The cells were incubated for 48 hours, then harvested and their luciferase expression measured. In this experiment there was no significant difference between the transfected cells (p > 0.05).

Figure 4-10 Viral Transduction of MDA 435 and 468 Cells. Ad-BAP-NA and Ad-BAP-NA-EGF were allowed to transduce MDA 435 and MDA 468 cells. The cells were then incubated for 48 hours, at which time the cells were harvested and measured for luciferase expression. In this graph, * indicates insignificant difference (p > 0.05), ** indicates p < 0.001, and *** indicates p < 0.001.
The MDA 468 cells were more easily transduced than the MDA 435 cells. Ad-BAP-NA complexed to EGF increased the transduction of EGFR positive cells MDA 468, while it did not increase the transduction of EGFR negative cells MDA 435. This experiment shows that biotinylated antibodies as well as biotinylated ligands are capable of increasing the breast cancer transduction by targeted adenoviruses.

In addition to breast cancer cells, the ovarian cancer cell line SKOV-3 also overexpresses EGFR and Her-2. To test if the previously made viral complexes are able to transduce SKOV3 cells better than untargeted virus, Ad-BAP, Ad-BAP-NA-EGF, and Ad-BAP-NA-Herceptin were allowed to transduce SKOV3 cells. The transgene expression was measured using the luminometer as shown in Figure 4-11.

Ad-Fiber-BAP complexed to EGF and Herceptin both increased the transduction of SKOV3 cells over Ad-Fiber-BAP. There is about a log difference between Ad-BAP-NA-Her and Ad-BAP-NA-EGF and another log difference between Ad-BAP-NA-EGF and Ad-BAP-NA. Both the targeted viruses transduced SKOV3 cells better than the untargeted virus. There is a significant difference between the transduction using Her-2 and EGFR targeting viruses which interestingly does not correspond to the receptor levels seen in cell binding studies shown in Chapter 3.
Figure 4-11 Viral Transduction of SKOV-3 Cells. SKOV3 cells were transduced with no virus, Ad-BAP-NA, Ad-BAP-NA-EGF, or Ad-BAP-NA-Her. After 48 hours, the cells were harvested and their luciferase expression measured. In this figure, * indicates $p < 0.0001$. 
In addition to the genetically modified Ad-BAP, our laboratory has developed methods to chemically conjugate ligands to Ad-WT using a bifunctional PEG linker. In this experiment peptides against skeletal muscle (hSh) and the two breast cancer peptides (231 and SKBr) were conjugated to Ad-PEG and allowed to transduce prostate cancer cells (DU 145), breast cancer cells (MDA 231 and SKBr), and human muscle cells (SKMC and HSMM). None of the peptides increased the transduction of the cells significantly above the levels of Ad-PEG. The results of this experiment are shown in Figure 4-12.

In the context of the PEGylated adenoviral vectors, the synthetically generated peptides selected breast cancer did not increase in transduction of MDA 231 or SKBr3 cells. The display of the peptides on the PEG molecules might allow them to interact in a manner that increases the avidity of the interaction between the cells and the peptides, but there is no corresponding increase in transduction with higher peptide amounts (data not shown). The peptide conjugated viruses do not warrant in vivo targeting experiments.
Figure 4-12 Ad-PEG Conjugated to Peptides Transduction. Peptides selected against skeletal muscle (hSh) and the two breast cancer peptides (231 and SKBr) were conjugated to Ad-PEG for transduction of prostate cancer cells (DU 145), breast cancer cells (MDA 231 and SKBr), and human muscle cells (SKMC and HSMM). The conjugated vectors as well as no virus, and Ad-WT were allowed to transduce the cells, then 48 hours later the cells were harvested and the percent of cells expressing EGFP were measured using the FACScan, in this experiment N=1.
4.5 Conclusions

Adenovirus can be produced in high titers and retargeted through chemical modification and covalent conjugation using the biotin/avidin interaction. During cesium chloride gradient purification, avidin can be added to the virus and free avidin purified from that attached to the virus. Targeting ligands can be added to the virus and purified through size exclusion column chromatography. These viral complexes transduce cells at least as well as the previous layering approach used in our laboratory and show promise as breast and ovarian cancer targeting gene therapy vectors. Chemical modification of the virus is also possible, but none of the peptides were sufficiently able to increase transduction of breast cancer cells to warrant more testing in their current form. The ability to make targeted viral complexes allows these gene therapy vectors to be injected into mice to test their *in vivo* targeting.
CHAPTER 5

In vivo evaluation of targeted adenoviral vectors

5.1 Abstract

Cancer gene therapy vectors must be able to transduce the cells or tissues of interest without background transduction in normal tissues. Nude mouse models allow the growth of human cancers as xenografts in subcutaneous and peritoneal sites and allow for whole body visualization of transgene expression. In this work, we demonstrate that targeted adenoviral vectors shown increased transduction in tumor cells to background cells in intratumoral and intraperitoneal injections.

5.2 Introduction

For cancer therapy using adenoviral gene therapy, the virus must be specifically targeted to cancer cells and its innate ability to infect other cells removed. In Chapter 4, targeted viral complexes were developed which showed increased transduction of breast and ovarian cancer in vitro over the uncomplexed virus. After the successful in vitro testing, their in vivo efficacy as gene therapy vectors could be tested. Xenograft breast and ovarian cancer tumors can be grown in nude mice because they are T-cell deficient. These tumors can then be used for in vivo testing of the different adenoviral complexes.

Determining the tissues transduced by gene delivery vectors is normally an exhaustive process requiring that the animals be sacrificed and their tissues and organs either be extracted for enzymatic assays or be sectioned to identify the specific cell types that are
targeted. These assays are quite laborious to the extent that one would actually need to section the whole animal to determine the true distribution of the vector and to ensure that gene delivery into unexpected sites is not missed. Given the labor involved in traditional assays, we tested transduction of targeted and untargeted adenoviral vectors by *in vivo* luminescence technology. This new technology is based on the ability of luciferase-transfected cells to metabolize luciferin substrate *in vivo*, and produce photons of light for detection (51, 52) by the very sensitive integrated Night Owl system.

5.3 Materials and Methods

**Materials**

Cell lines for this work were purchased from American Type Culture Collection (ATCC) in Manassas, VA. Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12), Dulbecco’s Modified Eagle Medium (DMEM), Minimum Essential Medium with Earle’s Balanced Salt Solution (EBSS), Fetal Bovine Serum (FBS), l-glutamine (l-glut), Penicillin-Streptomycin (P/S), and trypsin were purchased from Invitrogen in Carlsbad, CA. Herceptin was a gift from Dr. Malcom Brenner. Biotin-NHS was ordered from Vector Laboratories in Burlingame, CA. Neutravidin (NA) was purchased from Sigma Aldrich, St. Louis, MO. Biotinylated and unlabeled epidermal growth factor (EGF) and streptavidin-alexafluor 488 (SA488) were purchased from Molecular Probes in Eugene, OR. DG-10 desalting columns came from Biorad in Hercules, CA. Dialysis cassettes with 300 kDa molecular weight cut-off membranes were purchased from Spectrum Laboratories, Rancho Dominguez, CA. The luminometer
was purchased from Turner Biosystems in Sunnyvale, CA. The Night Owl Molecular
Light Imager and its associated Winlight 32 software were purchased from Berthold, Bad
Wildbad, Germany. General laboratory supplies were purchased through Fisher
Scientific and VWR.

**Cell Culture**

The breast cancer cell lines MDA 231, MDA 435, MDA 468, and SKBr3 and ovarian
cancer cell line SKOV-3 were cultured in DMEM/F12 with 10% FBS, 1% l-glut, and 1%
P/S. The human embryonic kidney cell line (HEK 293) which expresses adenoviral
genes E1 and E3 allowing for the propagation of replication incompetent adenovirus,
chronic myelogenous leukemia cell line (K562), and Chinese hamster ovarian cell line
(CHO) were grown in DMEM with 10% FBS, 1% l-glut, and 1% P/S. N3S cells, a
variant of HEK 293 cells which are able to grow in suspension, were maintained in EBSS
with 10% FBS, 1% l-glut, and 1% P/S. All cells were cultured in incubators at 37°C with
5% CO₂ and humid conditions.

**Viral Amplification**

Ad-Fiber-BAP was amplified in HEK 293 cells grown in DMEM with 5% FBS, 1% l-
glut and 1% P/S with 100μM d-biotin. Ad-Fiber-WT was amplified in HEK 293 cells
grown in DMEM with 5% FBS, 1% l-glut and 1% P/S. To remove the virus from the
cells after culture, cells were resuspended in 50mM Tris pH 8.0 and treated to three
freeze/thaw cycles. The cell debris was spun out of solution and the viral particles were
purified from the supernatant.
**Viral Purification**

The cell lysate from the viral amplification was layered on a cesium chloride gradient and spun for 3 hours at 20000 rpm in the ultra centrifuge. After the spin, the bottom viral band was removed from the tube, diluted to 4 ml with 50mM Tris pH 8.0 and layered onto another cesium chloride gradient and spun for 20 hours at 20000 rpm in the ultracentrifuge. Again the bottom viral band is removed and the virus is desalted and buffer exchanged into 500mM sucrose, 50mM Tris buffer in a DG-10 column.

**Viral Complexing**

The cell lysate from the viral amplification was layered on a cesium chloride gradient and spun for 3 hours at 20000 rpm in the ultra centrifuge. After the spin, the bottom viral band was removed from the tube, diluted to 4 ml with 50mM Tris pH 8.0, NA was added in 50 fold excess to the number of biotins per capsid, typically about 2mg, and incubated for 30min on ice. Then the virus was layered onto another cesium chloride gradient and spun for 20 hours at 20000 rpm in the ultracentrifuge. Again the bottom viral band is removed and the virus is desalted and buffer exchanged into 500mM sucrose Tris buffer in a DG-10 column. Biotinylated ligand was then added to the virus at 100 fold excess to the number of NA per virion. After a 30 min incubation on ice, the ligands were purified away by size exclusion on 400kDa beads.

**Subcutaneous Tumor Model**

Mouse xenograft models of SKBr3 human breast carcinoma will be initiated by the injection of 10^7 washed tumor cells injected subcutaneously (s.c.) into the flanks of 6
week old female nude mice. Tumors will be allowed to reach approximately 500 cm$^3$ in the mice prior to their use for vector targeting (typically occurs within 21 to 28 days).

**Intramuscular Tumor Model**

Xenograft tumor models of MDA 435 and MDA 468 human breast carcinoma will be initiated by the injection of $5 \times 10^6$ (MDA 435) and $10^6$ (MDA 468) washed tumor cells injected subcutaneously (sc) into the flanks of 6 week old female nude mice with one tumor type injected into each flank of the mice. Again, tumors will be allowed to reach approximately 500 cm$^3$ in the mice prior to their use for vector targeting (typically occurs within 21 to 28 days).

**Intraperitoneal Tumor Model**

Metastatic tumor models of SKOV3 human ovarian carcinoma will be initiated by the injection of $10^6$ washed tumor cells injected intra-peritoneal (i.p.) into the flanks of 6 week old female nude mice. Tumors will be allowed to grow for 21 days, as their volume is difficult to quantitate prior to sacrifice.

**Targeted Adenoviral Injections**

After tumor models were allowed to develop to the size or time set previously. S.c. or i.m. tumors were injected intra-tumorally (i.t.) with $10^9$ viral particles in 100$\mu$l PBS. The metastatic tumor model received the same viral dose injected i.p. The mice were left undisturbed for 72 hours at which point they were sacrificed and the luciferase expression was measured using the luminometer or the Night Owl.
**Homogenized Tissue Luminescence Measurement**

For s.c. and i.m. tumor models, mice were sacrificed and their livers and tumors were removed. The organs were then homogenized, the organ debris spun to the bottom of the tube. The supernatant was then measured for luciferase expression using the luminometer.

**Night Owl Luminescence Imaging**

The Night Owl Molecular Light Imager consists of a super-cooled CCD camera mounted in a light tight chamber. The imager is controlled by a computer system running the Winlight 32 software. Luciferin can be injected i.p. into mice and distributes systemically to sufficient amounts for transfected cells to be visualized throughout the body. Cells were transiently transfected with a plasmid carrying the luciferase gene. The luminescence of these cells was first measured in vitro, then known amounts of the cells were injected into mice and again the luminescence was measured. Cells were titrated to determine the limit of detection with the Nightowl. After 72 hours, the i.p. tumor model mice will be sacrificed, and injected with 1mg luciferin. The mice will then be imaged for 10 minutes for luminescence with skin, without skin, and just the organs from the peritoneal cavity. The images will then be analysed using the Winlight 32 software.
5.4 Results and Discussion

Increased Transduction of SKBr3 Tumors with Herceptin Conjugated Adenovirus

A preliminary experiment was performed with two mice in each group where SKBr3 cells that over-express Her-2 were used to form s.c. tumors. After 21 days, the tumors were injected with buffer, untargeted virus, or Herceptin conjugated virus. After 72 hours the mice were sacrificed, the tumors and select organs harvested and homogenized, and the transgene expression was measured through luminescence with the luminometer (Figure 5-1).

SKBr3 tumors were transduced much better by the Herceptin targeted virus than by the untargeted virus. There is some background transduction of the liver after intratumoral injection of adenovirus, but it is very low compared with the tumor transduction levels. With these positive results from the initial experiment, it was repeated increasing the number of mice to 5 per group. Again, mice were allowed to develop palpable tumors, and the tumors were injected it with no virus, Ad-BAP conjugated to only neutravidin (Ad-BAP-NA) or Ad-BAP conjugated to neutravidin and Herceptin (Ad-BAP-NA-Her). Three days after viral injection, the mice were sacrificed, the tumors and livers harvested, homogenized and measured for expressed luciferase using the luminometer (Figure 5-2).
Figure 5-1 Transduction Levels After SKBr3 Xenograft Infection of Adenoviral Vectors. SKBr3 xenograft tumors were grown subcutaneously in immunodeficient mice. Once palpable, the tumors were injected with no virus, Ad-BAP-NA, or Ad-BAP-NA-Her. Three days after viral delivery, the mice were sacrificed, their tumors (A) and livers (B) harvested and the luciferase expression measured. Each circle represents one mouse and the line is the average for the group, in this experiment N=2.
Figure 5-2 Transduction Levels After SKBr3 Tumor II Injection of Adenoviral Vectors. SKBr3 xenograft tumors were grown subcutaneously in immunodeficient mice and once palpable, they were injected intratumorally with no virus, Ad-BAP-NA, or Ad-BAP-NA-Her. Three days after viral delivery, the mice were sacrificed, their tumors (A) and livers (B) harvested and the luciferase expression measured. Each circle represents one mouse from a group, the line represents the group average, and # indicates that the data sets in each graph are not significantly different (p > 0.05).
When injected intratumorally, Ad-BAP-NA-her conjugated virus was able to transduce the breast cancer tumors slightly better than the Ad-BAP-NA virus. While Ad-BAP-NA does not have a targeting ligand, it should be noted that neutravidin itself confers an increase in transduction of cells as described in Chapter 4. Thus Ad-BAP-NA is perhaps not the best negative control virus for the experiment. The Herceptin conjugated virus also transduced the liver but not significantly more than the untargeted virus. The liver transduction levels are about a log lower than the associated tumor transduction. The transduction of the liver was not unexpected as the virus was not been completely CAR ablated to remove all background binding.

**Increased Transduction of MDA 468 Tumors with EGF Conjugated Adenovirus**

The increase in transduction with the Herceptin targeted virus was not significant in the second test, so another breast cancer tumor model utilizing MDA 468 cells was tested in mice to test the EGF targeted adenovirus. The mice were again injected with tumor cells sc and the tumors allowed to grow for approximately 20 days before they were injected it with wild type adenovirus, biotinylated adenovirus and EGF-targeted biotinylated adenovirus, as shown in Figure 5-3.
Figure 5-3 Transduction Levels After MDA 468 Xenograft Intratumoral Injection of Targeted Adenovirus. MDA 468 xenograft tumors were grown in nude mice. The mice were injected intratumorally with Ad-WT, Ad-BAP, Ad-BAP-NA or Ad-BAP-NA-EGF. Three days after the viral injections, the mice were sacrificed, the organs harvested and measured for luciferase expression. Each circle represents one mouse in the group, the line represents the average for a given group, and † indicates that there is no significant difference between the data sets in each graph (p > 0.05).
In marked contrast to *in vitro* data, there was no significant difference between any of the tumor transduction in any groups in this experiment and the lumens are very low as compared with other experiments. The liver transduction levels corresponded with those of the tumors. The tumors injected in this experiment were extremely small so there could be some error in viral delivery from that. This though does not explain where the vector went and which cells were transduced. The EGF targeted adenovirus did increase the transduction of the EGFR positive cells *in vitro*, but none of the viruses injected transduced the cells well *in vivo*. Instead of homogenizing all the organs for analysis, it would be ideal to have a system where the mice could be measured without being sacrificed and the organs harvested. For this reason the Night Owl was purchased and preliminary experiments done to test the suitability of the system.

**Sensitivity of the Night Owl**

Before the transduction of viral complexes can be measured in whole animals, detection limits of the Night Owl system must be determined. Preliminary data using transiently transfected cells (Figure 5-4) indicated that we would be able to use the system to detect luciferase expression from cells in mice. At low exposure times, 0.1 to 1 million luciferase-expressing cells were detected in mice and there was not a substantial loss of signal when the cells were placed in the subcutaneous or mesenteric space. Cell titration experiments indicated as few as 12,000 transfected cells could be detected with the Nightowl.
Figure 5-4 In Vivo and In Vitro Luciferase Imaging Using the Night Owl System. A) Imaging of mice injected with GFP or luciferase-transfected cells. B) 1 million CHO cells were transfected with GFP or luciferase plasmids, were injected subcutaneously in a rat, and then imaged for 1 minute. C) 100,000 luciferase-transfected CHO cells were injected into the mesentery of a rat at a depth of approximately 1 cm. This site was then imaged for 10 minutes. D) The indicated number of luciferase transfected cells in a 24 well plate were imaged for 5 minutes.
To test for tissue absorption and scatter of light in the peritoneum, CHO cells were transfected with a luciferase plasmid and 1 million cells were injected with luciferin into dialysis cassette with a 0.5x0.5 membrane window. This cassette was implanted into a euthanized animal under the mesentery and the animal was imaged on the Night Owl. As shown in Figure 5-4, the 560 nm light produced by luciferase is grossly distorted by absorption and reflection by the organs in the peritoneal cavity.

**In Vivo Transduction of Ad-Fiber-BAP-SA488-α-59**

After exploring the detection limits of the Night Owl luciferase assaying system, the transduction of the CD59 targeting adenoviral complexes was tested. Three million K562 cells in HBSS were injected into the right side of the peritoneum of female ICR mice. After 15 minutes, the mice were injected on the left side of the peritoneum with the indicated vectors. Mice that did not receive virus were injected with HBSS. Mice receiving virus were injected with approximately 5000 particles per cell of each vector. Unconjugated Ad-Fiber-BAP or Ad-Fiber-BAP-SA488-α-CD59 was injected in 200 μl of HBSS. One day after injection, the mice were anesthetized with avertin and imaged using the Night Owl. One milligram of D-Luciferin was injected into the mice after being anesthetized with Avertin. Four minutes after injection of D-Luciferin animals were evaluated for bioluminescence. The camera was positioned on an area of 150x150 mm at a sample height of 30 mm and autofocused using Winlight 32 software. To maximize sensitivity of camera, the image was taken at low resolution (5x5 pixel binning) for an exposure time of 5 minutes. A color overlay of luminescent signal was constructed over photo of animals using Winlight 32 software (Figure 5-5).
Figure 5-5 In Vivo CD59 Targeting Adenoviral Complexes. The viral complex was able to transduce K562 cells, in the peritoneum where first the K562 cells and then the virus were injected.
In this case, the untargeted vector gave no transduction (except at the injection site of one animal). In contrast, the Ad-BAP-SA-anti-CD59 complex mediated detectable transduction in all three of the animals, albeit at variable levels. These data suggested that a targeting complex could be functional in vivo at least in this simple, dispersed tumor cell model in the peritoneum.

**Targeted Transduction of SKOV-3 Cells with Herceptin and EGF Complexed Viruses**

To test this approach for ovarian cancer, SKOV-3 cells were injected into the peritoneal cavity of nude mice and allowed to form solid tumors for 21 days, at which point the mice were injected i.p. with no virus, Ad-BAP-NA, Ad-BAP-NA-EGF, and Ad-BAP-NA-Her. Three days after the vectors were injected, the mice were sacrificed, injected ip with luciferin and imaged in the nightowl (Figure 5-6). In this case, the SKOV-3 tumors were transduced by Ad-BAP-NA in 2 of the 5 mice. This contrasted with in vitro targeting where transduction by untargeted viruses was substantially lower than by targeted vectors (see Chapter 4). This suggested that either CAR-binding or neuravidin-mediated interactions were higher in the in vivo model than in vitro. The Herceptin targeted adenovirus appeared to transduce the SKOV-3 tumors better than the EGF targeted adenovirus or the untargeted virus. From the Herceptin targeted group 3 of 5 mice show transduction where in the EGF targeted group 2 of 5 mice show transduction of the SKOV-3 tumors. While transduction was observed in the tumors, none was observed in the livers or any other organ in the peritoneal cavity by gross imaging. The
lack of transduction of the organs other than the SKOV-3 tumors at the $1 \times 10^9$ particle dose was encouraging.

To better quantify the luciferase expressed in each tumor or liver, regions of interest were drawn around each organ in the 6 well dish and the Winlight software was used to quantify light production from luciferase from each area. These numbers are represented in Figure 5-7.

By this more quantitative analysis, the SKOV-3 tumors in 2 of the 5 mice were transduced by Ad-BAP-NA. Surprisingly, this transduction was substantially better than that by the EGF-targeted vector. This contrasted with \textit{in vitro} targeting where transduction by EGF was substantially better than by that by Ad-BAP-NA. These data suggest that neutravidin is increasing transduction by mediating non-specific interactions, whereas EGF appeared to be inactive in this \textit{in vivo} model. In contrast to EGF, the Herceptin effectively transduced adenovirus the tumors, but no better than Ad-BAP-NA. Liver transduction is 2 logs less than the tumor transduction. The overall transduction numbers were low for both the tumors and livers, so an increase in viral dose could possibly be tolerated.
Figure 5-6 Light Intensity From Luciferase/Luciferin in SKOV-3 Tumors and Livers After Ip Injection of Virus. Nude mice were injected with SKOV-3 cells intraperitoneally and tumors were allowed to develop for 21 days. The mice were then injected with 1x10⁹ viral particles of no virus (A), Ad-BAP-NA (B), Ad-BAP-NA-EGF (C), and Ad-BAP-NA-Her (D), 36 hours after viral injection, mice were injected with luciferin, sacrificed, imaged as whole mice, and then the tumors and livers were excised and imaged using the NightOwl. The mice and corresponding excised organs are labeled 1 to 5, with the excised livers on the left of each well and tumors on the right.
Figure 5-7 Lumens Measured After Adenovirus Injection. Nude mice were injected with SKOV-3 cells intraperitoneally and tumors were allowed to develop for 21 days. The mice were then injected with 1x10⁹ viral particles of no virus, Ad-BAP-NA, Ad-BAP-NA-EGF, and Ad-BAP-NA-Her, 36 hours after viral injection, mice were injected with luciferin, sacrificed, the tumors and livers were excised and imaged using the NightOwl. Regions of interest (ROI) were drawn around the tumors (A) and livers (B) from each mouse and the Winlight32 software was used to quantify the lumens emitted from each ROI. Each circle represents one mouse, the line represents the average value for each group of mice and the # indicates that there is no significant difference between the data sets in each graph (p > 0.05).
To increase the overall transduction, this experiment was repeated using $5 \times 10^9$ viral particles injected ip. In this case, the Ad-BAP with out neutravidin group was added to assess to what degree increased transduction by Ad-BAP-NA was mediated by neutraviding. The tumors were allowed to develop for 5 weeks prior to adenovirus injection then three days after the mice were again sacrificed and the luciferase expression measured using the Nightowl. The images taken are shown in figure 5-8. In this case, Ad-BAP and Ad-BAP-NA injected i.p both transduced SKOV-3 tumors well with 3 of 5 mice in each group transduced. The Herceptin targeted adenovirus transduced the SKOV-3 tumors well, but so did the WT and untargeted viruses. Unfortunately, in the Ad-BAP-NA-Herceptin group only 2 animals had overt tumor masses making comparison difficult. For this group, the fat pad where the tumors normally form was placed in the 6 wells where tumors were missing.
Figure 5-8 Light Intensity From Expressed Luciferase After Ip Injection of Adenovirus. Nude mice were injected with SKOV-3 cells intraperitoneally and tumors were allowed to develop for 21 days. The mice were then injected with $5 \times 10^9$ viral particles of Ad-WT (A), Ad-BAP (B), Ad-BAP-NA (C), and Ad-BAP-NA-Her (D), 36 hours after viral injection, mice were injected with luciferin, sacrificed, the peritoneal cavity was exposed and the mice imaged, then the tumors and livers were excised and imaged using the NightOwl. The mice and corresponding excised organs are labeled 1 to 5, with the excised livers on the left of each well and tumors on the right.
Figure 5-9 Lumens Measured in SKOV-3 Tumors and Livers After Adenovirus Injection. Nude mice were injected with SKOV-3 cells intraperitoneally and tumors were allowed to develop for 21 days. The mice were then injected with $5 \times 10^9$ viral particles of Ad-WT, Ad-BAP, Ad-BAP-NA, and Ad-BAP-NA-Her, 36 hours after viral injection, mice were injected with luciferin, sacrificed, the tumors and livers were excised and imaged using the NightOwl. Regions of interest (ROI) were drawn around the tumors (A) and livers (B) from each mouse and the Winlight32 software was used to quantify the lumens emitted from each ROI. Each circle represents one mouse, the line represents the average value for each group of mice, and the # indicates that there is no significant difference between the data sets in each graph (there were only two tumors in the Ad-BAP-NA-Her group, so that data set was not included in the statistical analysis).
Ad-WT transduced tumors in 4 of 5 mice while Ad-BAP-NA-Her transduced the tumors in 2 of 5 mice (but in 2 of 2 tumors for the mice that actually had tumors). There was not observed transduction in the liver or other organs in the peritoneal cavity by imaging. The increase in viral particle dose had a corresponding increase in signal from the Nightowl pseudo-color images. Unfortunately the experimental group with Herceptin targeted adenovirus did not have the same tumor formation seen in the other groups, with only 2 of the 5 mice in the group having tumors in the peritoneum. In addition to the pseudo-color images, the released lumens were again measured using the Winlight software and the results are presented in figure 5-9. The lumenessence from each tumor were measured showing that although more of the mice in the Ad-WT group were transduced, the overall signal was higher from the Ad-BAP and Ad-BAP-NA groups. The somewhat higher transduction by Ad-BAP-NA vs. Ad-BAP suggest that neutravidin itself does mediate increased transduction in the absence of ligand in both the tumor and liver. Given the lack of tumors in 3 of 5 mice in the Herceptin targeted group, no statements can be made about the transduction level of that group. The lumens released from the livers again are very low with a few outliers. Despite the 5 fold increase in the number of virions injected, there is no corresponding increase in liver transduction. The tumors were transduced, but not significantly better by the targeted virus than the untargeted virus. These results were confounded by the fact that 3 of the 5 mice injected with Ad-BAP-Herceptin appeared to not have any tumors.
5.5 Conclusions

The large increase in transduction seen *in vitro* did not translate into the *in vivo* system. Neither of the breast cancer models nor the ovarian cancer model showed significant increased transduction with the targeted virus models. This discrepancy may be due in part to the fact that the untargeted and neutravidin-conjugated vectors appeared to transduce tumors substantially better *in vivo* than *in vitro*. This suggests that CAR expression might be upregulated in these tumor cells after implantation *in vivo*. An alternate explanation may be that *in vivo* the vectors are binding blood factors like factor IX or complement that have recently been shown to increase transduction *in vivo*, but not *in vitro* in their absence. Alternately, the extended exposure of the tumor cells to the vectors *in vivo* might enable increased viral interactions with the cells via the RGD-integrin pathway, which may increase general transduction at the expense of the previously observed EGF or Her-2 mediated observed increases *in vitro*. The non-specific transduction observed from the untargeted adenovirus *in vivo* would likely be reduced with ablation of the CAR and blood factor binding sites in fiber and the integrin binding domains in penton base proteins prior to addition of new ligands to the vectors. This reduction would help increase the difference between targeted and untargeted adenovirus transduction, but it would not help increase the overall transduction of the targeted virus. Alternately, it is possible that the targeted viral complexes are just too large to penetrate the tumor cells well when injected into the tumor or into the peritoneum.
CHAPTER 6

In vivo near infrared optical imaging

6.1 Abstract

Optical imaging is a powerful tool for measuring real time, dynamic, in vivo biodistribution. Small molecule targeting ligands such as peptides and EGF can be chemically coupled with the fluorophores and their specific uptake into EGFR positive tumors can be measured. In addition to small molecules, larger molecules such as antibodies can be labeled with fluorophores and imaged in vivo. For gene therapy applications, fluorescent dye labeling of virions has the potential to allow tracking the real time path of the vector through the body independent of where it expresses transgenes.

6.2 Introduction

Imaging is an important part of cancer diagnosis, therapy, and prognosis. As the early detection of abnormalities within tissues improves, the overall cancer survival rates are increasing. Current imaging techniques include magnetic resonance imaging (MRI), molecular imaging, nuclear imaging (gamma imaging, positron emission tomography (PET), and single-photon emission computed tomography (SPECT)), and x-ray computed tomography (CT). Millimolar amounts of contrast agent are typically required for magnetic resonance and x-ray imaging, although recent improvements are decreasing this amount, while nanomolar amounts of the imaging agent are necessary for nuclear and optical molecular imaging (38).
Traditionally, our laboratory has worked with fluorescence imaging in the form of transgenes expressed from the gene therapy vectors. For the tracking of ligands, again the imaging system of choice is fluorescence for two main reasons. First, radioactive probes can require long imaging times and include exposure to radioactivity for the mouse and the researcher. Second, because it is less energetic, fluorescence is renewable and the tag can be re-energized in situ in the body. Each light event occurs on the order of a nanosecond, meaning that there are $10^9$ imagable events per second, making this a very powerful technique capable of sensing extremely low amounts of dye (39). Biological tissues have their own absorbance at different wavelengths, which are minimized around 1000 nm, making the near infrared (NIR) region of light a good choice for small animal imaging (39).

Light between the wavelengths of 700 and 1000 nm is classified as NIR and is capable of traveling up to several centimeters into tissue. NIR optical imaging is very sensitive and versatile, capable of real time, dynamic imaging (40, 41). It is an attractive region of light for imaging partially due to lower background auto-fluorescence than other fluorescent molecules such as fluorescein which emits around 520 nm and has high skin auto-fluorescence. NIR dyes such as Cy5.5 and IR800 with respective emission wavelengths of 710 nm and 830 nm are available with reactive groups for conjugation to cancer targeting ligands or molecules. The real time biodistribution of these NIR dye labeled antibodies and small targeting molecules can be imaged (42-45). This could also be valuable for screening cell-targeting peptides and other ligands to determine in vivo functionality and targeting before conjugating them to adenovirus. If antibodies can be
labeled with NIR dyes and tracked, perhaps larger entities such as virions could also be labeled and their in vivo biodistribution determined in real time. A method for virus tracking would provide the ability to measure the circulation path of the targeted and untargeted viruses in addition to the current method of determining where transgene expression exists.

Based on this, the feasibility of using in vivo optical fluorescent imaging to track cell-targeting ligands and targeted vectors in mouse models was tested. This effort is distinct from that of the previous chapter, since we aim to track protein ligands and viral particles rather than only where viral gene delivery occurs as is measured by luciferase imaging.

6.3 Materials and Methods

Materials

Cell lines for this work were purchased from American Type Culture Collection (ATCC) in Manassas, VA. Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12), Dulbecco’s Modified Eagle Medium (DMEM), Minimum Essential Medium with Earle’s Balanced Salt Solution (EBSS), Fetal Bovine Serum (FBS), l-glutamine (l-glut), Penicillin-Streptomycin (P/S), and trypsin were purchased from Invitrogen in Carlsbad, CA. Herceptin was a gift from Dr. Malcom Brenner. Biotin-NHS was ordered from Vector Laboratories in Burlingame, CA. Neutravidin (NA) was purchased from Sigma Aldrich, St. Louis, MO. Biotinylated and unlabeled epidermal growth factor (EGF) and streptavidin-alexafluor 488 (SA488) were purchased from
Molecular Probes in Eugene, OR. DG-10 desalting columns came from Biorad in Hercules, CA. IR-800-NHS was purchased from Licor in Omaha, NE. General laboratory supplies were purchased through Fisher Scientific and VWR.

**Cell Culture**

The breast cancer cell lines MDA 435 and MDA 468 were cultured in DMEM/F12 with 10% FBS, 1% l-glut, and 1% P/S. The human embryonic kidney cell line (HEK 293) which expresses adenoviral genes E1 and E3 allowing for the propagation of replication incompetent adenovirus was grown in DMEM with 10% FBS, 1% l-glut, and 1% P/S. N3S cells, a variant of HEK 293 cells which are able to grow in suspension, were maintained in EBSS with 10% FBS, 1% l-glut, and 1% P/S. All cells were cultured in incubators at 37°C with 5% CO₂ and humid conditions.

**In Vivo Optical Imaging**

The *in vivo* optical images were collected in collaboration with Dr. Shi Ke and Sunkuk Kwon from Dr. Eva Sevick-Muraca’s laboratory. *In vivo* fluorescence-based optical imaging was accomplished by illuminating the animal with light from a laser diode (488 nm at 20 mW for green fluorescent protein (GFP), 660 nm at 60 mW for Cy5.5, and 785 nm at 85 mW for IR800) expanded to an approximately 8-cm-diameter circular area. The re-emitted fluorescent light was collected by an image intensifier (model FS9910C, ITT Night Vision, Roanoke, VA) lens-coupled to a charge-coupled device camera (model CH350, Photometrics, Tucson, AZ). A detailed description of the *in vivo* optical fluorescence imaging system used in this study was given previously (53). The
resolution of the optical camera was 1024 x 1024 matrix pixels in 11.4 x 11.4 cm field of view. Filter sets used in this study included a bandpass filter (520 nm, 710 nm, and 830 nm center wavelength for GFP, Cy5.5, and IR800) to reject back-scattered and reflected excitation photons. Image acquisition was accomplished using V++ software (Digital Optics, Auckland, New Zealand). Data processing and analysis was accomplished using Matlab software (The MathWorks, Inc., Natick, MA). For all imaging sessions, the acquisition parameters were kept constant. The integration time for each image was 800 ms. For quantitative comparison, region-of-interest (ROI) was selected in tumor and opposite normal areas and fluorescence intensity in each ROI was measured.

**IR-800 Labeling of EGF**

EGF was resuspended at 200μg/ml in PBS at pH 7.4. IR-800 was added at a dye to molecule ratio of 5:1 and allowed to react for 1 hour at room temperature with mixing. The unreacted dye was removed using a DG-10 column and the EGF-IR-800 buffer exchanged into 50mM Tris pH 7.5.

**Viral Amplification**

Ad-Fiber-BAP was amplified in HEK 293 cells grown in DMEM with 5% FBS, 1% l-glut and 1% P/S with 100μM d-biotin. Ad-Fiber-WT was amplified in HEK 293 cells grown in DMEM with 5% FBS, 1% l-glut and 1% P/S. To remove the virus from the cells after culture, cells were resuspended in 50mM Tris pH 8.0 and treated to three freeze/thaw cycles. The cell debris was spun out of solution and the viral particles were purified from the supernatant.
IR-800 Labeling Virus

The cell lysate from the viral amplification was layered on a cesium chloride gradient and spun for 3 hours at 20000 rpm in the ultra centrifuge. After the spin, the bottom viral band was removed from the tube, diluted to 4 ml with 50mM Tris pH 8.0, and layered onto another cesium chloride gradient and spun for 20 hours at 20000 rpm in the ultracentrifuge. Again the bottom viral band is removed and the virus is desalted and buffer exchanged into 500mM sucrose phosphate buffer ph 7.5 in a DG-10 column. The virus was incubated with 33mg/ml psoralen for 30 minutes on ice under ultraviolet light to crosslink the DNA and to inactivate the virus for use outside a biosafety hood. The free psoralen was removed from the virus using a DG-10 column. IR800 was added to the virions at dye to vp ratios of 1:100000, 1:10000, and 1:1000 and allowed to react for 1 hour at room temperature. The virus was then separated from unreacted dye into a 500mM sucrose Tris buffer ph 7.5 in another DG-10 column.

Intramuscular Tumor Model

Xenograft tumor models of MDA 435 and MDA 468 human breast carcinoma will be initiated by the injection of 5x10^6 (MDA 435) and 10^6 (MDA 468) washed tumor cells injected subcutaneously (sc) into the flanks of 6 week old female nude mice with one tumor type injected into each flank of the mice. Again, tumors will be allowed to reach approximately 500 cm^3 in the mice prior to their use for vector targeting (typically occurs within 21 to 28 days).
Statistical Analysis

Statistical analysis was performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC) for Microsoft Windows. The data will be analyzed using the one-way ANOVA and the General Linear Model. The significance level was set at 0.05.

6.4 Results and Discussion

Theoretically, any wavelength of light can be used to image ligands or vectors in small animals. However, biological tissues have their own wavelengths of light absorbance making the use of some excitation and emission wavelengths less productive than others. For example, from 200 nm to 1000 nm there is strong absorbance by hemoglobin and melanin that decreases with increasing wavelength, shown in figure 6-1.

To determine directly which wavelengths of fluorescence might be optimal for tracking ligands and vectors in mouse xenograft models, we compared the levels of background fluorescence in mice at two common wavelengths for fluorescence emission, 520 nm for green fluorescent protein (GFP) and fluorescein and 710 for Cy5.5 (Figure 6-2). When unmodified mice were excited with a 488 nm laser, and emission was observed at 520 nm, there was marked level of autofluorescence mainly from the skin. When mice were illuminated with a 660 nm laser, observation of the emission at 710 nm demonstrated that this red-shifted wavelength was able to avoid the skin autofluorescence observed at green wavelengths. However, there was a new autofluorescence source observed in the intestines due to the chlorophyll in the mouse food. These images showed that each of these popular wavelengths for fluorescence both had problematic levels of background
fluorescence in mice. While the observed autofluorescence does not entirely preclude imaging at these wavelengths, it does set a high background level that translates into not being able to observe less than $0.5 \times 10^6$ GFP expressing cells or less than 0.01 nmol Cy5.5 at these wavelengths. When delivering the fluorescent protein or cells a significantly higher amount than the lowest observable must be delivered as the labeled compound will be diluted as it circulates throughout the mouse.

To determine if there was a dye with a better signal to noise ratio, we tested a dye further into the near infrared region, IR-800 which is excited at 785 nm and emits at 830 nm was investigated for its background autofluorescence levels as seen in Figure 6-3.

In this case, the autofluorescence from the skin and the food that were observed previously at 530 nm and 710 nm were not observed with near-infrared dye at 830 nm. The high signal to noise ratio of 0.01 nmol IR-800 makes it a more optimal imaging agent for situations where only a small amount of the compound being imaged is can be delivered either due to toxicity or difficulty in obtaining it. Although 0.01 nmol Cy5.5 was comparable to the autofluorescence, 0.01 nmol IR800 is significantly higher than the little to no autofluorescence observed at 830 mn, and a dose of 0.01 nmol could be diluted and still observed above background. For these reasons, IR-800 was used for the molecular imaging of EGF and virions.
**Figure 6-1 Biological Compounds and the Associated Absorbance at Different Wavelengths.** Hemoglobin and melanin contribute strongly to the absorbance at the lower wavelengths, while the water contribution to the absorbance increases with the wavelength.
Figure 6-2 Auto-fluorescence of a Mouse in 520 and 710 nm Wavelengths. Each image is an exposure of 800 ms, at 520 nm the skin auto-fluorescence is comparable to 0.5 x 10^6 GFP expressing cells and at 710 nm there is a fluorescent signal due to the chlorophyll in the animal food which is comparable to 0.01 nmol Cy5.5. The pseudo-color scale is 5 times greater for the GFP background as for the Cy5.5 background.
Figure 6-3 Mouse Autofluorescence at 830 nm Wavelength. Each image is an exposure of 800 ms, at 830 nm there is very slight autofluorescence, but when compared to 0.01 nmol IR-800, no significant autofluorescence is apparent.
Ligand Tracking by Near-infrared (NIR) Imaging of EGF to Tumors in Mice.

To test our ability to evaluate ligands by optical imaging, nude mice were injected with MDA 435 (EGFR negative) and MDA 468 (EGFR positive) tumors, and the tumors were allowed to grow for 4-6 weeks. After the tumors were established, mice were injected with 1 nmol of untargeted IR800 or EGF-IR800 and imaged over 40 minutes with each image taken by an 800 ms exposure. These individual frames were compressed into movies (data not shown) of approximately 5 minutes to watch the biodistribution of the dye over time. Selected images are shown in Figure 6-4. There is very little background signal from the mice before injection of dye. The tail vein injection of dye is seen in the tail, and the dye quickly disperses throughout the body as time passes. First it is seen in the chest C, then head H, liver, kidneys K, and the tumor T. After 4.4 seconds, some accumulation is apparent in the tumor in the both IR800 and EGF-IR800 injections, no accumulation is seen in the C225 pre-blocked animals. The EGFR is expressed in the mouse, especially in the skin, and the EGF used in these experiments is murine, so that could account for some of the signal seen in non-tumor regions in the mouse.
Figure 6-4 Real Time Imaging of IR800, EGF-IR800, and C225 Blocked Tumors With EGF-IR800 in Nude Mice. Nude mice were injected subcutaneously with MDA 468 cells and tumors were allowed to form for approximately one month. Then mice were injected into the tail vein with 1 nmol of IR800 (A), EGF-IR800 (B), or C225 24 hours prior to EGF-IR800 (C) and the uptake of dye into the tumor measured as a function of time. The MDA 468 tumor is in the right flank of the IR800 and EGF-IR800 mice, and the left flank of the C225 blocked mice. The top row of images shows the mice under white light conditions, the next image is the background fluorescence of the mice. The third image in each column is of the injection of dye entering the tail vein and each following image is 1.1 second after the previous (1.1, 2.2, 3.3, 4.4, 5.5, and 6.6 seconds).
After 4.4 seconds the accumulation of the dye in the tumor is apparent in both the IR800 and the EGF-IR800 mice, with the IR800 signal being higher in all locales than the EGF-IR800 due to reduced fluorescence in the EGF labeling process.

To better quantitate dye uptake into the tumors, fluorescence in the tumor areas were measured by MatLab. Although tumors were overtly visible in both cases by simple imaging, analysis by MatLab revealed significant differences in the specific uptake of the EGF-IR800 into the tumor as compared to IR800. In particular, a distinct difference in the rate of dye uptake by EGF-IR800 is observed compared to IR800 alone. (Figure 6-5). The signal from the IR800 was somewhat higher in the tumor region than in the background region, but the uptake rate was the same in the tumor as in the background, signifying a high degree of vascularization. The signal from EGF-IR800 was higher again in the tumor region than in the background and in addition, the EGF-IR800 uptake rate was higher in the tumor region than in the normal region, signifying a specific uptake. The observed non-specific uptake of the untargeted dye is probably due tumor vasculature which is known to be leaky. To determine if the EGF-IR800 uptake was actually mediated by EGF targeting, mice were injected with C225, an antibody known to block binding by EGF to EGFR (reviewed in (12)), before EGF-IR800 (Fig. 6-5C). C225 pre-blocking dropped the EGF-IR800 signal level to that of the background, lower than both IR800 and EGF-IR800, and the uptake rates of the tumor and background regions were comparable, demonstrating a specific interaction between EGF-IR800 and the EGFR. After measuring the uptake rate, the regions of interest were used to calculate the tumor to background ratios (Figure 6-6).
Figure 6-5 Tumor and Background Uptake of IR800 and EGF-IR800. Nude mice were injected subcutaneously with MDA 468 cells and tumors were allowed to form for approximately one month. Then mice were injected into the tail vein with 1 nmol of IR800 (A), EGF-IR800 (B), or C225 24 hours prior to EGF-IR800 (C) and the uptake of dye into the tumor measured as a function of time. The black lines represented the EGFR positive tumor region of interest (ROI) and the red or grey lines represented the background tissue region of interest (ROI). Each of these graphs represents one animal (N=1).
Figure 6-6 Tumor to Background (TBR) Ratios of IR800, EGF-IR800, and C225 Pre-blocked EGF-IR800. Nude mice were injected subcutaneously with MDA 468 cells and tumors were allowed to form for approximately one month. Then mice were injected into the tail vein with 1 nmol of IR800, EGF-IR800, or C225 24 hours prior to EGF-IR800 and the ratio between the dye signal in the tumor region of interest and the background region of interest was measured at different time points. The black bars represent EGF-IR800, the white bars represent IR800, and the grey bars represent C225 blocking with EGF-IR800. For EGF-IR800 and IR800 at 0 minutes, 40 minutes and 24 hours, N=3 and at 48 hours N=2 and for C225 preblocked EGF-IR800, N=4 for all time points. In this graph, * indicates p < 0.05, ** indicates p < 0.0001, and *** indicates p < 0.005, for all the other data sets there was no significant difference (p > 0.05), and no statistical analysis was done on the 48 hour data sets because N=2 for EGF-IR800 and IR800.
The tumor to background ratio (TBR) for EGF-IR800 continued to increase from 40 minutes after injection to 48 hours later, while the TBR for IR800 and C225 with EGF-IR800 remained close to 1 at 40 minutes and 24 and 48 hours. The TBR shows that C225 blocking drops the EGF-IR800 signal to the level of the IR800. The IR800 signal in the tumor was close to the signal in the background tissues, while the EGF-IR800 signal in the tumor was close to two times that of the background region.

These results can also be shown with pseudo-color pictures, such as these of the IR800 and EGF-IR800 injected mice as presented in Figure 6-7. The high EGF-IR800 and IR800 signal seen throughout the mouse at 40 minutes after injection was cleared from the IR800 animals at 24 hours but persisted in the EGF-IR800 mice. Persistent signal in other locals of the mouse by EGF-IR800 was likely due to the fact that EGFR expressed throughout the mouse and not just in the tumors. After 48 hours clearing of EGF-IR800 is seen from background mouse tissue but not from the EGFR over-expressing tumor.
Figure 6-7 Relative Clearing Times of IR-800 and EGF-IR800. Nude mice were injected subcutaneously with MDA 468 cells and tumors were allowed to form for approximately one month. Then mice were injected into the tail vein with 1 nmol of IR800 (A), EGF-IR800 (B), or C225 24 hours prior to EGF-IR800 (C) and the uptake of dye into the tumor measured as a function of time. The red circles in the white light images show the location of the EGFR positive tumor on the right and the background region on the left.
Virus Tracking by Near-infrared (NIR) Imaging of Adenovirus in Mice.

EGF-IR800 is a small molecule imaging ligand capable of binding to EGFR positive tumors in vivo. Given this, we next tested if this technology could also be used for tracking untargeted and targeted gene therapy vectors in vivo. In this case, the goal is to track the virions rather than only the subset of sites where these express transgenes like luciferase or GFP.

As an initial experiment, adenovirus virions were labeled with IR800 in ratios of 1:100000, 1:10000, and 1:1000 virus particle (vp) to dye molecules and free dye was removed by purification on CsCl gradients. When assayed on a fluorometer, the fluorescence of the virions was undetectable (data not shown). In contrast, but using Dr. Sevick-Muraca’s very sensitive imaging system, the varied levels of fluorophore labeled virus could be compared as shown in Figure 6-8. Virus labeled with increasing amounts of dye demonstrated increasing levels of fluorescence. The fluorescence of the most highly labeled virus (1:100000) was comparable to that produced by 0.01 nmol IR800. Earlier work demonstrated that 0.01 nmol of IR800 could be detectable over the background in nude mice. To test if we could track the labeled virions in vivo, 5 x 10^{10} Ad-WT-IR800 virions were injected by tail vein and were imaged in nude mice for 40 minutes and captured as a movie (data not shown). While the signal was relatively low, fluorescence could be observed initially at the injection site and the spreading to the chest cavity and head, and then accumulating in the liver as expected. Fluorescence also accumulated in the kidneys, which was unexpected for adenovirus. This suggested that free IR800 dye might have been injected or was being released from the virions.
Figure 6-8 Measurement of Virion Fluorescence. The tubes in the top row are 1 nmol, 0.1 nmol, 0.01 nmol and 0.001 nmol of IR800. The tubes in the bottom row are from left to right $2.5 \times 10^{10}$ vp in 100 µl, at dye:vp ratios of 1:100000, 1:10000, and 1:1000. The fluorescence of the virus labeled at 1:100000 (bottom left tube) is comparable to 0.01 nmol IR800 (top row second tube from the right).

Figure 6-9 Imaging of Ad-WT-IR800 in Nude Mice. Nude mice were injected intravenously in the tail vein with $5 \times 10^{10}$ viral particles of Ad-WT labeled 1:100000 with IR800. The images are 800 ms exposures taken 24 hours after the injection, anterior and posterior, then the mice were sacrificed, their organs harvested, and imaged ex vivo.
24 hours after virus injection, 800 ms exposure images were taken in the mice and then the organs were excised and another 800 ms exposure image was taken, as shown in Figure 6-9. In this case, Ad-WT-IR800 was observed in the liver and kidneys in the whole body imaging and in these excised organs, but not at other sites. The images are promising for the imaging of virions in mice, but need to be validated. The virions could have lost proteins which could account for some of the signal or there could be free IR800 which was not conjugated or fell off the virus.

In addition to tail vein injection, some work in our laboratory used on oral delivery for genetic vaccines. To image the orally delivered vectors, 5 x 10^{10} Ad-WT-IR800 were orally gavaged into nude mice, and then images were taken as shown in figure 6-10. At 5 minutes after administration, the IR800 signal is localized to the stomach with some signal beginning to appear in the intestines. After 40 minutes the signal has traveled a small distance into the intestines and is still present in the stomach. At 2 hours, the signal has moved further through the intestines. By 24 hours after administration there is no signal remaining in the mouse. It is not surprising that the material in the mouse intestines is turned over in under 24 hours. Anticipating this fact, at 24 hours, the mouse and its feces were collected and imaged (Figure 6-11). These images show that after 24 hours, the Ad-WT-IR800 signal is no longer detectable in the animal though it is present in the feces. It is unclear if there is some viral uptake that is undetectable in this system since the 0.01 nmol amount of virus delivered is close to the level of detection. The IR800 signal again could be due to labeled virions, labeled viral proteins that have fallen off the virion, or unconjugated dye.
Figure 6-10 Imaging of Orally Delivered Ad-WT-IR800. Each picture reflects an 800 ms exposure of the mouse at different time points before and after Ad-WT-IR800 oral gavage.

Figure 6-11 Imaging of Mouse Feces. After 24 hours the mouse feces were removed from the cage and an 800 ms exposure image was taken to show IR800 presence in the feces.
6.5 Conclusions

As advances continue to be made in molecular imaging, the diagnostic, therapeutic, and survival rates of cancer should continue to improve. IR-800 labeled small molecule targets, antibodies, and virions show promise for determining real time distribution of compounds throughout the body. EGF-IR800 accumulates in EGFR positive tumors to a greater extent than in the background tissues or in EGFR negative tumors. The accumulation of EGF-IR800 in EGFR positive tumors is specific because it can be blocked with C225, an antibody against EGFR. In addition to small molecules, adenovirus particles were successfully labeled with IR-800, and tracked in vivo. The stability of the virus-IR800 complex must be determined and whether the detected signals were due to intact virions, viral proteins which fall off the capsid during circulation, or if there were free dye contaminants present after the purification process must be investigated. One possible way to determine the free dye contribution to the IR800 signal is to use live virus, and measure first the IR800 biodistribution, and then compare that with the transgene expression profile.
CHAPTER 7

Conclusions and future directions

7.1 Ligand Discovery

The linear peptides selected from phage display library ON543 did not show high binding levels outside the context of phage. Two possible reasons for this are the display on phage constrains the peptides in an unknown manner which is not reproduced by the synthetically generated linear peptides or that the binding is increased due to the number of copies of the peptide displayed on the phage and their proximity in that display format. Context-specific constrained peptide libraries are being developed in our laboratory to address the first possible problem. Although avidity was taken into account in some of the experiments, the possibility exists that the phage display allows for the interaction between the peptides in a manner that was not reproduced in these experiments. Due to the lack of targeting from the selected peptides, and the high binding affinities shown by antibodies against EGFR and Her-2 as well as the small molecule ligand EGF, these molecules were used to re-target adenovirus for gene therapy.

7.2 Viral Complexing

Adenovirus can be produced in high titers and retargeted through chemical modification and covalent conjugation using the biotin/avidin interaction. During cesium chloride gradient purification, avidin can be added to the virus and free avidin purified from that attached to the virus. Targeting ligands can be added to the virus and purified through size exclusion column chromatography. These viral complexes transduce cells at least as well as the previous layering approach used in our laboratory and show promise as breast
and ovarian cancer targeting gene therapy vectors. Chemical modification of the virus is also possible, but none of the peptides were sufficiently able to increase transduction of breast cancer cells to warrant more testing in their current form. The ability to make targeted viral complexes allows these gene therapy vectors to be injected into mice to test their *in vivo* targeting.

### 7.3 In Vivo Viral Targeting

The large increase in transduction seen *in vitro* did not translate into the *in vivo* system. Neither of the breast cancer models nor the ovarian cancer model showed significant increased transduction with the targeted virus models. The transduction from the untargeted adenovirus could likely be reduced with the removal of CAR and integrin binding domains from the adenoviral fiber. The reduction would help increase the difference between targeted and untargeted adenovirus transduction, but it would not help increase the overall transduction of the targeted virus. It is possible that the targeted viral complexes are just too large to penetrate the tumors well when injected into the tumor or into the peritoneum.

### 7.4 Ligand and Virion Tracking

Molecular imaging of IR-800 labeled small molecule targets and virions shows promise for determining real time distribution of compounds throughout the body. EGF-IR800 specifically accumulates in EGFR positive tumors to a greater extent than in the background tissues or in EGFR negative tumors and can be blocked with an antibody against EGFR. Adenovirus particles can be labeled with IR-800, and tracked in vivo, but
more work is needed to determine the stability of the virus-IR800 compound and whether the detected signals were due to intact virions, viral proteins, or free dye contaminants.

7.5 Future Directions

The linear peptides selected against breast cancer cells were very low affinity when not displayed by the phage so context-specific constrained peptide libraries are being developed in our laboratory. These libraries will display the peptides within the framework of the virus for genetic insertion or another protein such as streptavidin which can be covalently conjugated to the virus for retargeting. Although avidity was taken into account in some of the experiments, the possibility exists that the phage display allows for the interaction between the peptides in a manner that was not reproduced in these experiments. Additional experiments could be done to affinity purify the existing peptides or to increase their avidity through a more rigorous exploration of the avidity interactions of the peptides.

The methods for retargeting the adenovirus involve chemical and covalent modification to add the targeting ligand. Although this method allows for the adding of any ligand to the virion after the purification of the virus, it does not allow for the use of replication competent virus as virions generated in vivo would not contain the targeting ligand. The addition of a tissue specific promoter would contain any gene expression or viral replication to the tissue of interest or the genetic addition of a targeting ligand to the virus could allow for the in vivo generation of targeted adenovirus.
Although the *in vitro* transduction of the untargeted virus is relatively low compared with the targeted virus, the *in vivo* transduction is comparable to the targeted virus. This could likely be reduced with the removal of CAR and integrin binding domains from the adenoviral fiber. The reduction would help increase the difference between targeted and untargeted adenovirus transduction, but it would not help increase the overall transduction of the targeted virus. It is possible that the targeted viral complexes are just too large to penetrate the tumors well when injected into the tumor or into the peritoneum. A genetically modified virus might be smaller and better able to penetrate the tumors.

To test whether the size of the modified virus is causing poor tumor penetration leading to low transduction, adenovirus particles can be labeled with IR-800, and tracked *in vivo*. Although proof of principle work is promising, more work is needed to determine the stability of the virus-IR800 compound. The signals detected could be from intact virions, viral proteins, or free dye contaminants, so more work must be done to determine exactly which signal was due to virions and which signal was not. In addition, work with active virions would allow for the simultaneous tracking of viral particles and transgene products for a more complete picture of the actual delivery path and locations of the virus.

Despite promising *in vitro* data, the *in vivo* use of targeted adenovirus did not result in the same level of increased transduction. To improve the transduction efficiency of the targeted adenovirus, the native tropism of the virions should be removed and alternative
targeting methods and ligands should be explored. The ability to track the biodistribution of the virions in real time will allow for faster testing of targeting ligands and viral complexes for better targeted gene therapy.
Bibliography


