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Binding, internalization, and transgene expression of an adenoviral vector retargeted to HER3/4

by

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Abstract

Adenoviruses (Ads) have been well studied for use in cancer gene therapy. However, low levels of the primary receptor, coxsackie-adenovirus receptor (CAR), in tumor cells has been shown to be a factor in low transgene expression. To increase Ad infection of breast cancer cells we constructed a human Ad5 targeted to HER3/4 receptors by the insertion of the HER3/4 ligand, the HRG EGF-like domain. These growth factor receptors are overexpressed on breast cancer, as well as other cancer cells.

Here, we have shown higher transgene expression levels after infection of breast cancer cells expressing high levels of HER3/4 by the modified virus, compared to the wild-type binding virus. Furthermore, we have shown expanded tropism of the modified virus to Chinese hamster ovary cells that are refractory to infection by the wild-type binding virus. Competition with either the HRG EGF-like domain or soluble Ad virus fiber knob supported these results. However, gene transfer to a breast cancer xenograft model was not improved by the addition of the heregulin (HRG) EGF-like domain.

We compared binding and internalization of the modified virus to that of the wild-type binding virus. As expected, the wild-type virus bound and was taken up into CAR+ cells within 10 min. The modified virus was similar in CAR+ cell lines. Surprisingly, in CAR- cells, very little binding or internalization of the modified virus was detected within 10 min. When re-assessed under stringent conditions used in binding and internalization assays, there was no detectable reporter gene expression after infection of CAR- cells with either virus. Moreover, fluorescence

microscopy demonstrated that longer incubation times increased internalization of the modified virus into CAR- cells, consistent with the original transgene expression assays. Thus, the modified virus internalization into CAR- cells appears to be delayed compared to internalization of the wild-type binding virus.

We have shown differences in binding, internalization, and gene expression after modification of Ad to bind to HER3/4, in addition to CAR. Further study and modifications of this vector should result in an effective gene therapy vector for breast or other cancers.

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Abreviations and Nomenclature

A488 – Alexa 488 dye

aa – amino acids

Ad – adenovirus

Ad2 – adenovirus serotype 2, subgroup C

Ad5 – adenovirus serotype 5, subgroup C

Ad12 – adenovirus serotype 12, subgroup A

Ad35 – adenovirus serotype 35, subgroup B2

AdLuc(HRG-fiber) – the virus targeted by HRG EGF-like domain, also encoding the reporter gene luciferase

AdLuc(wt-fiber) – the control virus encoding the reporter gene luciferase and wild-type binding fiber, also called AdLC8c-luc

AdLC8c-luc – called AdLuc(wt-fiber) in this document

AKT – serine/threonine kinase, also known as protein kinase B (PKB)

BAP – biotin acceptor protein

CCAC – Canadian Council on Animal Care

CAR – coxsackie-adenovirus receptor

CHO – Chinese hamster ovary cells

CHO-al2 – CHO cells transfected with human α2 integrin, used as a control for CHO-CAR

CHO-al2/HER3 – CHO cells transfected with human α2 integrin and HER3

CHO-CAR – CHO cells transfected with CAR

CHO-CAR/HER3 – CHO cells transfected with CAR and HER3

CHO-NT – CHO cells not transfected with any exogenous surface receptor

CMV – cytomegalovirus

CPE – cytopathic effect

CPZ – chlorpromazine

DMEM – Dulbecco's Modified Eagle Medium

DT – diphtheria toxin

EEA1 – early endosome antigen 1

EGF – epidermal growth factor

eGFP – enhanced green fluorescent protein

EGFR – epidermal growth factor receptor

ER – estrogen receptor

ErbB3 – human epidermal growth factor receptor family 3 (HER3)

FGF2 – fibroblast growth factor 2 (basic)

FGFR – fibroblast growth factor receptor

FX – vitamin K-dependent blood coagulation factor X

GEF – guanine nucleotide exchange factor

GFP – green fluorescent protein

HEK – human embryonic kidney cells

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER – human epidermal growth factor receptor family

HER2 – human epidermal growth factor receptor family 2 (ErbB2, neu)

HER3 – human epidermal growth factor receptor family 3 (ErbB3)

HER4 – human epidermal growth factor receptor family 4 (ErbB4)

HRG – heregulin

HSPG – heparin sulfate proteoglycans

hTERT – human telomerase reverse transcriptase

HVR – hexon hypervariable regions

ifu – infectious units

IGF1 – insulin-like growth factor 1

IGF1R – insulin-like growth factor 1 receptor

IHC – Immunohistochemisty

ITR – inverted terminal repeat

kb – kilobase

Kd – dissociation constant

kDa – kiloDalton

LRP – lipoprotein receptor-related protein

MEM – Minimum Essential Medium

MOI – multiplicity of infection

mTOR – mammalian target of rapamycin

NHS – N-hydroxysuccinimide

NLS – nuclear localization site

NPC – nuclear pore complex

NRG – neuregulin

OTC – ornithing transcarbamylase

PBS – phosphate buffered saline solution

PBS++ – phosphate buffered saline solution with additional calcium and magnesium

PE – phycoerythrin

PEx - Pseudomonas exotoxin

PFU – plaque forming units

PH – pleckstrin homology

PI3K – phosphatidylinositol-4,5-bisphosphate 3-kinase

PI3KCA – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PIP2 – phosphatidylinositol-4,5-bisphosphate

PIP3 – phosphatidylinositol(3,4,5)-triphosphate

PMSF – phenylmethylsulfonyl fluoride

PR – progesterone receptor

PSA – prostate specific antigen

PTEN – phosphatase and tensin homolog

pTP – adenoviral precursor terminal protein

RCA – replication competent adenovirus

RGD – arginine-glycine-aspartic acid tripeptide sequence

RLB – reporter lysis buffer

RLU – relative light units

s-knob – soluble wild-type Ad fiber knob

TFP – tetrafluorophenyl

TORC1 – target of rapamycin complex 1

TP – adenoviral terminal protein

ts 1 – temperature sensitive mutant of Ad2

TSC1/2 – tuberous sclerosis protein 1/2

vp – viral particles

CHAPTER 1: Introduction

1.1 Breast Cancer

1.1.1 Breast cancer background

Breast cancer is the most common cancer in Canadian women, and the second leading cause of cancer death among women (6). 20-30% of breast cancer patients develop metastatic disease, which remains difficult to treat (7).

Breast tumors have been classified in multiple ways, and some classification schemes, such as ER+ (estrogen receptor) or HER2+ (human epidermal growth factor receptor family 2 (ErbB2, neu)), can result in different treatment options (8). A recent review outlined six breast cancer subtypes, which differ based not only on ER/PR (progesterone receptor)/ HER2 status, but also on other important growth related genes and ultimate tumor prognosis (7). These subtypes are: basal-like, HER2-enriched, normal breast-like, luminal A, luminal B and claudin-low (7). Another recent article included an additional subtype, called molecular apocrine (9). The number and composition of the subtypes remains controversial. For example, the so called triple-negative breast cancers (ER-/PR-/HER2-) are further subdivided by Eroles *et. al.* primarily into the basal-like and claudin-low subtypes (7). However, though these two subtypes are comprised mostly of triple-negative breast cancers, some of the cancers in each of these groups would be positive for some of the receptors ER, PR and/or HER2. This results from the use of a large number of other genes to stratify the cancers, as opposed to simply ER, PR and HER2. Additionally, the other breast cancer subtypes also contain a small percentage of the triple-negative breast tumors (7). Thus, many changes to breast tumor subtype classification, as well as changes in cut-off points to determine gene

amplification or protein expression have made comparisons between different studies difficult (8). Furthermore, there are also some questions as to the existence of some of the groups, for example the normal breast-like group, as it may be due to contamination of normal breast tissue in samples (7; 10). As a result, when looking at breast cancer subtypes, it is essential to describe how the breast tumor samples were stratified; and which genes or proteins were used.

1.1.2 Breast cancer PI3K pathway overview

There are many signalling pathways which have been shown to be important in breast cancer. One such pathway is the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/ AKT (protein kinase B, PKB)/ mammalian target of rapamycin (mTOR) pathway (Figure 1.1, reviewed in (11)).

The PI3K pathway is implicated in cell growth and survival, and is one of the pathways activated by growth factors (7). Activation of growth factor receptors, such as the HER2/HER3 (ErbB2/ErbB3) heterodimer, results in tyrosine kinase activation of the receptor, phosphorylation of the intracellular domain and activation of signalling pathways, including that of PI3K (7). The PI3K regulatory subunit, p85, binds to phosphotyrosine motifs and releases inhibition of the p110 catalytic subunit (11). PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol(3,4,5)-triphosphate (PIP3), (12; 13) which recruits AKT to the plasma membrane (14-16). AKT activation results in multiple downstream events, including activation of the mTOR containing compound target of rapamycin complex 1 (TORC1) (17-19). There are also many inhibitors of this

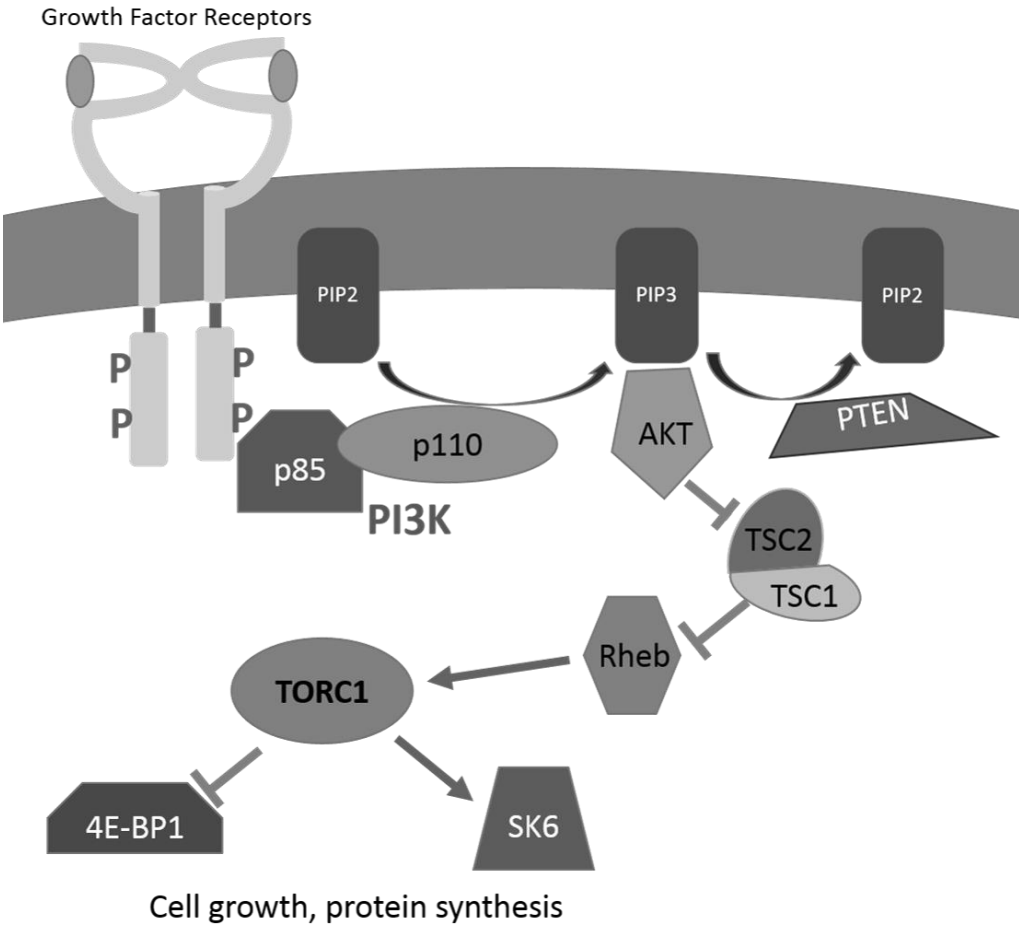


Figure 1.1: *PI3K-AKT signalling downstream of a growth factor receptor.* Phosphatidylinositol-3 kinase (PI3K) is activated downstream of growth factor receptor (such as the HER2/HER3 heterodimer). The regulatory subunit of PI3K (p85) binds to phosphotyrosines located on active receptors. The p110 catalytic subunit phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to PIP_3. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), opposes PI3K activity by dephosphorylating PIP_3 to PIP_2. Proteins with pleckstrin homology (PH) domains are recruited to the membrane to bind PIP_3, such as AKT and PDK1. AKT is phosphorylated by PDK1 and PDK2 to become fully active, at which point AKT moves from the membrane to the cytoplasm and nucleus to phosphorylate many downstream proteins. AKT has an effect on many pathways, including activation of the mTOR pathway, leading to cell growth and proliferation. (Modified from Meier *et al.* (2005)) (1)

pathway, including the well-known tumor suppressor phosphatase and tensin homolog (PTEN), a phosphatidylinositol 3' phosphatase (20).

Multiple proteins within the PI3K pathway can be mutated resulting in increased pathway activation. Activating mutations of PI3K (21-24), as well as loss of function mutations in the negative regulators PTEN and tuberous sclerosis protein 1 and 2 (TSC1/2) (7; 25) have been produced. Interestingly, in breast cancer, PI3K catalytic subunit α (PIK3CA) mutations are found in most of the subtypes described in Section 1.1.1 (26), however, the highest frequency of PIK3CA mutations is found in the rare subtype, metaplastic breast cancer (11). Other PI3K pathway changes tend to cluster more with specific subtypes, such as the loss of PTEN within the basal-like subtype (11).

There have been many attempts to inhibit this pathway, with only modest success (27; 28). Targeting this pathway can be difficult, since mTOR inhibition has been shown to increase PI3K activity through a negative feedback loop (7; 25).

1.1.3 Breast cancer treatments

As with many types of cancer therapy the risk of recurrence and death must be balanced with treatment-related adverse effects, in order to give the optimal treatment to the patient. The specific treatments depend on tumor staging and characteristics, and increasingly on gene expression profiles (29). Additionally, there are differences with treatment recommendations nationally or locally, and these are constantly changing (29; 30). However, in general, Canadian data for breast cancer treatments are similar to other countries or regions (31).

Most US women with breast cancer undergo surgery (mastectomy or breast-conserving surgery) (32). The last 30 years has seen a shift towards breast conservation treatment, however, mastectomy is still recommended for about one-third of European women (29). Other than surgery, treatments administered include radiation, chemotherapy and hormone therapy (32).

Chemotherapy can be either neoadjuvant (prior to the main therapy) or adjuvant (post-operative), but is not always required (30). Chemotherapy is given in many different regimens, depending on the tumor profile (29). Anthracyclines are recommended for most patients, while taxanes may also be of significant benefit (29). Trastuzumab (Herceptin), a monoclonal antibody targeted to HER2, is recommended for patients with HER2 overexpressing or amplified tumors (29).

Radiation therapy is often given prior to hormone therapy, after surgery and chemotherapy (29; 30). As with other treatments, radiation therapy can differ between patients, depending on tumor profiles (29).

Endocrine therapy is generally administered if at least 1-10% of tumor cells are positive for ER by immunohistochemistry (IHC), though this may vary by region (29; 30). The choice of agent for endocrine therapy depends on the diagnosis and the women's menopausal state (30).

Unfortunately, at this time metastatic breast cancer (stage IV) is considered an incurable cancer, but some of the treatments outlined above, in addition to new treatments being tested, can allow women with this disease to retain an acceptable quality of life (30).

However, since breast cancer remains the second leading cause of cancer deaths in Canadian women, there remains a need for novel treatments, especially in some breast tumor subtypes, such as basal-like, HER2-enriched and claudin-low (6; 7).

1.1.4 Breast cancer gene therapy

Gene therapy is the therapeutic transfer of nucleic acids into cells (33). In cancer, this can take the form of replacement of a defective gene, such as a tumor suppressor gene, activation of the immune system towards tumor cells, or specific killing of tumor cells, among other actions (34; 35). The most common target of gene therapy is cancer, accounting for about 64% of clinical trials (36). Many viruses are being examined for use as vectors for delivery of therapeutic genes; however the most common virus examined for use in gene therapy is adenovirus, accounting for almost one quarter of clinical trials (36). Viruses can be modified to selectively replicate in tumor cells. These oncolytic viruses can be further modified by arming with additional transgenes (37). Adenoviruses (Ads) have also been tested as oncolytic viruses in breast cancer (33; 37).

1.2 Adenovirus

1.2.1 Adenovirus background

Ads were first discovered associated with respiratory infections and isolated from adenoid tissues in the 1950s (38; 39). Ad infections can range from asymptomatic to lethal, but are usually benign in people with normal immunity (34; 40; 41).

There are over 60 human Ad serotypes, with a select few primarily studied for gene therapy use (41; 42). These viruses are divided into six subgroups (A to G), based on characteristics ranging from hemagglutination properties to DNA sequence homology (35; 41; 43; 44). Within the viral subgroups, Ads tend to cluster into tropism for similar cell types or cellular receptors, which mediate viral binding and internalization (41). Subgroup C adenoviruses, including adenovirus serotype 5 (Ad5), are the most extensively characterized, and the most well studied for use as gene therapy vectors (45). This document will focus primarily on Ad5, and any other Ad serotype will be specifically named. Ad2, another well studied subgroup C Ad, with 95% sequence similarity with Ad5, will also be frequently mentioned.

1.2.2 Adenovirus genome and capsid structure

Ad5 contains a double stranded DNA genome of approximately 36 kb, which encodes around 39 identified proteins, and possibly many more (40; 46). The early genes are required for transactivation of the other viral genes and for viral genome replication, while the late genes are primarily structural proteins (35; 45).

The Ad5 capsid is a non-enveloped, icosahedral structure, 90-100 nm in diameter, with pseudo T=25 symmetry (40; 47; 48). The capsid is formed primarily by three major proteins: the facets by trimers of hexon protein, the vertices by pentamers of penton base protein, and the fiber protein, which protrudes from the vertices (49-52). The knob domain of fiber and the penton base protein play roles in Ad binding and internalization, respectively (41). The capsid can be divided into 252 protein subunits, or capsomeres: 240 hexon trimers and 12 penton pentamers (53). The

capsid also contains five minor proteins (proteins VI, VIII, IX, IIIa and IVa2), which stabilize the capsid structure (34; 49-52; 54; 55). Additional encapsidated proteins are associated with the DNA genome: terminal protein (TP), protein VII, mu and protein V (34).

1.2.3 Adenovirus life cycle

Ad infection, or the Ad life cycle, is a process that usually begins with cell entry and culminates in production of new viral particles. It is often measured by gene expression, viral genome replication or production of infectious viruses. The Ad life cycle takes between 24-36 hours (hrs) to complete and can produce up to $1x10^4$ infectious virions per infected cell (Figure 1.2) (45). This life cycle contains many steps; a brief outline of which is described here, while viral binding and endocytosis will be examined in greater detail in later sections (Sections 1.2.5, 1.2.6, and 1.2.8). The binding of Ad to cells is thought to occur by a two-step process (41; 56). Primary Ad binding is to the coxsackie-adenovirus receptor (CAR) *via* fiber knob (57-63). This binding is thought to bring the virus into close proximity to the cell to allow for the secondary binding of penton to integrins ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) (57-63). Secondary binding generally initiates clathrin-mediated endocytosis (64-68).

Capsid disassembly begins soon after binding, and in some situations fiber was shown to be released from virion at cell surface, though fiber remains associated with the cell (69; 70). The virus escapes from the endosome, evading degradation by the lysosome (69; 71). The cellular PKC activation has been linked to endosomal

escape, and recent evidence has linked an interior capsid protein, pVI, to endosome lysis (70; 72; 73). This protein is released from the capsid during disassembly (72). The virus is transported by the microtubule network to the nuclear pore complex, with further capsid destruction en route (74-76). Binding of virus to different receptors may result in alternative intracellular pathways (77-79). Nevertheless, Ad was shown to accumulate perinuclearily about 60 minutes (min) after infection (80). The capsid is fully disassembled at the nuclear pore complex (NPC), and the protein complexed DNA genome enters the nucleus (69; 81-83).

Once inside the nucleus, early genes (E1, E2, E3 and E4) are expressed (34). The E1 gene products are required for transcription of the other early genes, and ultimately trigger genome replication (34; 45). About 8 hrs post infection, replication is initiated (35; 84). The viral DNA is synthesized by strand displacement in one continuous strand, using the viral precursor terminal protein (pTP) as a primer (35; 84; 85). Replication can be initiated at either viral inverted terminal repeat (ITR), and the displaced strand can also serve as template for replication by the formation of a panhandle structure (85-87).

Following DNA replication, late gene expression occurs. Proteins under the control of the major late promoter are primarily structural proteins, which self-assemble and package the genome into the nucleus (45; 85). The virus is released by cell lysis, and can then spread to other cells (35; 45).

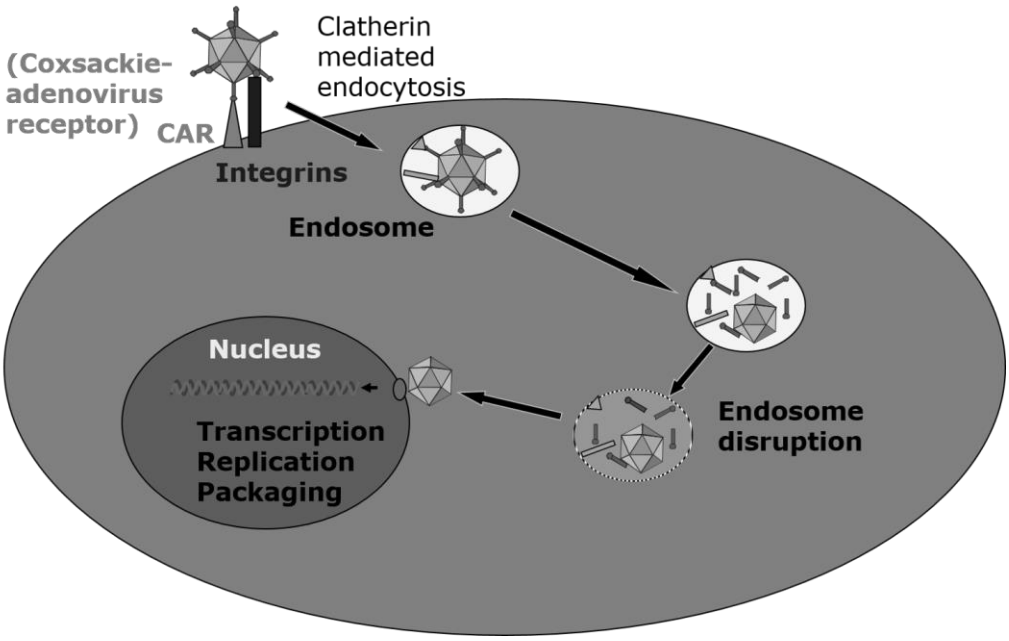


Figure 1.2: *Wild-type adenovirus life cycle.*

The virus first binds to its primary cell surface receptor, CAR, through the knob domain of the fiber protein. The second interaction of the virus occurs through viral penton base binding to integrins (primarily $\alpha_v\beta_3$ and $\alpha_v\beta_5$) on the cell surface, which triggers clathrin-mediated endocytosis of the virus. The capsid is partially disrupted and escapes to the cytosol by lysis of the endosome, where it is transported rapidly to the nuclear pore complex. The capsid undergoes further disassembly and releases the genome into the nucleus. Inside the nucleus, the viral genes are transcribed and viral DNA replication and assembly of new virus occur. (Adapted from Bilboa *et al*. (1998) (2))

1.2.4 Adenovirus as a cancer therapeutic

Ad is well studied as a gene therapy vector (36; 88). This virus has been investigated as an antitumor virus almost since its discovery. Unmodified Ad was used to treat cervical cancer in 1956, where treatment resulted in some tumor necrosis in more than half the patients between four and ten days after virus administration (34; 89; 90). However, in this case survival was not significantly extended, and all patients eventually succumbed to cancer (89; 90). Since that time, modified Ads have been used for cancer therapy, including first generation gene therapy vectors, helper-dependent gene therapy vectors and oncolytic Ads.

1.2.4.1 First generation adenovirus vectors

Various modifications have been made to the Ad genome to make it more useful as a gene therapy vector. First generation Ad vectors contain deletions in the E1 region, which render the viruses replication-defective (35; 91). These vectors must be replicated in E1 complementing cell lines such as human embryonic kidney (HEK)-293 cells (35; 92; 93). Furthermore, other viral genes can be deleted from the Ad genome in order to incorporate large transgenes, since Ad genomes larger than around 38kB in size are less efficiently packaged (92; 94). The E3 region is often deleted from first generation Ad vectors in order to increase the space for transgene insertion, since the E3 region is not required for viral replication *in vitro* (34; 35). Together, these deletions result in a cloning capacity of up to 8.3 kb (35).

Despite the fact that first generation Ads generally do not complete the entire viral life cycle in host cells, transgenes encoded by the viral genome can be expressed at high levels when controlled by strong promoters.

Many different transgenes have been inserted into the viral genome. For cancer gene therapy some of these include: tumor suppressor genes (*e.g.* p53), genes encoding prodrug activating enzymes (*e.g.* HSV-TK, to activate ganciclovir) or immunomodulatory genes to trigger immune reactions to the tumor (34; 95-98). In 2003, a p53 encoding Ad delivery vector, Gendicine, achieved regulatory approval in China (99; 100). This vector appears to be effective, with minimal side effects (99-101).

Despite the deletion of the E1 genes in first generation Ad vectors, there is some leaky expression of viral genes which can lead to the induction of an anti-viral immune response (35; 102; 103). In 1999, there was a death associated with an Ad clinical trial for a genetic defect (ornithine transcarbamylase deficiency, OTC) (104). The patient, Jesse Gelsinger, had been given a large dose of the virus into the liver blood supply, which resulted in a systemic immune response to the vector (104). This case highlighted the need to balance risks with potential clinical benefit, something that must be considered with any therapy, not just gene therapy. Increasing Ad targeting could help mitigate some of these risks (see Section 1.2.11).

1.2.4.2 Helper-dependent adenovirus vectors

In order to decrease the immunogenicity of Ad vectors and increase the cloning capacity, a helper-dependent or "gutless" Ad vector was created (5; 35; 105). This

virus contains the minimum viral sequences required in *cis* for replication and packaging of the viral genome, and thus requires a helper virus to provide proteins needed in *trans* for genome replication and capsid production (34; 35). These viruses have a coding capacity of up to 37 kb, though the viral genome must be stabilized with "stuffer" sequences if the inserted sequence is less than 27 kb (106).

1.2.4.3 Adenovirus as an oncolytic therapy

Oncolytic viruses replicate in tumor cells, resulting in cell lysis and death (107). Targeting these viruses for selective replication in tumor cells is expected to make a more effective tumor treatment (107; 108). Oncolytic Ads with deletions in E1B-55K have reached commercialization in China (Oncorine, H101), and phase III clinical trials in the US (Onyx015) (100; 101; 109). There was evidence of patient response to this virus, however some of the studies did not include many patients or long term survival data (101). Both H101 and Onyx015 are generally administered intratumorally (101).

These viruses (H101 and Onyx015) were originally thought to replicate selectively in tumor cells lacking p53, however later evidence has questioned this (34; 109). Certain tumor cells expressing wild-type p53 were permissive to Onyx015 replication, while other p53 negative tumor cell lines required E1B-55K in order to replicate Onyx015 (110; 111). It appears that Onyx015 tumor selectivity is actually related to another E1B-55K function, late Ad mRNA export (112; 113). Deletions of both E1B-55K and E1B-19K have also been studied (114; 115). Other manipulations to target oncolytic Ads include deletion of virus-associated RNAs

(VA-RNAs) and deletions of part of the E1A binding site for Rb (Delta-24) (116; 117).

1.2.4.4 Transcriptional targeting of adenovirus

Ads can also be rendered oncolytic by placing the E1A gene under the control of a tumor specific promoter (98). For example, the human telomerase reverse transcriptase (hTERT) or the prostate specific antigen (PSA) promoters (98; 118; 119). Our lab is examining the utility of the mammaglobin promoter as a breast cancer selective promoter (120). This promoter controls the expression of mammaglobin, a protein of unknown function discovered by an increase of RNA levels in breast cancer biopsies relative to normal breast tissue (121).

Transcriptional targeting is also useful for targeting the expression of a transgene encoded by first-generation or helper-dependent Ads (108). This targeting should aid in limiting transgene expression in non-tumor cells, thus should decrease the risk of toxicity resulting from this therapy.

1.2.4.5 Advantages of adenovirus as a gene therapy vector

There are many advantages to the use of Ad as a gene therapy vector, compared to other gene therapy vectors. These include: infection of quiescent cells, infection of many cell types (broad tropism) and lack of integration into the host genome (40; 122-126). More important for a gene therapy vector targeting cancer are: the relative ease of manipulation compared to other viruses, the ability to produce high

titre stocks, and the accommodation of relatively large inserts (up to 8kb in a first generation virus) (40; 122-124).

1.2.4.6 Disadvantages of adenovirus as a gene therapy vector

Unfortunately, there are also disadvantages to using Ad for gene therapy, including pre-existing immunity to Ad5, and high immune response to the Ad vector itself (discussed in Section 1.2.10.1) (33). A virus-induced inflammatory response is provoked by tissue macrophages and activated dendritic cells, and this may lead to elimination of Ad and reduced antitumor efficacy (33). This effect is inherent in the virus, as the innate and adaptive immune responses combine to clear the wild-type Ad (33; 127). Furthermore, Ad has distinct liver tropism, which can result in side effects if the liver is not the target organ, such as in breast cancer therapy (discussed in detail in Section 1.2.10.3) (128).

Ubiquitous expression of the primary and secondary Ad receptors can also pose a problem (64; 129; 130). CAR and integrins are expressed on many cell types (see Sections 1.2.5.1 and 1.2.6.1); allowing infection of Ad in many cell types (122-124). Furthermore, lack of CAR expression on tumor cells can decrease Ad infectivity in these tumor cells, decreasing effectiveness of Ad as a tumor therapy (88; 101; 131-136). This can be circumvented by retargeting Ad to receptors overexpressed on tumor cells.

In order to fully understand the requirements for Ad retargeting, first wild-type Ad binding and receptor mediated internalization must be examined in detail.

1.2.5 Adenovirus primary receptor

Ad utilizes a two-step process for infection: first binding to the primary receptor, CAR, to concentrate at the cell surface (41; 56). Then the binding to the secondary receptor, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin, is thought to be required for viral internalization (41; 56).

1.2.5.1 Coxsackie-adenovirus receptor (CAR)

CAR is the primary receptor for Ad subgroup C, along with several other Ads, and the coxsackie B virus (40; 41; 57; 60). This receptor is important for viral attachment (57; 60), and its presence has been shown to be necessary for infection (137; 138). CAR is a 46 kilodalton (kDa) type I transmembrane protein of immunoglobulin superfamily that is normally found in the tight junction (42; 57; 137; 139-141). The normal function of CAR is in binding to CAR on another cell, resulting in cell-cell adherence (141; 142). CAR appears to be important in development, and overexpression of murine CAR in mice can lead to cardiomyopathy through MAPK pathway activation (41; 143; 144). Interestingly, CAR activation has also been shown to suppress tumor cell proliferation (145).

The CAR protein is expressed in many tissues including brain, liver, heart, lungs, kidneys, and the mRNA is also present in many different cell types (41; 60; 126; 130).

1.2.5.2 Fiber structure and CAR binding sites

Ad5 fiber is a homotrimeric protein of 186 kDa which contains three domains: tail, shaft and knob (42; 146-149). The N-terminal tail length is 44 amino acids (aa), and this domain acts to anchor the fiber protein to penton on the vertices of the virus (146-149). The 355 aa β-spiral fiber shaft joins the tail to the knob domain (146-149). The shaft length varies with Ad serotype, and this may influence the ability of the fiber knob to bind CAR (150-152). The C-terminal, globular knob is essential for fiber trimerization, and necessary and sufficient for CAR binding (58; 146-149; 153). The fiber knob has a three-bladed propeller structure, with each blade formed by two antiparallel β-sheets connected by flexible loops (Figure 1.3) (58; 150; 153). CAR is known to bind on the lateral surface of the knob, between adjacent monomers, with a very high affinity (dissociation constant, Kd = 1.7 nM) (Figure 1.3) (4; 64; 140; 154; 155). The AB loop of the fiber knob has the most interaction with CAR, with the DG loop also contributing to CAR binding (4; 150). Interestingly, CAR binding to Ad fiber is stronger than CAR to itself (149; 156). The fiber knob binds to CAR with potentially three receptors per knob (4; 154; 155), however both the existence and the necessity of clustering of CAR is unclear (42). The activation of CAR does not appear to be required for viral internalization, since receptors lacking the C-terminal tail and transmembrane domain still function in viral internalization (157-159). Though a single extracellular CAR domain, the D1 domain, appears to be sufficient for binding to knob, the other receptor domains are likely required for cellular function (156; 157). Activation of CAR can play a

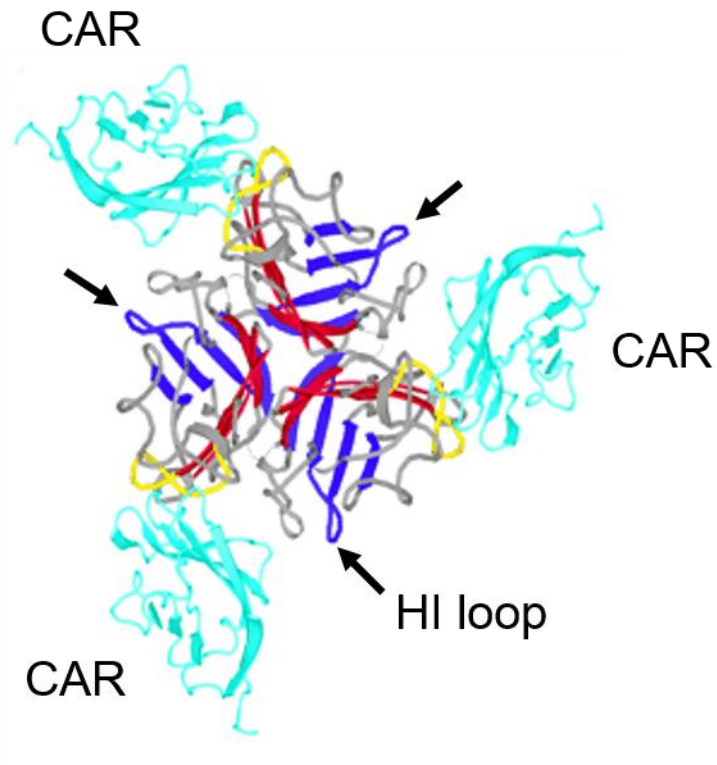


Figure 1.3: *Fiber knob trimer complexed with CAR.*

Three CAR receptors are shown in aqua. The trimeric knob portion of Ad12 (subgroup A) is shown at the center, in grey, red, blue and yellow. The CAR binding site (AB loop) is in yellow, and each CAR receptor can be seen interacting with one knob trimer. The HI loop is found on the exposed surface of each molecule of the trimer (indicated by arrows, in blue), at a distance from the CAR binding site. The HI loop is one of the common sites used for retargeting the adenoviral capsid. (Modified from Bewley et al. (1999) (4))

role in viral infection, however, as viral activation of CAR can lead to an inflammatory response in human respiratory cells (159; 160).

Fiber has other effects on infection, in addition to CAR binding. Viral escape from the endosome may be dependent on release of fiber, based on information gained from a temperature sensitive Ad2 mutant, *ts 1* (161; 162), though recent evidence indicates that endosomal escape is related to the release of pVI from the interior of the capsid (72). Furthermore, fiber can also affect intracellular trafficking; Ad5 containing Ad35 (subgroup B2) fibers are not released from the endosome in the same manner as wild-type Ad5 (163). The CAR binding of free Ad fiber may also play a role in viral spread, *via* disruption of CAR-dependent intracellular junctions (41; 137). Though CAR has been shown to be important for Ad infection in many cell lines, it is not essential. Subgroup C Ads can bind and enter cells using only the secondary receptors, however this is not necessarily as efficient (164; 165).

1.2.6 Adenovirus secondary receptor

1.2.6.1 Integrins ($\alpha_V\beta_3$ or $\alpha_V\beta_5$)

The secondary receptor for Ad5 is $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin (64-67). These receptors are members of large family of heterodimeric adhesion molecules expressed on the surface of many cell types (41; 60; 126). They normally mediate cellular adhesion to extracellular matrix, and regulate many important biological processes, including proliferation and apoptosis (166; 167).

1.2.6.2 Penton structure and integrin binding sites

In Ad5, and all other Ads except Ads 40 and 41 (subgroup F), penton binds to integrins by an RGD (arg-gly-asp) motif, which is likely located at the ends of flexible loops (41; 64; 168-172). Though this RGD location may help keep the motif away from the fiber shaft, there may still be steric hindrance from the nearby fiber protein (172; 173). Steric hindrance has been shown to limit integrin binding to Ad12 (subgroup A), though the RGD loop of Ad12 is known to be less flexible than that of Ad2 (174). The interaction between penton and integrins (Kd = 55 nM) is of lower affinity than that of fiber-CAR (175). Up to 5 receptors can bind to each homopentamer, thus binding of Ad causes clustering and activation of integrins, which signals cytoskeletal changes important for viral internalization (175-178). Integrin binding may also lead to destabilization of penton, affecting the release of fiber from the capsid and further capsid destabilization (174).

Binding to and activation of integrins are required for clathrin-mediated endocytosis of the virus in most cell lines (62; 64). The activation of the receptor activates PI3K, and other downstream proteins, and this pathway activation is also required for viral internalization, as PI3K inhibitors can inhibit Ad gene delivery (63; 179). PI3K activity was previously shown to be triggered by penton and not fiber (62; 165). Interestingly, there is evidence that binding to $\alpha_v\beta_5$ integrin, as opposed to $\alpha_v\beta_3$ integrin, may also play a role in viral endosomal escape (65; 180). However, a recent paper demonstrated that increased expression of $\alpha_V\beta_3$ integrin resulted in increased Ad transduction (181). Thus, the precise role of specific integrin dimers in Ad internalization in all cell lines is not fully established.

It has been shown that viruses with mutated integrin binding sites can enter cells, though this appears to be delayed (66; 163). The mechanism of this entry is unclear, however it may be related to multiple CAR recruitment (41; 182). It has been shown that Ad internalization into some cells occurred through other mechanisms, such as factor X mediated liver internalization (183), further discussed in Section 1.2.10.3. However, these additional entry mechanisms likely do not play a major role in internalization of unmodified Ad into most tumor cells.

Macropinocytosis, another internalization mechanism, is also activated by penton binding to integrin, among other activation mechanisms (184). The role of macropinocytosis in viral internalization, though linked, is not clear (further discussed in Section 1.2.8.3).

1.2.7 Non-canonical adenovirus binding and infection

Natural infection at the apical side of polarized cells may require other factors, such as those released from activated macrophages, including the chemokine CXCL-8 (185). This allows the localization of CAR and $\alpha_v\beta_3$ integrin on the apical surface, and subsequent adenoviral entry (185).

Subgroup C adenovirus also binds to alternate receptors that can mediate infection in some cell lines. Ad5 knob interacts with MHC I, but the region of interaction does not appear to overlap with the CAR binding site, and this receptor alone is not sufficient to mediate infection after overexpression in hamster cells (186; 187). VCAM-1 (41; 188-190) and lactoferrin (191-193) can also enhance Ad infection in cells expressing these receptors. Furthermore, binding to blood components also

enhances scavenging by macrophages, including Kupffer cells, the resident macrophages in the liver (reviewed in (193)). The KKTK sequence in the fiber shaft is also important, as mutation will modify Ad tropism (41; 194; 195). It was thought this motif interacts with heparin sulfate proteoglycans (HSPG), and mutation of this site can reduce virus delivery to the liver (41; 194-196). More recently, it was shown that hexon binding to Factor X is important in Ad internalization into hepatocytes (183). This will be discussed in detail in Section 1.2.10.3, adenovirus liver tropism.

1.2.8 Internalization of adenovirus

Adenovirus enters cells primarily by binding to integrin, which results in clathrin-mediated endocytosis, however other internalization mechanisms have been seen in specific situations. Other internalization mechanisms may also play a role in internalization of retargeted adenoviruses, depending on the receptor targeted.

1.2.8.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is a type of receptor-mediated endocytosis (197). Activation of receptors, such as integrins, allows the recruitment of clathrin to the plasma membrane through clathrin-dependent accessory proteins (such as Eps15) or adaptor proteins (such as AP-2) (56; 197). A clathrin-rich region, called a coat, is assembled at the membrane prior to induction of membrane curvature (197). Further downstream endocytic events are likely not clathrin-specific, as the proteins important in these events (*e.g.* dynamin) are also important in other clathrin-

independent endocytic mechanisms (197). Dynamin regulates the constriction and budding of clathrin-coated pits, and thus is important in fission of newly formed coated pits from the plasma membrane (198; 199). PI3K can activate rac and cdc42 to induce actin polymerization (63; 165), which can play a role in clathrin-mediated endocytosis in some situations, including viral internalization (197; 200). Prior to fusion with a lysosome for degradation or recycling of the receptor to the cell surface, the endosome fuses with a sorting endosome (201; 202). This process is regulated by Rab5 and EEA1 (201; 202).

Inhibition of clathrin-mediated endocytosis can be achieved by dominant negative proteins, such as K44A-dynamin and DN-Esp15 (203-205). Potassium depletion, AP-2 inhibition and chlorpromazine (CPZ) treatments are other methods of inhibition (206-208). Both CPZ and potassium depletion are thought to prevent or remove the clathrin lattices from the cell membrane (207; 209; 210). CPZ additionally appears to assemble clathrin and AP-2 complexes on the endosomal membranes (207; 210). Unfortunately, these inhibitors do not always inhibit clathrin-mediated endocytosis specifically, since many endocytic mechanisms share many characteristics (see review (208)). For example, dynamin has been shown to play a role in non-clathrin internalization, including caveolin-mediated internalization and some types of macropinocytosis (184; 211). Furthermore, most clathrin-mediated internalization inhibitors also inhibit internalization of fluid phase markers (macropinocytosis), making it difficult to distinguish these two internalization mechanisms (208). However, since macropinocytosis appears to be

unimportant in Ad2 infection, being able to distinguish these two internalization mechanisms may be less important in this thesis (203).

1.2.8.2 Internalization of adenovirus by clathrin-mediate endocytosis

Ad5 has been shown to enter cells primarily by clathrin-dependent endocytosis (56; 68; 212). Early electron microscopy studies showed Ad2 and Ad5 within clathrin-coated pits (213-215). Others have shown the entry of Ad2 and Ad5 to be through coated or uncoated vesicles (56; 69; 216-218).

Inhibition of proteins important in clathrin-mediated internalization has been shown to inhibit adenoviral internalization or infection. Fluorescently- or radioactively-labelled Ad internalization was inhibited by dominant negative proteins: DN-Esp15, clathrin fragments, K44A-dynamin, and DN-Rab5 (reviewed in (56; 165)) (203; 217-219). Many of these dominant negative proteins also inhibit infection, as measured by transgene expression (56; 165; 203; 217-219). Clathrin and Esp15 inhibition are specific to clathrin-mediated endocytosis, while dynamin and Rab5 are downstream proteins important in both clathrin-dependent and some types of clathrin-independent endocytosis.

Disruption of the actin cytoskeleton by cytochalasin D inhibits Ad entry and infection (165; 214). Dominant negative versions of the actin-polymerization-inducing GTPases rac and cdc42 also decrease Ad entry (63; 165). Ad binding results in other cytoskeletal rearrangements, including filopodial extensions, lamellipodia formation and membrane ruffling (63). This has been attributed to penton binding to integrins, however fiber interactions may also be important since

there is an increase in filopodia with the addition of soluble fiber protein to cells (42).

Signalling is also important in Ad entry, especially by the PI3K pathway (63; 165). PI3K activation was shown to be important in virus internalization, but not attachment (62; 165). The adaptor molecule, p130CAS, linking src and PI3K, is also important for Ad entry (165; 220).

Ad binding may also activate pathways not required for internalization. For example, p125FAK can act upstream of both PI3K and MAPK, and is activated upon Ad entry. However, dominant negative forms of FAK do not inhibit Ad internalization (62; 165), suggesting that the virus has alternate mechanisms for activation of the PI3K pathway. Interestingly, although the MAPK pathway is activated during infection, it does not appear to be important for internalization (62; 221).

Growth factor receptor activation could circumvent the need for integrin binding, by activating PI3K to mediate Ad internalization (165; 179).

1.2.8.3 Adenovirus and macropinocytosis

Macropinocytosis is the non-receptor mediated internalization of fluid and other molecules found on or near the surface of the plasma membrane. This process is activated by integrin binding or EGF stimulation, among other signalling pathways (56; 222; 223). The PI3K pathway has been shown to be important in multiple stages of macropinocytosis (184). Membrane ruffling is triggered by PI3K, and

when the ruffles collapse they form uncoated vesicles called macropinosomes (56; 224).

Ad2 was shown to activate macropinocytosis in many different cell lines (203). Similar to clathrin-mediated Ad internalization, α_v integrins and Rho GTPase activity were shown to be required for Ad-dependent macropinocytosis formation (63; 64; 203). However, macropinocytosis was shown to occur even with inhibition of virus internalization by K44A dynamin 1, a dominant negative inhibitor of clathrin-mediated internalization (42; 203; 217-219). Furthermore, an amiloride derivative has been shown to inhibit macropinocytosis without inhibiting all Ad internalization (203). Although, the amiloride derivative does inhibit Ad endosomal escape (203).

Since macropinocytosis can occur under conditions which inhibit Ad infection (K44A dynamin), this process is not thought to be important in Ad2 or Ad5 internalization (203). However, macropinocytosis has been implicated as an important internalization mechanism for Ad3 (subgroup B1) and Ad35 (subgroup B2) (225; 226). In conclusion, though macropinocytosis occurs frequently with Ad infection, there are other entry mechanisms that appear to play more important roles in subgroup C entry, such as clathrin-mediated endocytosis.

1.2.8.4 Internalization of adenovirus by other mechanisms

Though most Ad internalization is thought to be clathrin-mediated, mechanisms such as caveolar internalization may play a role in specific situations. Caveolae are "flask-shaped invaginations of the plasma membrane" which contain caveolin-1

(56; 227). Formation of caveolae requires a stimulus (such as ligand binding), actin, Rho GTPase, and dynamin (228-230). After formation, caveolae can fuse with endosomes or go to other cellular locations, such as the endoplasmic reticulum (231; 232). Ad internalization is linked with caveolin-mediated internalization in lymphocytes (212). Furthermore, the addition of bovine Ad4 knob appears to change the internalization of Ad2 to caveolin-mediated in CHO cell lines (68). One important line of evidence linking internalization of these Ads to caveolin is the inhibition of caveolae formation by dominant negative caveolin-1 or knockdown of caveolin-1, and subsequent decrease in gene expression (68; 212; 233; 234). This and other mechanisms of Ad internalization have not been fully examined in many cell lines.

1.2.9 Detection of adenovirus binding and internalization

Adenoviral infection is often measured by transgene expression or viral replication, which is the ultimate goal of viral delivery and therapy. However, these measurements do not address the actual internalization mechanism, and can only detect those infections which result in the measured downstream outcome (*e.g.* gene expression). Results of measurements of radioactive particle internalization were not equivalent to those of reporter gene expression (green fluorescent protein, GFP) in a study of an FGF2-retargeted Ad (235). More recent internalization assays have used fluorescence to detect viral particles, either through genetically modified capsid proteins (*e.g.* pIX-GFP), or covalent labeling of the Ad capsid with a fluorescent molecule (68; 236). However, there has been difficulty in distinguishing

the real signal of a low number of infectious particles from "biological noise" of a high number of non-infectious particles (237). The effect of these non-infectious particles on infectious viral gene expression is unclear. It is important to investigate virus binding and internalization *in vitro*, to be able to more easily understand the more complex environment of *in vivo* viral vector administration.

1.2.10 In vivo delivery of adenovirus

The goal of any gene therapy vector is generally delivery of the transgene to the proper cells within a patient. Two challenges that are important to consider for Ad therapy *in vivo* are Ad immunogenicity and Ad liver tropism.

1.2.10.1 Adenovirus immunogenicity

As previously mentioned one difficulty with *in vivo* delivery of Ad vectors is pre-existing immunity to Ad5. It has been estimated that 40-80% of people have neutralizing antibodies to Ad5, depending on the population studied (129). Additional mechanisms cause rapid clearance of Ad from blood (238). Some of these include innate immune responses by macrophages or dendritic cells and the activation of complement proteins in the blood (239-243). Other mechanisms include adaptive immune responses, such as neutralizing antibodies in pre-exposed individuals, and uptake of the virus and infection of other body organs, especially the liver (239; 244).

1.2.10.2 Modification of adenovirus immunogenicity

One method of preventing or decreasing Ad immunogenicity is by genetic modification, such as mutations in hexon or swapping hexon from different serotypes (245), as this is the most immunogenic Ad capsid protein (246). Another method is using various types of shielding for the Ad particle (247-250), including polyethylene glycol (PEG) (251) or other polymer coats (239). However there is some evidence that shielding is not enough to prevent Ad immunogenicity (248; 252). Another method to reduce neutralization by antibodies is to inject the virus directly into the target tissue, such as into the tumor for cancer gene therapy. Though this strategy should increase the amount of virus at the primary site, this does not aid in targeting metastatic tumors.

1.2.10.3 Adenovirus liver tropism

Another important challenge to Ad use *in vivo* is its innate liver tropism. Ad has been shown to be taken up by the liver, including hepatocytes and Kupffer cells (liver macrophages) (193; 238; 253-255).

Internalization into the liver was recently shown to be mediated primarily by vitamin K-dependent blood coagulation factors through binding to lipoprotein receptor-related protein (LRP) and heparin sulfate proteoglycans (HSPGs) (163; 256). Previous studies demonstrated that neither CAR nor integrin appear to be important for entry into hepatocytes (194; 195; 257; 258). The most efficient vitamin K-dependent blood coagulation factor for Ad binding appears to be factor X (FX) (256). Ad5 was shown to bind to FX (Kd = 229 pM) with a 40-fold stronger

affinity than to CAR (183). The Ad protein which binds to FX is hexon, and this binding is reduced or eliminated when the hypervariable region of hexon is modified (183). Hexon modification of Ad may affect the route of intracellular transport, even though it doesn't seem to affect attachment or internalization, except with regards to FX mediated internalization (183). HSPGs also interact with Ad through factor IX, FX or complement binding protein-4 (163; 259).

In contrast to the hexon studies, a more recent study has implicated fiber in a more prominent role in FX mediated internalization (260). Ad35 liver transduction is four orders of magnitude less efficient than Ad5, and modified Ad5 vectors containing fibers from other serotypes often show less hepatotropism, despite the presence of Ad5 hexon (260-264). In a study using an Ad5 virus with an Ad35 fiber (Ad5/35), FX was shown to inhibit transduction (determined by GFP expression) by inhibiting intracellular trafficking of the virus (260). In addition, this study confirmed the interaction of FX with Ad5 hexon and the enhancement of Ad5 (not Ad5/35) infection of HSPG-expressing cells (260). Additionally, the failure of transduction in this study may be linked to the Ad35 fiber in Ad5/35, since the addition of Ad35 fiber to Ad5 has previously been shown to affect Ad5 intracellular transport without FX (163).

1.2.10.4 Decreasing adenovirus liver tropism

Since Ad binding to blood coagulation factors appears to be mediated by hexon hypervariable regions (HVR), mutating these regions may prevent binding to FX (183). Decreased liver internalization has been demonstrated following i.v.

injection of mice with a virus containing a large insertion in HVR5 (183). Another group has shown decreased liver tropism using vectors encoding different peptide modifications of hexon HVR (265). Recently, specific point mutations in Ad5 hexon hypervariable regions were shown to decrease hepatocyte transduction *in vivo* (266). Additionally, X-bp, a snake (*Deinagkistrodon Acutus*) protein that binds human and murine FX with high affinity, has also been shown to block FX mediated Ad liver transduction in mice (259).

1.2.11 Adenovirus targeting

CAR expression is low in many tumors and cancer cell lines, which leads to decreased infection by Ad (88; 131-136; 193). Furthermore, decreasing CAR expression has been associated with increasing tumorigenicity and malignancy in an LNCaP prostate cancer progression model (267; 268). Additionally, low Ad infection in the more progressed cell line can be increased by the restoration of CAR levels (267).

Thus, use of Ad for gene therapy could benefit from retargeting of the virus to the target cells, such as cancer cells. Transcriptional targeting can be used to restrict gene expression (see Section 1.2.4.4), but modification of the virus capsid is likely essential for gene transfer to low CAR expressing cells. This can be achieved by genetic retargeting of the virus, swapping fibers with other Ad serotypes, or various strategies to conjugate the virus with a targeting molecule. Additionally, there is a need to detarget the virus to prevent internalization in the liver, as well as other normal cells expressing the wild-type virus receptors CAR and integrins.

1.2.11.1 Adenovirus detargeting

Prevention of Ad binding to CAR and integrin has been proposed to be important for retargeting Ad (269; 270). A substantial effort has been made to knock out wild-type Ad binding by multiple mechanisms (271). Genetic mechanisms of ablation have been used, including ablation of CAR-knob interactions (154). The Mizuguchi lab is one of several to show successful liver tropism reduction by modification of CAR and integrin binding (272-274). In contrast, others have shown that penton modifications that alter integrin binding have little effect on liver transduction (194; 275). This is consistent with the more recent studies showing the importance of hexon binding to blood factors in liver tropism (183). Thus, as previously mentioned, detargeting Ad liver tropism may require interfering with hexon interactions with blood factors (see Section 1.2.10.4).

Bispecific molecules targeting the virus to other receptors often inhibit CAR binding, at least partially, by preventing knob interactions with CAR, and can also be considered as a detargeting strategy (193) (see Section 1.2.11.4).

1.2.11.2 Genetic retargeting of adenovirus fiber

Genetic modifications in multiple locations of several Ad proteins have been examined for retargeting utility, including fiber, hexon and pIX (276-285). Incompatibility of insertions in the viral proteins can be a limiting factor in targeting ligand incorporation, including disruption of viral protein structural integrity and reduction in ligand targeting capacity (193).

Of the viral sites chosen for genetic modification, fiber knob is the most common (286). One reason for this is that fiber knob is located relatively far from the surface of the viral capsid, and thus has the potential to be free of at least some steric hindrance which may affect insertions closer to the capsid surface. Steric hindrance has been reported with the RGD binding domains in Ad12, and this appears to prevent all five RGD motifs in a penton base from binding integrin at the same time (174).

Within the fiber knob there are multiple options for insertion of a peptide for retargeting. A popular insertion site is the C-terminal domain of fiber knob. This location has been shown to allow relatively efficient physical interaction with receptors (284; 287; 288). Successfully incorporated targeting motifs include RGD motifs and heparin-binding polylysine motif (K7 or K20) (193; 284; 287; 289; 290). However, thus far, only ligands up to 30 aa in size have been inserted, other than the non-structural biotin acceptor protein (BAP) (284; 287; 288).

Other targets for insertion include fiber knob loops which link two antiparallel β-sheets (150). Many of these loops occur on the surface of fiber knob, and thus likely have the ability to incorporate foreign ligands with less structural limitations than other sites (150). Lord *et. al.* (2006) tested the effectiveness of the insertion of an RGD containing peptide (9 aa) into the loops between β-sheets C and D, H and I, and I and J (291). They demonstrated that the most effective insertion point for binding $\alpha_v\beta_3$ integrin was the HI loop, followed by CD, and lastly IJ (291).

The flexible HI loop, connecting the H and I β-strands, is particularly attractive for modification, since this loop is oriented away from the viral particle, is not required

for CAR binding and does not contribute to trimer assembly (Figure 1.3) (148; 150; 276; 277; 292). The HI loop has been a focus for insertion of small peptide ligands, including FLAG, RGD, polylysine, and transferrin receptor binding peptides (276-278; 286; 291-294). Furthermore, the insertions of RGD or polylysine have been shown to be effective for tumor transduction *in vivo* (295-297). HER2, a receptor overexpressed in many breast cancer cells, has also been targeted using an affibody (small protein affinity ligand) inserted into the HI loop of a CAR-ablated fiber (193; 298). Thus, the HI loop of fiber knob remains an attractive target for ligand insertion.

1.2.11.3 Genetic retargeting of other adenovirus proteins

Other Ad capsid targets, such as hexon and pIX, are located at or relatively near the surface of the capsid, and binding of modified capsid surface proteins to alternate receptors may be sterically inhibited by the presence of fiber (276-285; 299). Despite this, Ads have been successfully targeted by fusing the C-terminus of pIX to many ligands, including polylysine and a single-domain antibody (llama heavy chain only) against CD66c (280; 300). The insertion of the latter, a relatively large molecule, successfully enhanced vector targeting to CD66c (300). Interestingly, the insertion of the biotin acceptor protein (BAP) into the C-terminus of pIX did not allow binding to biotinylated ligands (301). However, other large molecules, such as GFP and enhanced green fluorescent protein (eGFP), have been genetically inserted successfully into the C-terminus of pIX, to allow for visualization of the

virus (236; 302). It appears that the type of ligand inserted may greatly affect the presentation of the ligand on the viral surface (193).

Despite its location on the capsid surface, hexon is attractive as a targeting protein, since this is the most abundant protein on the viral surface (193). Insertion of an RGD containing peptide has been shown to increase gene transfer to aeortic smooth muscle cells, which are not efficiently infected with unmodified virus (283). However, another study modifying hexon with a different RGD peptide into showed no gene expression in different cell lines (286). The hexon variable regions can incorporate relatively large ligands, including the 71aa BAP protein (193; 303). As with pIX, ligand choice may be important in successful incorporation into hexon (193). Due to other roles for hexon in infection, insertion of a targeting ligand into hexon may also decrease liver transduction and immunogenicity of the virus which could be beneficial for some gene therapy application (183; 193; 246).

1.2.11.4 Other adenovirus targeting

There are also non-genetic mechanisms that have been examined to retarget Ad. Adapter molecules linking the virion, generally fiber knob, to the alternate receptor have been used to target Ad (304; 305). They generally have the advantage of decreasing Ad binding to CAR (193; 306).

An early adapter molecule used fibroblast growth factor 2 (FGF2, basic FGF) bound to a neutralizing Ad antibody (307; 308). This was shown to redirect viral tropism from CAR to fibroblast growth factor receptor (FGFR) (306), increasing gene expression after infection with the FGFR targeted virus compared to non-

targeted viruses (306; 309; 310). A later study showed that FGF2 targeting is required in *cis* (bound to vector) to increase gene expression (235).

Ad has been targeted to EGFR by the use of an engineered binding site on the Ad capsid and a corresponding one on epidermal growth factor (EGF) to target the virus to EGFR (269). In another study, Ad was targeted to HER2 by the fusion of a trimeric HER2 antibody to the CAR ectodomain (311). This bispecific molecule decreased virus-encoded luciferase expression in HEK-293 cells, allowed binding to HER2 expressing cells, and showed increased luciferase expression in most HER2-positive cells with the exception of the ovarian tumor cell line SKOV-3 (311). Thus, in some cases factors in addition to receptor binding, such as virus internalization or entry into the nucleus, may affect expression of the reporter gene. Another important consideration is that, as with many of the bispecific-molecule-targeted Ad vectors, this vector likely retained integrin binding (311). As a result, these vectors could potentially utilize wild-type adenoviral internalization mechanisms for cell entry.

However, this is not the case for all bispecific retargeting mechanisms. Melanoma cells lacking α_v integrins have been effectively targeted by EGFR and IGF-1R ligands fused to anti-penton antibody (62; 179). The length of the bispecific molecule may influence bridging between penton and the target receptor. Additionally, internalization of such viruses is likely independent of the wild-type mechanism of penton binding to integrin, as α_v integrin is not expressed on the target cell, and penton itself is likely bound by the bispecific molecule.

The use of bispecific molecules has been somewhat limited due to potential instability (279; 305; 311; 312). Thus, this method may not be as effective at altering virus tropism, or at eliminating CAR binding as genetic modifications are. Ads have also been successfully targeted to EGFR after shielding the vector with poly hydroxypropylmethacrylamide (pHPMA) polymers to block neutralizing antibodies, with either EGF or cetuximab (anti-EGFR antibody) covalently linked to the polymer (313; 314). Cetuximab targeting demonstrated that EGFR-targeted internalization does not require activation of the receptor, since this antibody does not activate EGFR. This strategy does still require an exogenous moiety added to the virus prior to administration, and therefore is potentially unstable, as are bispecific molecules bound to Ad.

Other targeting strategies, including swapping of Ad5 fiber with fibers from other Ad serotypes, primarily subgroup B fibers, have been shown to result in transduction of cells expressing the cognate receptor for the new fiber (reviewed in (193; 315)). Fibreless Ads have also been modified by fusing a targeting moiety to exogenous trimerization domains, for example that derived from the Moloney murine leukemia virus, which replaces fiber in the capsid structure (316). This platform has been used to add peptides, affibodies, or other targeting ligands (*e.g.* RGD), to the vector (317). An affibody fused to fibrin has been used successfully to target a knobless Ad to HER2 (318). Unfortunately, the complete deletion of fiber, or other extensive capsid modifications can result in lower virus production due to problems in capsid assembly (193). Thus, there are advantages to

maintaining the virus structure as much as possible, to retain effective encapsidation, virion assembly, and virion maturation.

1.2.11.5 Internalization of retargeted adenoviral vectors

Redirection through non-native targets could have unanticipated intracellular effects (235), and this may affect viral infectivity at different stages from internalization to transgene expression. Retargeting Ad to FGFR was shown to influence virus binding and trafficking, detected by changes in transgene activity or accumulation of radioactively labelled virus (235). However, other factors affecting other life cycle stages were also likely involved because the changes in gene expression could not be entirely accounted for by viral internalization (235). The same paper also demonstrated second messenger signalling after virus binding, but this signalling was not mediated by FGFR activation (235). Tumor targeting using PEG shielding and a peptide ligand was also recently shown to modify the endocytic profile of Ad (319). Significant inhibition of reporter gene activity was shown with a macropinocytosis inhibitor, as well as inhibitors of clathrin-mediated endocytosis and lipid rafts, although the latter two also inhibited unmodified Ad.

1.2.11.6 Targets for adenovirus cancer gene therapy

In many cases, the goal of cancer gene therapy involves the expression of a transgene by cancer cells. Expanding adenovirus tropism is required for such cancer gene therapy, due primarily to low expression of CAR on tumor cells (131-133; 193). Targeting with RGD or polylysine has been popular, but since the target

receptors are expressed on many cells, these ligands do not necessarily mediate cancer-selective transduction (150). In contrast, receptors overexpressed on tumor cells, for example growth factor receptors, may make an excellent new target for Ad. Overexpression of growth factor receptors has been commonly found to be a driver in tumor growth (165). Furthermore, growth factor receptors often activate signalling pathways similar to integrin, allowing for the potential for these factors to replace the function of integrin in Ad internalization (165). This includes the PI3K pathway, shown to be important in wild-type Ad internalization (165).

Retargeting of Ad to FGFR has resulted in both increased internalization and transgene expression (235). In this case signalling by FGFR or through PI3K was not required (235). Furthermore, with Ad targeted to EGFR or EpCAM, CAR or integrin signalling were not needed, nor were receptor ligands used in this situation, so there was likely no signalling present (304). Surprisingly, inhibition of integrin binding has been shown to enhance FGFR retargeted Ad internalization (306).

Thus, targeting Ad to a growth factor receptor is a viable strategy for gene transfer to tumor cells. For breast cancer, HER3 may provide an excellent target receptor for an Ad gene therapy vector.

1.3 HER3 Receptor

1.3.1 HER3 overview

HER3, an 180kDa glycoprotein, was first identified in 1989, as a transmembrane receptor tyrosine kinase (320; 321). This receptor is also known as ErbB3, and is a member of the human epidermal growth factor receptor family (HER), which also

includes the prototypic member EGFR, as well as HER2 (neu or ErbB2) and HER4 (ErbB4) (321). The entire receptor family has a similar mode of action, where ligand binding induces homo- or heterodimerization, an increase in tyrosine kinase activity, phosphorylation of the receptor, recruitment of effector proteins, and downstream signalling (322-325). HER3 is overexpressed in breast and other cancers, (321; 326) and thus would make a potentially useful target for gene therapy.

1.3.2 HER expression and role in breast development

A recent review described the roles of the HER family in normal and malignant breast biology (327). HER3, as well as HER2 and HER4, also play important roles in neural and cardiovascular system development and maintenance (321; 327). All HER family members play a role in mammary development, primarily during puberty, pregnancy and lactation (327). Increased expression of HER3 is seen in mammary tissue during pregnancy, where HER3 signalling through PI3K plays a role in morphogenesis (321; 327; 328). HER3 was also shown to be important for the maintenance of the luminal phenotype of breast epithelium (329).

HER4 is important for lobuloalveolar development, and HER4 activation and downstream signalling through STAT5 was shown to be required in the breast during lactation (327; 330). A soluble fragment of HER4 that can localize to nuclei and mitochondria seems to mediate HER4 functions (327; 331).

1.3.3 HER3 ligands

Heregulin (HRG, also known as neuregulin, NRG) 1 and 2 (including α and β forms of each) are ligands for both HER3 and HER4, though not other receptors in this family (332-334). These ligands bind through their EGF-like domains (55 aa) to the receptors (333-337). Ligand binding is thought to stabilize the receptor in an open conformation, allowing interaction between receptors (323-325; 338). Either homo- or heterodimerization of receptors is generally thought to be required for receptor activation and signaling (339; 340).

HRG binding to HER3 was enhanced when HER3 was dimerized with HER2 (337; 341). Furthermore, HRG induced phosphorylation of HER2 in breast cancer cells (335). Initially, this result led to the incorrect conclusion that HRG bound to HER2 (335), though this was later disproven (336; 342-344). However, as a result of the activation of HER2 (neu), HRG is also called neu differentiation factor (333; 335). HER2 dimerizes with HER3, and both receptors become phosphorylated in the presence of HRG (345; 346). HER2 itself has no known ligands (332), and appears to be in a receptor conformation that is able to dimerize constitutively (332).

Surprisingly, HRG has recently been shown to bind to $\alpha_v\beta_3$ and $\alpha_6\beta_4$ integrins, but the binding of HRG to HER3 (Kd = 1.9nM) is 70-fold stronger than to integrin (347; 348).

In conclusion, HRG binds to HER3 or HER4, inducing homo- or heterodimerization with other receptors, including HER2, and initiates a transmembrane signal through receptor activation (342; 349; 350).

1.3.4 HER3 activation

HER3 is part of a family of receptor tyrosine kinases, but this receptor is unique in that it has been shown to have no tyrosine kinase activity (351). Early studies using a chimeric EGFR-HER3 receptor demonstrated that EGF binding to the EGFR extracellular domain resulted in phosphorylation of the HER3 C-terminal domain, which resulted in mitogenic activity (352). Furthermore, the same chimeric receptor was shown to have less phosphorylation than the other family members after EGF addition, and less phosphorylation of other downstream proteins (353). Thus, the receptor likely requires dimerization with another family member to be fully activated by phosphorylation of the C-terminal tyrosine and to signal effectively (330; 340). Additionally, HER3 itself has been shown to be phosphorylated by HER2 (345). More recently, HER3 was phosphorylated after HRG addition to a cell line expressing exogenous HER3 and EGFR, while EGFR was not phosphorylated, since HRG does not bind EGFR (346). Interestingly, a recent paper has demonstrated that HER3 is not completely kinase dead, but the HER3 kinase activity is about 1000-fold less active than the EGFR kinase (354). Therefore, HER3 would still require other family members for effective signalling (354; 355). Thus, though HER3 is unable to form homodimers which are effective for signaling, HER3 does retain the ability to transphosphorylate its own intracellular domain to a limited extent (340; 354).

Activation of HER3 is also affected by intracellular factors, in addition to ligand binding and phosphorylation by its dimerization partner. A recent study has shown a role for cytohesins, Ras-like small GTPases, in activation (phosphorylation) of

EGFR and HER3 (356). Cytohesins were shown to interact directly with EGFR (356). Furthermore, inhibition of cytohesins by chemical antagonism or knockdown decreased EGFR and HER3 activation, and overexpression increased this activation (356).

The phosphorylated C-terminal domain of HER3 acts as a binding site for PI3K, grb2, shc, src, and other signaling molecules (330). PI3K is one of the most well studied of the downstream pathways. Six binding sites for PI3K have been identified on the HER3 C-terminal domain, and the p85 subunit itself has been shown to bind to HER3 (321; 357). PI3K binding is rare in the HER family. The only other member to bind PI3K directly is HER4, which contains only one binding site (358). Furthermore, PI3K, and not PLCγ or GTPase-activating proteins, can substitute for HER3 activation (359). Evidence from systemic profiling of phosphotyrosine interaction sites suggests that different downstream pathways are activated by receptors in this family depending on which receptor partners are dimerized (327).

1.3.5 HER family endocytosis

Most studies on endocytosis of HER family receptors have concentrated on EGFR, and much less is known about other family members.

1.3.5.1 EGFR endocytosis

EGF binding to EGFR has been shown to result in rapid endocytosis into clathrin coated pits (360). Early studies have shown rapid clustering of ligand-receptor

complexes which are internalized and ultimately degraded in the lysosome (361). Direct interaction of the receptor with AP-2 and the clathrin-associated protein complex was shown (362). A di-leucine motif in the EGFR C-terminal domain was shown to be important for this interaction (132). There is evidence that only dimerization of the receptor is required for internalization, and not receptor activation or downstream signalling (363; 364). However, there remains some controversy in the EGFR endocytosis field, as other studies have shown activation to be important in endocytosis (360).

EGFR was shown to be the only HER family member to be rapidly internalized (365), while the other receptors in the family were shown to be endocytosis impaired (353).

1.3.5.2 HER2 and HER4 endocytosis

The details of HER2 internalization remains somewhat controversial. An early study showed that HER2 can slow down EGFR internalization, and that HER2 does not contain an internalization signal in the cytoplasmic tail (361). Other studies have shown HER2 is not endocytosed or delivered to endosomes (366; 367). There is also evidence that HER2 is not associated with the clathrin adaptor protein AP-2 (368). In contrast, a more recent study has shown HER2 to complex with adaptin, clathrin, Esp15 and dynamin2 (369). Other studies have shown that HER2 is endocytosed, but rapidly recycled back to the plasma membrane, which may explain at least some of the contrasting data (36; 370; 371). A recent study has also shown that the internalization signals in the receptor dimer must be identical (*e.g.*

homodimers) to result in internalization (372). This study also showed HER2 internalization after the binding of a HER2 inhibitory peptide EC-1 bound to GFP (372). This peptide, EC-1, was previously shown to bind to the extracellular domain of HER2 and inhibit phosphorylation (373). This internalization appeared to be clathrin-mediated in the ovarian cancer cell line SKOV-3, but was not induced in the breast cancer cell line SKBR3. This study also linked phosphorylation of HER2 to internalization (372).

There is very little information regarding HER4 internalization specifically. An early study has shown that with a chimeric EGFR containing the HER4 intracellular domain, there was little downregulation of the receptor from the cell surface and no change in the receptor half-life with the addition of EGF (353).

In summary, there remains conflicting data regarding HER2 internalization and no conclusive evidence of HER4 internalization, in contrast to EGFR. Although there may be some variability between different cell lines and conditions, there is little doubt that differences do exist in internalization mechanisms between the receptors in this family.

1.3.5.3 HER3 endocytosis

HER3 was shown to be taken up slowly after HRG binding (374). Since this internalization is slower than EGFR, the internalization mechanisms may not be the same (353; 374-376). Internalization of HER3 does appear to be dependent on the C-terminal tail, as with other receptors of this family. In an experiment with a HER3-EGFR chimera, the chimera with the HER3 intracellular domain showed a

three-fold lower internalization rate than EGFR (353). However, the receptor half-life and the rate of downregulation from the cell surface were closer to those of EGFR than other receptors tested (353). Furthermore, in this experiment endogenous HER receptor levels were low, indicating that the HER3 intracellular domain may not require other receptors as partners to mediate internalization (353).

A recent study examined HER3 internalization in porcine aortic endothelial cells (PAE) expressing exogenous HER3 and EGFR and in endogenously expressing breast cancer cell lines (SKBR3 and MCF-7) (346). They demonstrated ligand independent internalization of HER3 and colocalization with early endosome antigen 1 (EEA1). Additionally, they showed an increase of HER3 on the cell surface following clathrin siRNA inhibition (346). Furthermore, they demonstrated that though HER3 does not inhibit endocytosis of radioactive EGF in cells expressing both EGFR and HER3 to the same degree as HER2 does in cells expressing EGFR and HER2, there appears to be some inhibition when cells are stimulated simultaneously with both HRG and EGF (346).

Other recent studies show HER3 may play a role in the prevention of HER2 internalization into SKBR3 cells induced by the HER2 inhibitor EC-1 (see also Section 1.3.5.2) (372).

HRG itself is taken up inside cells, as shown in a study in which 80% of cells show internalization of an HRG-GFP fusion protein within 30 min in the breast cancer cell line MDA MB 453 (375). Though HRG was shown to be degraded after internalization in a separate experiment, internalization of HRG was not as rapid as that of EGF in the SKBR-3 breast cancer cell line (376).

Thus, though there is evidence that HER3 is endocytosed, this is likely slower than EGFR, and may not be ligand dependent.

1.3.6 Receptor recycling and/or receptor degradation

EGFR degradation was shown to be lysosomal and mediated by the E3-ubiquitin ligase, Cbl (360; 377). In contrast, the E3-ubiquitin ligase Nrdp1 (neuregulin receptor degradation protein -1) was shown to mediate HER3 ubiquitination and subsequent degradation (378-380). There are conflicting reports of lysosomal degradation of HER3 (374; 376; 379). One study has shown only partial inhibition of HER3 degradation by the lysosomal inhibitor chloroquinone (376). More recent evidence suggests that Nrdp1 diverts HER3 to the lysosome after HRG stimulation (379). Furthermore, whether or not lysosomal degradation is induced by ligand has also been debated (346; 374; 376; 379). If the HER3 degradation pathway is not ligand induced, it is likely a basal degradation pathway (376). This idea is supported by the shorter half-life of HER3, when compared to EGFR (353; 374-376; 381). Additionally, there is also evidence for recycling of HER3 back to the cell surface, as this can be inhibited by the recycling inhibitor monensin (374).

1.3.7 HER nuclear translocation

There are putative nuclear localization sites (NLS) on all EGFR family members, and a review was recently published outlining the currently understood role of each family member in the nucleus (369; 382).

Nuclear localization of EGFR has been shown in several tissues, and has been associated with poor prognosis in multiple tumor types, including breast cancer (383-388). Nuclear localization of both EGFR and HER2 has been linked to cancer therapy resistance (389-391).

Nuclear HER2 is involved in transcriptional activation of genes related to cancer, including COX-2 (392-394). Interestingly, though HRG treatment can increase nuclear HER2, a kinase deficient HER2 does not localize to the nucleus (369; 392-394).

A cleaved form of HER4, 4ICD, has been shown to be active intracellularly, and is also thought to act in the nucleus in a complex with STAT5a (331; 395; 396). Surprisingly, 4ICD has been linked to both shorter patient survival and improved response to therapy, thus its role remains ambiguous (382; 397; 398). Full length HER4 has been seen in the nucleus of some normal cells (399; 400).

Full length HER3 has also been observed in the nucleus, though its role remains unclear (321; 401; 402). In pancreatic cancer, low nuclear HER3 appears to predict higher risk of recurrence (403; 404). A truncated form of HER3 has also been detected in the nucleus, and has been linked to activation of the Cyclin D1 gene promoter (405).

1.3.9 HER3 expression and role in cancer

HER3 and the other EGFR family members are all known to play a role in cancer, to some extent, though their roles and levels vary (327).

1.3.9.1 Other family members' role in cancer

EGFR and HER2 are overexpressed in many cancers, such as lung and breast cancers (326; 406). These receptors are considered cancer drivers, though HER2 has been shown to require HER3 to drive breast cancer (358; 407). Interestingly, HER3 is not thought to drive cancer alone (358; 407).

Contrary to the other family members, there has been some evidence that HER4 expression might be a marker for good prognosis in some cancers, through induction of apoptosis (408; 409). However, the full role of HER4 in different cancers is still not entirely clear (326; 406). HER4 expression has been linked to positive ER status in breast cancer, and it is possible that HER4 expression is regulated by estrogen (327). Full length HER4 and the cleaved variant (4ICD) might also have different functions from each other in breast cancer (410).

1.3.9.2 HER3 role in cancer

HER3 has been shown to be overexpressed in breast and ovarian cancers, among other cancers (321; 326; 411-417). Furthermore, HER3 is often overexpressed in tumors which overexpress HER2 (321; 326). The HER2/3 complex has been shown to be an important breast cancer driver, and is considered the most oncogenic dimer of the HER family (321; 418-420). Additionally, HER2-overexpressing transgenic mice have been shown to overexpress HER3 at the protein level (421).

However, a recent review has found the prognostic value of HER3 expression in breast cancer as inconclusive, because some studies have demonstrated HER3 association with poor prognosis, and some with good prognosis (422-424). HER3

expression has also been associated with acquired resistance to hormone therapy using tamoxifen and fulvestrant in the ER-positive MCF-7 breast cancer cell line (425). Interestingly, unlike the rest of the HER family, there are no reported mutations of HER3 coding sequences in human malignancies, only overexpression of HER3 mRNA or protein (358; 426; 427).

1.3.9.3 Cancer signalling pathways

EGFR family members can activate many signalling pathways depending on which family members are activated. HER3 contains six PI3K binding sites (321), and the PI3K pathway is activated by HER3 phosphorylation in the HER2/3 heterodimer (358; 428). Activation of the PI3K pathway has been linked to breast tumors (321). The ras/MAPK pathways can also be activated through HER2 phosphorylation (358; 428). Src activation has been shown to be important for HER2/3 downstream effects in a murine fibroblast model system, possibly by stabilizing the HER2/3 dimer in breast cancer cells (418).

1.3.10 HER family inhibition

1.3.10.1 Inhibition of HER signalling

Kinase inhibitors have been developed which target the HER family in general (*e.g.* neratinib), or certain members specifically (*e.g.* the EGFR targeted gefitinib) (327; 429-431). Since HER3 has been shown to lack an effective kinase, these inhibitors are generally not thought to act on HER3 directly (351). However, the inhibition of the kinase active partner of a HER3 heterodimer may prevent HER3 signalling.

Specifically, prevention of signaling by HER2/3 dimers is an important strategy for breast cancer therapy (321; 418). Unfortunately, inhibitors are more effective at decreasing HER2 autophosphorylation than HER2 phosphorylation of HER3, which can undermine the efficacy of such therapy (426; 432; 433).

Other mechanisms of HER family inhibition have been examined, including ectodomain-binding monoclonal antibodies. The best known of these is trastuzumab, a HER2 targeted humanized mouse antibody, which was shown to induce regression of breast tumors with HER2 amplification (427; 434). Another HER2 targeting antibody, pertuzumab, has been shown to act through prevention of HER2 binding to HER3, and can disrupt ligand induced PI3K signalling (427; 435). Furthermore, there are two HER3 targeted antibodies, MM-121 and AMG-888, but these antibodies are still in early clinical trials (427; 436).

1.3.10.2 Resistance to HER inhibition by HER3 activation

One proposed mechanism of resistance to HER family inhibition is through activation of signaling by other HER family members (327). HER3 overexpression has been linked to resistance to the EGFR inhibitor gefitinib, as well as other inhibitors (437). This increase in HER3 levels corresponded with AKT reactivation (437). Interestingly, inhibition of signalling pathways other than EGFR, notably PI3K, did not increase HER3 levels (437). The authors did not identify the pathway downstream of EGFR that is responsible for the increase in HER3 (437). There are also other mechanisms by which HER3 is able to mediate resistance to HER family

inhibition, which generally result in increased HER3 signalling, but do not involve HER3 overexpression (433).

1.3.11 HER3 as a target for gene therapy

HER family members have been used as Ad binding targets, mentioned previously in Sections 1.2.11.2 to 1.2.11.4 (179; 304; 311).

HRG has also been used as a targeting moiety. Both HRG α and β1 (splice variants of the HRG gene) have been fused to the viral envelope glycoprotein, gp70, of the Moloney murine leukemia virus (438), to successfully target the virus to breast cancer cells expressing HER3 and HER4 (438). HRG fused to the Ad penton protein was also used to target non-viral gene delivery resulting in gene expression in MDA-MB-453 breast cancer cells (439).

In summary, HER3 is overexpressed on many breast cancer cell lines and tumor samples. Therefore, it is a viable candidate receptor for retargeted Ad gene therapy. Examining the binding and internalization of a retargeted Ad, as well as gene expression would be important in understanding how such a virus would function biologically and clinically.

1.4 Summary of thesis

Our lab previously constructed a virus, AdLuc(HRG-fiber), targeted to the HER3 receptor, as the initial step in generating a platform for breast cancer targeted Ad gene therapy (see Methods Section 2.2.1, and our previous publication (3)). This virus was targeted to HER3/4 by inserting the coding sequence of the EGF-like

domain of HRG into the HI loop of the fiber knob. We demonstrated that this insertion did not impede fiber trimer formation or viral replication in the packaging cell line, HEK293 (Figure 1.4 and 1.5) (3). The insertion in the HI loop was not expected to affect viral binding to CAR, and as a result, this retargeted virus was expected to bind to and infect cells expressing HER3, HER4, CAR and/or integrin. A control virus, AdLuc(wt-fiber), was expected to only bind to and infect cells expressing CAR and/or integrin.

In this thesis, Chapter 3 describes our characterization of this virus through infection of breast cancer and other cells differing in surface expression of the receptors HER3/4 and CAR *in vitro* and *in vivo*. Chapter 4 examines the binding of the retargeted virus to cells, and the internalization mechanism of the retargeted virus, compared to the wild-type binding virus.

We are the first to show expanded tropism by an HRG-modified Ad into cells not normally infected by wild-type binding Ad. These included cells expressing high levels of the HRG receptors HER3 and HER4, as well as other cells. This expanded tropism provides some resistance to competition by soluble Ad fiber knob. Surprisingly, this expanded tropism did not translate to a mouse xenograft model.

We also examined the binding of the retargeted virus to cell lines expressing either CAR, HER3, neither or both receptors. Surprisingly, there was very little binding to any cell line, except those expressing exogenous CAR, despite high levels of gene expression after infection with AdLuc(HRG-fiber). Similarly, internalization of either virus was not high, except in CAR^+ cells. Also, AdLuc(wt-fiber) internalization was higher than AdLuc(HRG-fiber) internalization in most cell

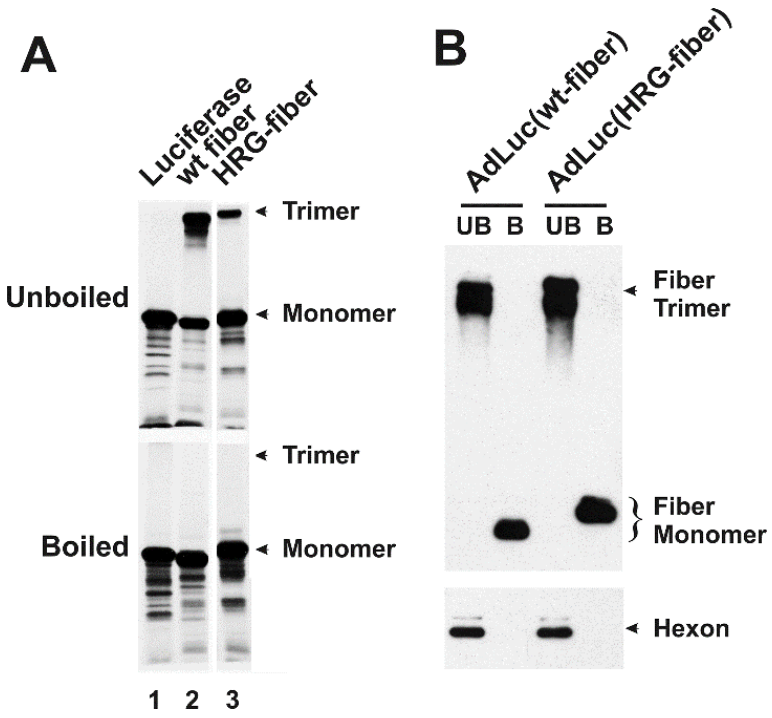


Figure 1.4: *Expression of wild-type and modified fiber proteins.*

(A) *In vitro* translation and trimerization assay. Plasmid expression vectors encoding wt fiber, fiber modified by insertion of the EGF-like domain of HRG into the HI-loop (HRG-fiber), or the monomeric firefly luciferase, as a control, were translated *in vitro* in the presence of ^{35}S-methionine. The resulting products were either loaded without boiling (top panel) or boiled for 5 minutes prior to loading (bottom panel) and resolved by SDS-PAGE. The positions of fiber monomer and trimer are indicated to the right. (B) Western blot analysis of fiber content in purified AdLuc(HRG-fiber) and AdLuc(wt-fiber) virions. Viral particles (2.5×10^9 viral particles (vp) per lane) were either loaded directly onto the gel (without boiling, lanes marked UB), or boiled for 5 minutes prior to loading (lanes marked B) and resolved by SDS-PAGE. Following transfer to PVDF membranes, samples were probed for fiber protein (top panel) and for hexon protein (bottom panel). The positions of fiber trimers and monomers are indicated to the right. The anti-hexon antibody did not recognize denatured hexon (3).

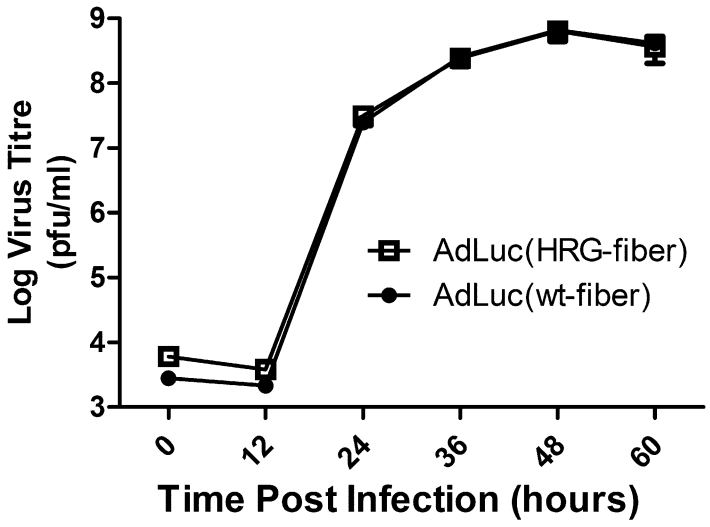


Figure 1.5: *Growth curves for AdLuc(wt-fiber) and AdLuc(HRG-fiber).*
Monolayers of HEK293 cells were infected (in duplicate) with indicated viruses at a multiplicity of infection (MOI) of 5 plaque forming units (PFU) per cell and cells were harvested at indicated times post infection. Following the release of cell-associated virus by two cycles of freeze/thaw, the viral titer in each sample was determined by plaque assay on HEK293 cells. Error bars are equal to one standard deviation and most are smaller than the symbols (3).

lines, which is opposite from gene expression experiments. Repeating a gene expression assay in a similar manner to the internalization assay yielded results similar to the internalization assay.

Microscopic examination of internalization was more consistent with transgene expression, with the number of AdLuc(HRG-fiber) inside the cell higher than AdLuc(wt-fiber). Colocalization of virus with receptor was as expected, with AdLuc(wt-fiber) colocalizing primarily with CAR, while AdLuc(HRG-fiber) colocalized with both HER3 and CAR.

Ultimately, our data demonstrated AdLuc(wt-fiber) and AdLuc(HRG-fiber) binding and internalization into CAR+ cells after 10 min at room temperature or 37°C. This results in high levels of transgene expression. AdLuc(HRG-fiber) binding and internalization into CAR- cells appeared to occur between 10 min and 30 min at 37°C, but high transgene expression and expanded virus tropism require long exposure times (potentially up to 30 min) to be observed.

CHAPTER 2: Materials and Methods

2.1 Cell culture

2.1.1 General cell culture

All cell lines used are adherent human breast carcinoma, unless otherwise noted. BT549a, BT549b, MDA-MB361, MDA-MB-453, SK-BR-3, T47D and ZR-75-1 cells were cultured in RPMI medium 1640 (31800, Invitrogen). CHO (Chinese Hamster Ovary) cell lines and derivatives were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM, 12800-082, Gibco, high glucose is 4.5 g/L). The mouse breast cancer cell line MT1A2 (derived at McMaster University, Hamilton, Ontario, Canada) (440), the human ovarian cancer cell line SKOV-3, the human glioblastoma cell line U118MG, the human rhabdomyosarcoma cell line RD, and the human cervical cancer cell line HeLa were maintained in DMEM. MCF7, MDA-MB-231, MDA-MB-468 and A549 (human lung carcinoma) cells were cultured in either RPMI or DMEM high glucose. HEK-293 cells (human embryonic kidney cells transformed with the left end of Ad) (93) were cultured in Minimum Essential Medium (MEM, 61100, Gibco). All media were supplemented with 10% fetal bovine serum (12483, Gibco), PSF (Antibiotic-antimycotic, 15240, Gibco) and 200 μM L-glutamine (25030, Gibco).

Additionally, two separate isolates of BT549 cells (here designated BT549a and BT549b) were used, which demonstrated different CAR levels (Figures 3.1 and 3.2, and Table 3.1). These differences may be explained by the different methods used to detect the receptors, or differences in expression levels at the time of measurement. Thus, we used the receptor levels measured at the time of each infection to categorize the cell lines.

2.1.2 CHO cell lines

A series of stable CHO transfectants were used for multiple experiments. CHO-NT (not transfected with any exogenous receptor) was acquired from Dr. Zhixiang Wang (University of Alberta); CHO-CAR (transfected with the coxsackie-adenovirus receptor (CAR)), and CHO-al2 (transfected with an unrelated receptor, human α2 integrin) were acquired from Dr. Jeffrey Bergelson (University of Pennsylvania) (57).

The CHO-al2/HER3 and CHO-CAR/HER3 stable cell lines were generated as follows: CHO-al2 and CHO-CAR were transfected with plasmid phErbB3 encoding HER3 (from Open Biosystems, MHS1010-98051190, Human MGC Verified FL cDNA (IRAT), clone ID 6147464) using Lipofectamine 2000 (11998-019, Invitrogen). Cell lines were sorted by flow cytometry into individual wells of 96-well plates based on HER3 expression, and stable clones were selected with 100 μg/ml Hygromycin B (10687-010, Invitrogen). Expression of receptors was detected by flow cytometry (see Figure 2.1, flow cytometry methods can be found in Section 2.8). We selected clones F3 for CHO-al2/HER3 and C12 for CHO-CAR/HER3 for further use. Elsewhere these clones will be referred to as CHO-al2/HER3 and CHO-CAR/HER3, respectively.

2.2 Viruses

2.2.1 Virus construction and amplification

The viruses used in the following experiments were the control virus AdLuc(wt-fiber), also called AdLC8c-luc (5), and AdLuc(HRG-fiber) (3) (Figure 2.2).

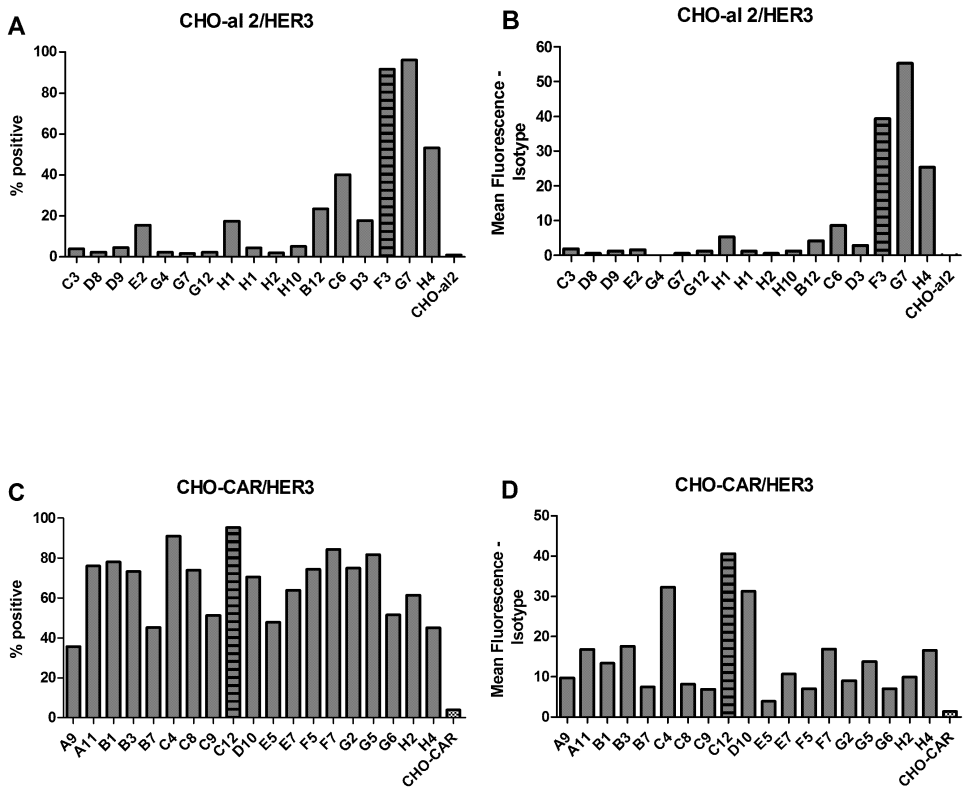
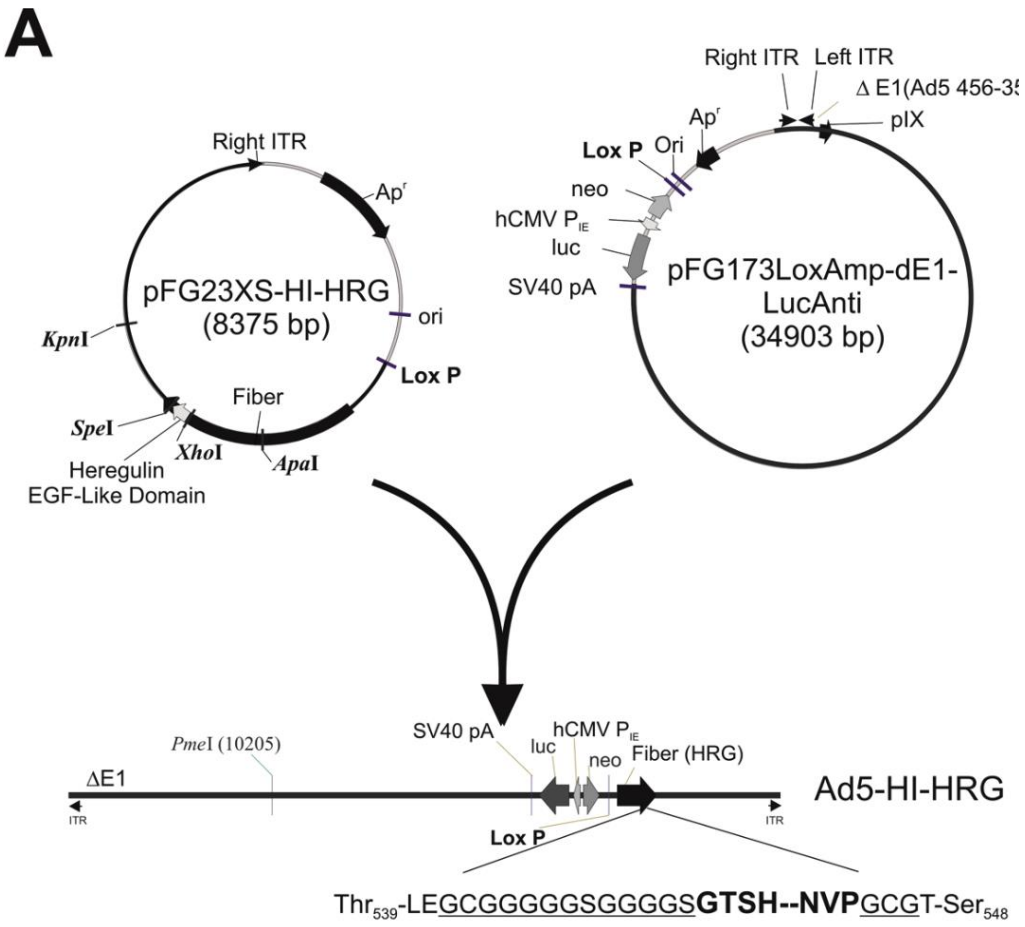


Figure 2.1: *HER3 receptor levels in HER3 transfected CHO cells.*

(A and B) CHO-al2 cells and (C and D) CHO-CAR cells were transfected with a HER3 encoding plasmid and sorted for HER3 expression into individual wells of 96-well plates. Stable clones were selected with Hygromycin B and then tested for HER3 expression by flow cytometry. The proportion of cells positive for HER3 are shown in (A and C), while the relative number of receptors on the cell surface are shown in (B and D). The clone names are shown on the X axis, compared to either (A and B) CHO-al2 or (C and D) CHO-CAR. The clones used in future experiments are F3 for CHO-al2/HER3 and C12 for CHO-CAR/HER3 (striped bars).


A
Right ITR
Ap^r
pFG23XS-HI-HRG
(8375 bp)
ori
Lox P
Fiber
KpnI
SpeI
XhoI
ApaI
Heregulin
EGF-Like Domain
Right ITR
Left ITR
Δ E1(Ad5 456-3
pIX
Ap^r
Lox P
Ori
neo
hCMV P_IE
luc
SV40 pA
pFG173LoxAmp-dE1-
LucAnti
(34903 bp)
PmeI (10205)
SV40 pA
hCMV P_IE
luc
neo
Fiber (HRG)
ΔE1
ITR
ITR
Lox P
Ad5-HI-HRG
Thr539-LEGCGGGGGSGGGGSGTSH--NVPGCGT-Ser548

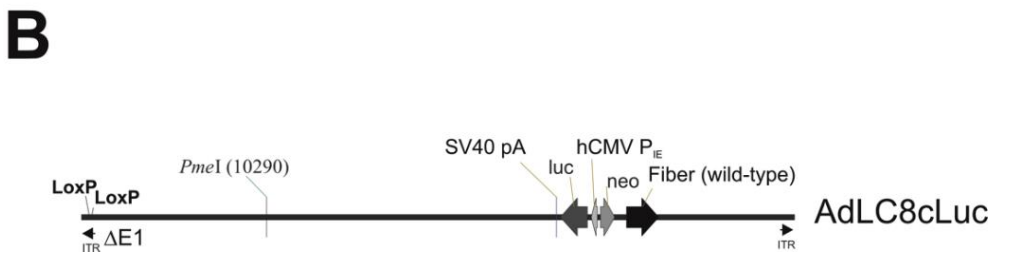

B
PmeI (10290)
SV40 pA
hCMV P_IE
luc
neo
Fiber (wild-type)
LoxP
LoxP
ITR
ΔE1
ITR
AdLC8cLuc

Figure 2.2: *Schematic diagram of the recombinant plasmids and the rescue strategy employed.*

(A) Structure of AdLuc(HRG-fiber) (previously designated Ad5-HI-HRG) and its parent plasmids. The amino acid sequence of the fiber modification is shown below the schematic of the recombinant virus. Ad5 fiber sequence was modified only between Thr_{539} and Ser_{548} (see also Figure 2.3). The amino acid sequence corresponding to the EGF-like domain of HRG-α are in bold, while the linker sequences are underlined and the amino acids between GTSH and NVP of HRG-α are represented by two dashes. The full amino acid sequence of the insert can be found in Figure 2.3. (B) Structure of the control virus AdLuc(wt-fiber) (previously designated AdLC8c-luc [(5)]). Open reading frames and regulatory sequences are indicated by broad arrows. Adenovirus sequences are indicated by narrow black bars.

AdLuc(HRG-fiber) was constructed by Mabrouk Elgadi (McMaster University), and the construction is outlined in Figure 2.2. The viruses are both first generation adenoviruses, containing deletions in the E1 and E3 regions (E1 deletion positions 456 to 3525 and E3 deletion position 28138 to 30465). The luciferase reporter gene was inserted in place of the E3 region, under control of the human cytomegalovirus (CMV) immediate early promoter. The differences between the two viruses are (1) the insertion of the coding region of the EGF-like domain of heregulin (HRG) into the HI loop of fiber knob, flanked by two linker sequences, in AdLuc(HRG-fiber), (2) a loxP insertion in the E3 region of AdLuc(HRG-fiber), and (3) insertion of loxP sites flanking the packaging sequence in AdLuc(wt-fiber) (AdLC8c-luc) (Figure 2.2 and 2.3).

2.2.2 Virus titration

The viruses were amplified and purified using standard methods (441). Briefly, the viruses were amplified in 20 to 40, 150 mm plates of HEK-293 cells, which compensate for the E1 deletion in the viruses. Once the cell monolayers showed complete cytopathic effect (CPE), the cells were harvested and the virus was purified by cesium chloride banding (441). The virus was titred by plaque assay (441) or Adeno-X rapid titre kit (632250, Clontech). The Adeno-X titration method uses antibody detection of the late viral protein hexon as a marker for infection. The titre of an AdLuc(wt-fiber) prep previously titred by plaque assay was about two-fold higher using the Adeno-X method. The resulting titres are noted as pfu/ml (plaque forming units, titred by plaque assay), or ifu/ml (infectious units, titred by

ACA *ctc gag* gga tgc gga ggt gga gga gga tcc ggc gga
Thr Leu Glu Gly Cys Gly Gly Gly Gly Gly Ser Gly Gly

ggt ggc agc **ggt acc agc cat ctt gta aaa tgt gcg gag**
Gly Gly Ser **Gly Thr Ser His Leu Val Lys Cys Ala Glu**

aag gag aaa act ttc tgt gtt aac gga ggg gag tgc ttc
Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe

atg gtg aaa gac ctt tca aac ccc tcc aga tac ttg tgc
Met Val Asn Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys

aag tgc caa ccc ggg ttc act gga gca aga tgt act gag
Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu

aat gtg ccc ggg tgc ggt *act* *AGT*
Asn Val Pro Gly Cys Gly Thr Ser

Figure 2.3: *Final sequence of HRG insert and flanking fiber gene in AdLuc(HRG-fiber).*
The HRG sequence is in bold, the linker sequences are underlined and the XhoI and SpeI restriction digest sites are italicized. The sequence begins at the Ad fiber amino acid Thr_{539} and ends at Ser_{548} (see also Figure 2.2).

Adeno-X). The number of virus particles per ml (vp/ml) was calculated using absorbance of the diluted viral preparation at OD_{260} (442). A table of the viral preparations used, their titres and particle numbers is found in Table 2.1. Virus preparations 1, 2, and 4 were titred by plaque assay, while preparations 3 and 5 were titred by Adeno-X kit. The viruses were also confirmed to contain less than one replication competent adenovirus per 1e8 pfu or ifu vector, tested using the replication competent adenovirus (RCA) test (443).

2.2.3 Fluorescent labeling of virus

Antibody labeling kits were used to label virus with either Cy2 (FluoroLink™-Ab Cy2 labelling kit, PA 32000, Amersham Biosciences) or Alexa 488 (A488, Alexa Fluor® 488 monoclonal antibody labeling kit, A20181, Molecular Probes). Both dyes have absorption spectra that peak at approximately 488 nm (Cy2 - 489 nm, A488 - 494 nm), and emission spectra that peak at 506 nm (Cy2) or 519 nm (A488). Cy2 is a bifunctional N-hydroxysuccinimide (NHS)-ester, while A488 is a tetrafluorophenyl (TFP) ester moiety. Both esters react with primary amines of proteins to label proteins with the fluorescent dye. In order to conjugate the dye to the virus, a procedure similar to that outlined in Leopold et al. (74) was used. The virus was first diluted to a concentration of 1e12 vp/ml with phosphate buffered saline solution (PBS) ++ (PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.1 g/L $MgCl_2 \cdot 6H_2O$) +10% glycerol. The Cy2 dye was resuspended in 500 µl 0.1 M sodium bicarbonate, while the A488 dye was resuspended in 100 µl of 0.2 M sodium bicarbonate. A range of dye dilutions

Number	Virus	pfu/ml or ifu/ml	vp/ml	Ratio of vp to pfu or ifu	Figures
1	AdLuc(wt-fiber)	4.00E+09	1.69E+12	423	3.3B, 4.11
2	AdLuc(wt-fiber)	6.00E+09	2.44E+12	407	3.4, 3.6, 4.2
3	AdLuc(wt-fiber)	2.62E+10	3.96E+12	151	3.8
4	AdLuc(HRG-fiber)	1.40E+09	1.14E+12	814	3.3B, 3.4, 3.6, 4.2, 4.11
5	AdLuc(HRG-fiber)	8.75E+09	2.55E+12	292	3.8

Table 2.1: *A table of unlabelled viral preparations used in this thesis and the figures displaying the data in which the different preparations were used.*

were tested (see Section 4.2.1.), and ultimately the virus was mixed with the reactive dye in a 1:1 ratio (usually 50 µl of each solution), and incubated at room temperature for 30 min (Cy2) or 1 hr (A488), with mixing. Two µl of 0.1 g/ml glycine was added to stop the reaction (Cy2 only), and the entire mixture was transferred to a Slide-A-Lyzer MINI dialysis device (10,000 molecular weight cut-off, 0.1 ml maximum volume, 69576, Thermo Scientific) for dialysis. The solution was dialysed against two changes of 500 ml of buffer (10% glycerol, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl) over 24 hrs at 4°C. After removal from dialysis, glycerol was added to the virus to a final concentration of 30% and the virus was stored at -80°C until use.

The number of dye molecules per viral capsomere was calculated for each labelling reaction (Table 2.2). For Cy2 labelling, the molar concentration of the dye was calculated by dividing the absorbance at 489 nm (after subtracting the background at 430 nm) by the extinction coefficient of Cy2 provided by the manufacturer (150,000 $cm^{-1}M^{-1}$). This was converted to molecules of dye per ml using Avogadro's number (6.022e23 molecules per mole). The number of virus particles per ml (vp/ml) was calculated using absorbance of the diluted viral preparation at 260 nm (after subtracting the background at 430 nm). The dye to protein ratio was then calculated by dividing the molecules of dye per ml by the number of viral capsomeres per ml (number of virus particles per ml multiplied by 252 capsomeres per virion (53)).

For A488 labelling, the molar concentration of the dye was calculated by multiplying the absorbance at 494 nm (after subtracting the background at 430 nm)

Dye	Virus	Dye to Capsomere Ratio	Figure used
Cy2	AdLuc(wt-fiber)	3.1	4.1
Cy2	AdLuc(HRG-fiber)	1.9	4.1
A488	AdLuc(wt-fiber)	37.0	4.5, 4.6
A488	AdLuc(HRG-fiber)	39.1	4.5, 4.6
A488	AdLuc(wt-fiber)	66.6	4.7
A488	AdLuc(HRG-fiber)	34.4	4.7

Table 2.2: *A table of labelled viral preparations used in this thesis and the figures they were used in.*

by the dilution factor and dividing by the extinction coefficient of A488 provided by the manufacturer (71,000 $cm^{-1}M^{-1}$). This was converted to molecules of dye in the total solution using Avogadro's number (6.022e23 molecules per mole). The total number of viral particles in solution was calculated based on dilution of the viral stock (eg. 50 µl of 1e12 vp/ml is 5e10 vp total in solution). The dye to protein ratio was then calculated by dividing the total molecules of dye by the total viral capsomeres.

The labelling procedures were performed in parallel with virus without dye present to control for the effect of the labelling procedures on the virus. The labelled and unlabelled virus were compared for infectivity using a limiting dilution assay (also called endpoint method) (444 p70-72). Briefly, a 96-well plate of HEK-293 cells was infected with a virus at dilutions between 1e-3 and 1e-13, with one column per dilution. The wells were scored for presence or absence of infection (CPE). For this method, it is essential that there be one dilution where all wells are infected and one where all wells are not. The titre was calculated by graphing the ratio of wells with visible CPE at day 10 or 11 against the log of the viral dilution. GraphPad Prism (GraphPad Software, Inc.) analysis software was used to determine the tissue culture infectious dose at 50% of maximum (TCID50) using a log(agonist) vs. dose response curve (445). The addition of the dye made very little difference in infectivity in HEK-293 cells. However, we cannot conclude that this procedure did not affect binding to other receptors not expressed on HEK-293 cells (*e.g.* HER3) or infectivity in other cell lines.

2.3 Flow cytometry

2.3.1 Receptor detection by flow cytometry

Cells were trypsinized, washed twice in PBS and resuspended at 4e6 cells/ml in blocking buffer (1% human serum in PBS). For HER3, HER4 or integrin detection, 1e5 cells were aliquoted into tubes and stained with 10 µl antibody (HER3, phycoerythrin (PE)-conjugated mouse monoclonal anti-human ErbB3, clone 66223, FAB3481P, R&D Systems; HER4, PE-conjugated mouse monoclonal anti-human ErbB4, clone 182818, FAB11311P, R&D Systems; or α_v integrin, PE-conjugated mouse monoclonal anti-human CD-51, clone P2W7, FAB1219P, R&D Systems) or isotype control (PE-conjugated mouse IgG_1, IC002P, R&D Systems) for 30 min at room temperature in the dark. For CAR detection, the antibody used was 20-100 µl of supernatant harvested from mouse hybridoma cells (RmcB, ATCC# CRL-2379). The cells were washed with PBS + 0.5% BSA. If the primary antibody was unconjugated, cells were then stained with 10 µl PE-conjugated secondary antibody (Goat $F(ab')_2$, F0102B, R&D Systems) for 30 min, and washed again. The cells were resuspended in 500 µl PBS + 5% formaldehyde and stored at 4°C before fluorescence was detected by a flow cytometer (FACSCalibur, BD Biosciences).

2.3.2 Flow cytometry analysis

Each cell line was analysed separately, since cell lines vary in properties such as cell size and intracellular complexity. For each cell line, only live cells in single cell suspension were analysed, by gating cells based on forward and side scatter

(size and intracellular complexity). Mean fluorescence is the average fluorescence of the entire sample, after subtractions of the mean fluorescence for the isotype or control sample.

To calculate percent of cells positive for receptor detection, isotype or negative control samples were used to set gates (at 1% positive) for positive fluorescence. The percent of cells positive for fluorescence was determined by the percent of cells in the gate on a histogram.

To calculate percent of cells positive for fluorescent virus detection, the percent of cells inside a gate was used. This gate was placed on a dot plot of fluorescence relative to a control fluorescence channel so that only about 1% of the control sample is positive, on average. The control fluorescence channel is a fluorescence channel which was predicted to be negative based on the emission spectrum of the fluor used, and is therefore expected to remain relatively consistent for a given cell line. An example of this analysis can be seen in Figure 4.5.

2.4 Western blot analysis of receptor expression

2.4.1 Cell line receptor detection by Western blot

To detect cellular proteins, total cell lysates were prepared from washed cells by incubation on ice for 20 min in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml of each of aprotinin, leupeptin and pepstatin). Cleared lysates were assayed for protein using a Micro-BCA kit (Pierce, Rockfort, IL, USA). In all, 50 μg total protein (100 μg for HER4 and CAR

analyses) were boiled for 7 min in sample buffer before resolution by SDS–8% PAGE. Resolved proteins were electro-transferred to polyvinylidene difluoride membranes. Blocked membranes were probed for HER2, HER3, HER4 (rabbit polyclonal antibodies anti-neu, C18 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-ErbB3, C17 (Santa Cruz Biotechnology Inc.); anti-ErbB4 (Lab Vision Corp., Kalamazoo, MI, USA), human CAR (mouse monoclonal antibody RmcB; Upstate USA Inc., Billerica, MA, USA) and β-actin (mouse monoclonal antibody AC15; Sigma-Aldrich Co.). Signals were detected using either horseradish peroxidase-conjugated anti-mouse IgG or horseradish peroxidase-conjugated anti-rabbit IgG antibody (Pharmingen) and ECL Western detection reagents (Amersham Bioscience).

2.4.2 Receptor expression in mouse xenografts and tissue samples by Western blot

40 μg of protein for each sample was heated at 95°C in loading buffer (final concentration: 3% glycerol, 1.5 M Tris-HCl pH 6.8, 0.4% SDS, 30 ng/ml bromophenol blue, 40 mM 2-mercaptoethanol) for 5 min, prior to resolution by SDS-9% PAGE (Bio-Rad Mini-PROTEAN apparatus, running buffer). Resolved proteins were electro-transferred to nitrocellulose membranes in transfer buffer (3.03 g/L Tris base, 14.4 g/L glycine, 20% v/v methanol) for 90 min at 350 amperes on Bio-Rad Mini-PROTEAN apparatus. Blocked membranes (5% milk, 0.1% Tween 20 in PBS) were probed for the human proteins HER3, CAR or β-actin (anti-ErbB3 (C17), sc385, Santa Cruz Biotechnology, Inc.; mouse monoclonal antibody

RmcB). Signals were detected using either HRP-conjugated goat anti-mouse IgG (115-035-003, Jackson ImmunoResearch Laboratories, Inc.) or HRP-conjugated anti-rabbit IgG antibody (11-035-144, Jackson ImmunoResearch Laboratories, Inc.), and ECL Western detection reagents (Amersham Bioscience).

2.5 In vitro infection of monolayer cultures

Cells were plated in growth medium at an amount to be 80-90% confluent at the time of infection. For cells used for luciferase assays, the cells were plated in a 24-well plate. The cells in the carcinoma panel (Figure 3.3) were plated at 1e5 to 2e5 cells per well, and CHO derivative cell lines were plated at 2e5 or 2.5e5 cells per well. 2-3 wells were counted the day of the infection and used to calculate the number of infectious viruses per cell (multiplicity of infection, MOI). Virus was diluted in PBS++ and 100 µl was used to infect the cells. The virus was allowed to adhere to the cells for 30 min at 37°C before the wells were washed twice with PBS and fresh medium was added. The cells were incubated for the times indicated, prior to analysis.

2.6 Luciferase assay

2.6.1 Luciferase assay of in vitro infections

After infection as described in Section 2.5, the infected cells were incubated for 24 hrs (breast cancer cells) or 48 hrs (CHO cell lines) at 37°C, then washed twice with PBS. The cells were lysed by the addition of 200 µl of reporter lysis buffer (RLB)

and incubation at room temperature for 20 min. The plate was either freeze-thawed or stored at -70°C until further analysis was performed.

After thawing, the lysed cells in RLB were removed from the wells by pipetting and transferred to microfuge tubes. The lysate was centrifuged at 12,000 x g for 2 min at 4°C, and the supernatant was transferred to fresh tubes. The amount of luciferase in each lysate was detected using a luminometer (FLUOstar Optima, BMG Laboratories) and a luciferase detection kit (E4030, Promega), according to kit instructions. Briefly, 20 µl of lysate, or an appropriate dilution in RLB, was added in duplicate to a white 96-well plate. 100 µl of luciferase substrate was dispensed by the luminometer into a single well immediately before the light emission was measured in relative light units (RLU) at five different gains. A standard curve of luciferase (QuantiLum® Recombinant Luciferase, E170A, Promega) was used to confirm that detection was in the linear range of the luminometer and to convert RLU to µg luciferase. After analysis the lysate was stored at -70°C.

2.6.2 Luciferase assay of in vivo infections

The luciferase assay of mouse tissue lysates were performed in a similar manner, except the lysis procedure differed (see Section 2.8.2).

2.7 Competition assays

2.7.1 Competition assays with breast cancer cell lines

To generate soluble fiber knob protein, the sequence of Ad5 fiber corresponding to the fiber knob (lysine 398 to glutamic acid 581) was PCR-amplified and inserted into the bacterial expression plasmid pET15-b (Novagen, Darmstadt, Germany). The resulting plasmid, pET15-Ad5knob, was transferred to Escherichia coli BL21 (DE3) pLysS (Novagen). After induction with 1 mm isopropylthio-b-galactoside for 4 hrs at 37°C, the protein was extracted from the cells under native conditions and purified using Ni-NTA agarose column (Qiagen, Toronto, ON, Canada). Following extensive dialysis against PBS, the protein concentration was determined using a BCA kit (Pierce).

In all, 2 μg of soluble wild-type Ad fiber knob (s-knob) were added to 2 x 10^5 cells in 24-well dishes and the cells were incubated at room temperature for 20 min. Virus was then added to the cells (100 vp per cell) and the incubation was continued for an additional 30 min at room temperature. The medium was then removed and the cells were washed twice with PBS and overlaid with complete medium. The cells were incubated for 24 hrs, and then luciferase activity was determined as above.

For HRG competition experiments with cancer cell lines, 2 x 10^5 cells in 24-well plates were overlaid with cold Dulbecco's modified Eagle's medium/F12 (Invitrogen) media containing 0.25 mg of recombinant human HRGα EGF-like domain (R&D Systems) and incubated on ice for 30 min. Virus (100 vp per cell) was then added and incubation was continued for an additional 30 min on ice. The

cells were then shifted to room temperature for 10 min before washing twice with PBS. Following the addition of the medium, the cells were incubated for 24 h at which time luciferase activity was determined as above (Section 2.6.1).

2.7.2 Competition assays with CHO cell lines

CHO-NT, CHO-al2 and CHO-al2/HER3 were infected as in Section 2.5, except prior to virus infection the cells were incubated with 100 µl PBS supplemented with recombinant human HRG1-α EGF-like domain (296-HR, R&D Systems) at 5 µg/ml for 30 min on ice. Additionally, the MOI of 1 PFU/cell used for virus infection was calculated based on the cell number plated (2.5e5 cells/well). Ten microliters virus diluted in PBS++ was added without removal of the HRG, and the cells were incubated for an additional 30 min on ice, then 10 min at room temperature (to allow for viral internalization). The wells were washed twice with PBS and fresh medium was added prior to incubation at 37°C for 48 hrs. The procedure for luciferase assay (Section 2.6.1) was then performed.

2.8 Mouse tumor model and in vivo infection

2.8.1 Mouse tumor model

All animal experiments were carried out in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the local animal care committee of the Cross Cancer Institute. 5×10^5 or 2×10^6 ZR75.1 human breast cancer cells were suspended in 25% Matrigel (356234, BD Biosciences) in PBS and injected into each of two contralateral abdominal mammary fat pads of 10

female NIH-III mice. An estrogen pellet (17β-estradiol, SE-121, Innovative Research of America) was also implanted subcutaneously in each mouse at the same time, to facilitate tumor growth. Animals were housed in sterile surroundings with standardized light/dark cycle and access to food and water *ad libitum*. Tumor growth was measured in three dimensions using digital calipers and the size calculated using the formula for the volume of a typical ellipsoid: Tvol = π/6 x width x length x depth (446).

2.8.2 Intratumoral injection of virus

After 40 days, the mice were separated into two groups with approximately equal tumor volumes. One tumor per mouse was injected with 3 x 10^8 pfu of either AdLuc(HRG-fiber) or AdLuc(wt-fiber) (5 mice each). This was repeated twice over a total of one week. Mice were sacrificed 2 days after last viral injection, and tumors and livers were harvested, flash frozen in liquid nitrogen, and stored at -80°C until analysis.

2.8.3 Mouse tissue analysis

Mouse tissues were homogenized by dissociation (GentleMACS, Miltenyi Biotec) in lysis buffer RLB (1 ml per 0.1 g of tissue). Lysates were frozen at -80°C, then thawed and centrifuged. The cleared supernatant was transferred to fresh tubes, and protease inhibitors (PMSF (P7626, Sigma) and protease inhibitor cocktail (P-8340, Sigma)) were added to a 100 μl portion for use in Western blot analysis. Both this Western blot portion and the remaining sample were stored at -80°C. Protein

concentrations were detected by BCA assay (Pierce® BCA Protein Assay Kit, #23225, Thermo Scientific). Samples were analyzed for reporter gene expression by luciferase assay (Section 2.6.1) and receptor expression levels by Western blot (Section 2.4.2).

2.9 Binding assays

2.9.1 Fluorescent virus binding (flow cytometry)

CHO cell lines were trypsinized, washed twice in PBS and resuspended in single cell suspension at 2e6 cells/ml in PBS + 0.5% BSA + 0.1% azide. 1e5 cells (50 µl) were aliquoted into tubes and 10 µl of virus was added (final MOI 1e5 vp/cell). Virus and cells were incubated 30 min on ice, then 10 min at room temperature. The cells were washed with PBS + 0.5% BSA, and resuspended in 500 µl PBS + 0.5% formaldehyde (v/v) and stored at 4°C before fluorescence was detected by a flow cytometry as described in Section 2.3.2.

2.9.2 Cells binding to immobilized virus

In order to test binding between cells and virus, virus was bound to plates and the amount of cells that bound to virus was measured by a metabolic assay as follows. The day before the experiment, 50 µl of virus (1e10 viral particles per well) in PBS++ was added to wells in 96-well polystyrene high-binding plates (Costar, #9018, Corning Inc.). Plates were sealed and stored overnight at 4°C. The next day the plates were rinsed with PBS, coated in 100 µl blocking buffer (PBS + 0.1% BSA), then incubated for 1 hr at room temperature to block non-specific binding.

The cell lines indicated were trypsinized, counted and diluted in PBS to a final concentration of 1e6 cells per well (50 µl). Blocking buffer was removed from the plates and the cells were added to the plate on ice and incubated for 1 hr, before transferring to 37°C for 10 min. The wells were washed with PBS, before the addition of 200 µl of PBS and 20 µl of resazurin sodium salt (440 µM, R7017-5G-Sigma). The plates were incubated for 4 hrs at 37°C before measurement of fluorescence (excitation 570 nm, emission 585 nm) by a plate reader (FLUOstar). A standard curve of each cell line from 5e5 to 3.9e3 cells per well was used to calculate the cell number bound to each well. This curve was set up and run on the same plate as the experimental samples, however, in this case the cells were not washed off the plate.

2.10 Internalization assays and inhibition by CPZ

2.10.1 Fluorescent virus internalization (flow cytometry)

CHO cell lines were trypsinized, washed in PBS + 0.5% BSA + 0.1% Azide or PBS alone and resuspended at 1e6 cells/ml in Krebs-HEPES buffer (140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 11.7 mM glucose, 0.2% BSA, 5 mM HEPES, pH 7.4), with or without the clathrin-mediated internalization inhibitor chlorpromazine (CPZ, 25 µg/ml, 70 µM, C8130, Sigma-Aldrich). 5e4 cells (50 µl) were aliquoted into tubes and incubated for 30 min at 37°C, before the addition of 10 µl of virus (final MOI 1e4 vp/cell). Virus and cells were incubated 30 min on ice, then 10 min at 37°C. The cells were washed with PBS, then trypsinized (0.5% in Versene, 0.21 mM, 15090-046, Gibco) for 10 min at 37°C to

allow removal of virus not taken up by the cells. The cells were then washed with PBS again, and finally resuspended in 500 µl PBS + 5% formaldehyde and stored at 4°C before fluorescence was detected by flow cytometry as described in Section 2.3.2.

A488 labeled transferrin (T13342, Molecular Probes) was used as a control for internalization and inhibition of clathrin-mediated internalization by CPZ.

2.10.2 Virus luciferase assay with CPZ inhibition

A procedure similar to Section 2.10.1 was performed, except that the final resuspension was not in PBS + 5% formaldehyde, but in 1 ml of medium and plated into a 24-well plate. The cells were incubated for 48 hrs at 37°C, and the procedure for luciferase assay (Section 2.6.1) was then performed. A BCA assay (Pierce) was also performed to determine protein concentration of the samples.

2.11 Immunofluorescence Assays

2.11.1 Fluorescent virus internalization (microscopy)

This procedure is similar to Section 2.5, except cells were plated, at an amount to be 70% confluent on the day of the infection, in a 24-well plate containing a sterile coverslip in each well. Only the cell lines CHO-al2/HER3 and CHO-CAR were used in this experiment. Cells were counted the day they were plated to determine cell number per well for MOI calculations. Fluorescently labeled viruses (Section 2.2.3) were used for infection. Cells were incubated with virus for 30 min on ice, then incubated for 10 min or 30 min at 37°C, before they were washed three times

with PBS. The cells were fixed on coverslips in 4% paraformaldehyde (P6148-500G, Sigma-Aldrich) in PBS and washed with 20 mM glycine in PBS, prior to permeabilization with 0.4% Triton X-100 (R06433, BDH) in PBS. The cells were washed three times in PBS, and blocked in 4% BSA in PBS. The coverslips were then inverted onto 20-30 µl of primary antibody in PBS, and incubated for 1 hr at room temperature. Coverslips were then washed three times with 1 x PBS, prior to incubation with secondary antibody coupled with a fluorophore. Coverslips were washed three times with PBS and a final time with distilled water, prior to mounting on slides using a 90% glycerol-PBS based medium containing an anti-fade (1 mg of paraphenylenediamine/ml and 0.5 mg DAPI/ml). CHO-al2/HER3 cells were probed from HER3 expression using the primary antibody C-17 (ErbB3, SC-285, Santa Cruz) at 1:200 dilution, and a Cy3 conjugated donkey anti-rabbit antibody (711-165-152, Jackson ImmunoResearch Laboratories, Inc.). CHO-CAR was probed for CAR expression using undiluted supernatant harvested from mouse hybridoma cells (IgG1, RmcB, CRL-2379, ATCC), and a Cy5 conjugated donkey anti-mouse antibody (715-175-150, Jackson ImmunoResearch Laboratories, Inc.). The sample was then imaged on a Zeiss 710 LSM equipped with a Plan-Apochromat 40x/1.3 Oil DIC M27 objective.

Images were processed using Imaris Software (7.6.0, Bitplane Scientific Software). A surface was created based on the red channel signal (receptor: CAR or HER3) to represent the edge of the cell. This surface was smoothed to area level detail of 1.00 µm, with absolute threshold adjusted to a level appropriate to the size of the cell, making sure all cells present in the image are included. This surface was also set to

70% transparency, to make visualization of the virus inside the cell clearer. Spots were created to represent the virus, using an estimated diameter of 0.5 μm. This size is bigger than individual virus particles (~90 nm), but is appropriate because the resolution for a conventional light microscope for green and red fluorphores is approximately 250-300 nm and 300-350 nm, respectively. By using a larger sphere to represent the virus, we can be more accurate when judging a virus signal as inside or outside the cell. We also included a category of "on the edge" of the cell surface to include virus signals which were not clearly inside or outside the cell. Some of the virus signals appeared bigger than 0.5 μm. When determining whether the virus signal is outside, inside or on the edge of the cell, the surface and spots previously created were used. The cells were viewed in 3D from many angles and each sphere containing virus particles was put into one of the categories above. In order to determine colocalization of the virus (green channel) with the receptor (red channel), each channel was automatically scaled and colocalization was quantified above a threshold value. Usually the threshold is determined by the mean of a background area plus two standard deviations, in order to separate signal from background. Based on a visual inspection of the colocalization module of the Imaris Software, we chose to take a threshold of 10% of maximum intensity of each channel in each stack. We did this consistently with every stack acquired. This threshold was more stringent than mean plus two standard deviations of background, and gives an impression of the colocalization of the two signals.

CHAPTER 3: Insertion of heregulin into the adenovirus fiber protein expands viral tropism *in vitro*, but not following intratumoral injection in mice

Portions of this chapter have been published previously (3).
S. MacLeod performed all experiments in this chapter, except as noted below.
M. Elgadi performed experiments in Figures 3.2, 3.3A, 3.5, with assistance from G. Bossi and U. Sankar.
K. Agopsowicz performed mouse xenograft injections, virus injections and tumor removal for Figures 3.7 and 3.8, and assisted with cell staining in Figure 3.1.
D. Sharon assisted with the Western blots in Figure 3.9.
A. Pisio contributed to the generation of the CHO-al2/HER3 and CHO-CAR/HER3 cell lines.
F. Graham and M. Hitt contributed to the design of experiments and analysis of results.

3.1 Introduction

Research during the past two decades has underscored the potential of adenoviruses (Ads) as gene delivery vehicles.(88) Subgroup C human Ads (exemplified by the most widely used Ad5 and Ad2) attach to and enter host cells in a two-step process.(56) The initial recognition/attachment step is mediated by interactions between the knob component of fiber protein on the Ad capsid and the extracellular domain of the coxsackie adenovirus receptor (CAR) on the host cell surface.(57-61) This attachment is followed by a second interaction between the RGD motif in the penton base of the virus capsid and cell surface integrins (secondary Ad receptors), which allows viral entry *via* receptor-mediated endocytosis. (64-67) Both the primary and secondary Ad receptors are expressed on a wide range of tissues leading to the observed broad tissue tropism of Ads. (125; 126) Unfortunately, a large number of tumor cells appear relatively refractory to Ad infection because of limited surface expression of the primary Ad receptor. (88; 131; 132; 134; 135) This observation has been a driving force behind recent intensive efforts to generate Ad viral vectors with altered tropism.

Fiber has been a major target of most attempts to alter Ad tropism. The highly flexible HI loop of fiber knob, oriented away from the viral particle, is not required for CAR binding and probably does not contribute to trimer assembly, making the HI loop a potential site for insertion of targeting ligands. To overcome suboptimal expression of CAR in cancer cells and to demonstrate the utility of the HI loop to accommodate relatively large targeting ligands, we sought to expand the viral tropism by insertion of the epidermal growth factor (EGF)-like domain of

heregulin-α (HRG) into the HI loop. HRGα, also called neuregulin and Neu differentiation factor, (333; 335) binds directly via the EGF-like domain (334; 336; 337) to HER3/ErbB3 and/or HER4/ErbB4, members of the EGF receptor family, which then homodimerize or heterodimerize with other family members to initiate a transmembrane signal. (342; 349; 350) An HRG-targeted virus may be valuable for the treatment of a number of human malignancies, including breast and ovarian carcinomas, in which members of HER receptor family are frequently overexpressed. (321; 407; 447)

Here, we show that the modified virus was more efficient than control virus in *in vitro* gene transfer to cells expressing the cognate receptors for HRG. Moreover, competition experiments with recombinant HRG or soluble knob clearly demonstrated that the modified virus was capable of entering cells *via* a novel HRG-mediated pathway in addition to the native CAR-dependent mechanism. Addition of the HRG EGF-like domain to the virus fiber knob confers on the virus the ability to infect cell lines poorly infected by virus with native capsids, and this infectivity is at least partially dependent on HRG binding. In contrast, we did not observe a difference in gene transfer between the modified virus and the control virus following intratumoral injections in a mouse breast cancer xenograft model. We suggest that this loss of differential infectivity could be due to the high local concentrations of virus resulting from intratumoral injections. We propose that it may be possible to improve this differential by increasing virus dispersion intratumorally either by modifying virus delivery or by using conditionally replicating virus.

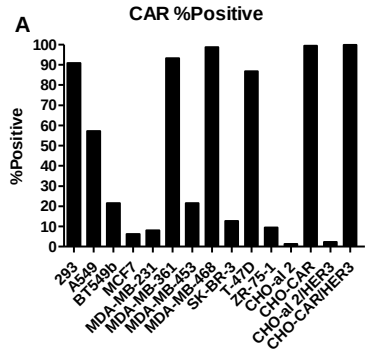
3.2 Results

3.2.1 Cell surface receptor expression in cell lines

In order to determine whether viral binding, internalization and gene expression is correlated with expression of receptors, cell surface expression of receptors were measured by flow cytometry using antibodies as described in section 2.3.1. CAR levels were measured because CAR is the primary Ad receptor; and CAR levels are linked to viral infectivity (40; 41; 57; 137; 138; 448). Likewise, α_v-integrin forms the common half of the secondary Ad receptor, $\alpha_v\beta_3$ or $\alpha_v\beta_5$, so α_v-integrin levels could affect viral internalization and ultimately gene expression (62-64; 67; 449; 450). HER3 and HER4 levels were measured since these are the receptors the retargeted Ad, AdLuc(HRG-fiber), is expected to bind, and they may mediate internalization of this virus. Receptor expression was measured in a panel of breast cancer cell lines, in addition to control cell lines and CHO cell lines transfected with relevant human receptors (Figure 3.1). Both the proportion of cells expressing the receptor and the relative number of receptors per cell were measured, as both of these values may have important effects on virus infection in cells.

3.2.1.1 Expression of CAR

A high degree of variability in cell surface CAR was observed. A high proportion of cells expressing CAR was detected in three out of nine breast cancer cell lines (Figure 3.1A). These cell lines also expressed a larger number of receptors per cell, compared to the other breast cancer cell lines, and were considered CAR positive (Figure 3.1B). The cell lines which expressed low numbers of receptors per cell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MDA-MB-361
MDA-MB-453
MDA-MB-468
SK-BR-3
T-47D
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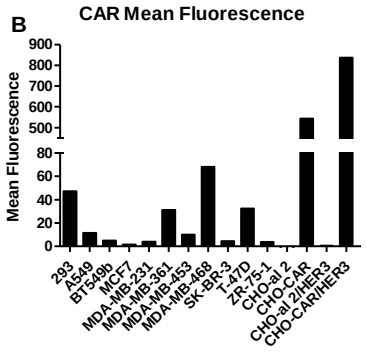
CHO-al 2
CHO-CAR
CHO-al 2/HER3
CHO-CAR/HER3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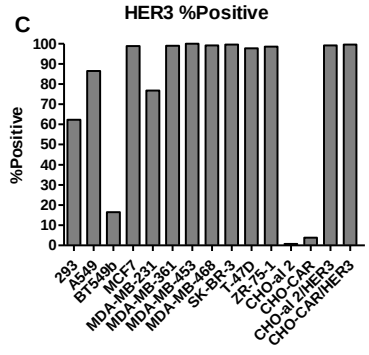
CHO-CAR
CHO-al 2/HER3
CHO-CAR/HER3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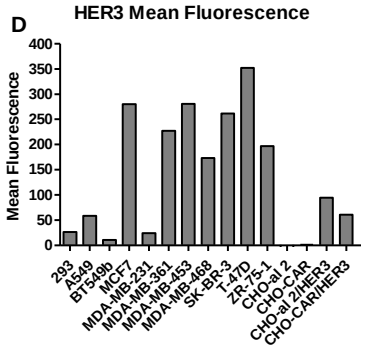
CHO-al 2
CHO-CAR
CHO-al 2/HER3
CHO-CAR/HER3


D
HER3 Mean Fluorescence
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BT549b
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MDA-MB-361
MDA-MB-453
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SK-BR-3
T-47D
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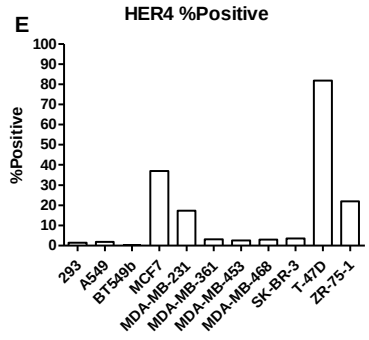
CHO-al 2
CHO-CAR
CHO-al 2/HER3
CHO-CAR/HER3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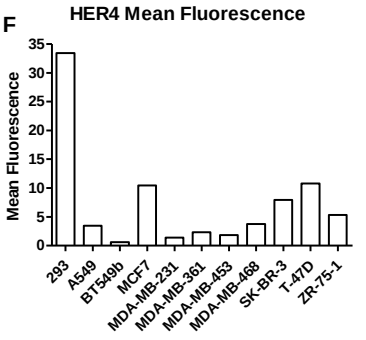
T-47D
ZR-75-1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HER4 Mean Fluorescence
Mean Fluorescence
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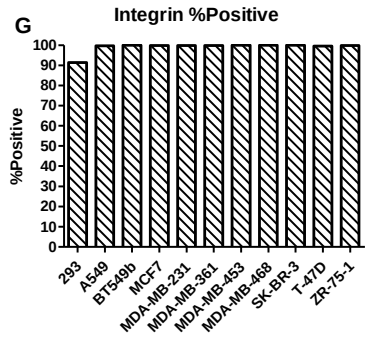
ZR-75-1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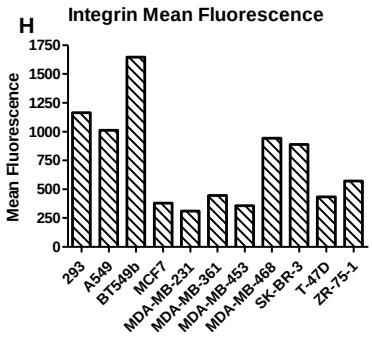
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MDA-MB-361
MDA-MB-453
MDA-MB-468
SK-BR-3
T-47D
ZR-75-1

Figure 3.1: *Cell surface expression of receptors in various cell lines, measured by flow cytometry.*

Variability in receptor expression can be seen both in the proportion of cells expressing the receptor (% positive: A, C, E, G) and in the relative number of receptors on the cell surface (mean fluorescence – isotype control: B, D, F, H). Both values are shown for each cell line for the receptors CAR (A, B), HER3 (C, D), HER4 (E, F) and α_v-integrin (G, H). CHO cell lines were not tested for HER4 or integrin expression. All cell lines are breast carcinoma except for the control cell lines 293 (human embryonic kidney) and A549 (lung carcinoma), and the CHO cell lines transfected with the human receptors indicated. Parts of this figure were previously presented in MacLeod *et. al.* (2012) (3).

generally also had a low proportion of cells which expressed the receptor, and as a result were considered CAR negative in future experiments.

Unsurprisingly, both CAR transfected CHO cell lines expressed this receptor on close to 100% of cells. These cell lines also had a larger number of receptors per cell, and this number was much higher than any other cell line (8 to10 fold higher). The magnitude of the difference in receptor expression compared to the positive breast cancer cells may be an important consideration in viral infection experiments. Additionally, CHO-CAR/HER3 had more receptors per cell than CHO-CAR. The CHO cell lines which were not transfected with CAR were negative for CAR expression, in agreement with previous results (57).

3.2.1.2 Expression of HER3

HER3 expression was also variable in the different cell lines, and a large number of HER3 receptors were detected in seven out of nine breast cancer cell lines on more than 97% of cells (Figure 3.1C and 3.1D). As a result, these cell lines were considered positive for HER3. BT549b cells had both a low receptor concentration and a low proportion of cells expressing the receptor, and was considered negative for HER3. Oddly, MDA-MB-231 cells had a low receptor concentration per cell, but more than 70% of cells expressed these receptors. Due to the low receptor concentrations, MDA-MB-231 cells were considered negative for HER3 expression.

Of the CHO cell lines, those which were transfected with HER3 expressed this receptor on almost 100% of cells. However, though the number of receptors

expressed on the surface of these cells was higher than HER3-negative cell lines, it was noticeably less than any of the HER3 positive breast cancer cells. As a result, though these cell lines were also considered positive for HER3, the low receptor concentration may lead to lower levels of viral infection in these cell lines. Regardless, the numbers of HER3 receptors per cell in the HER3 transfected CHO cell lines were similar to each other.

3.2.1.3 Expression of HER4

HER4 expression was also variable, and the relative number of HER4 receptors per cell on most cell lines was low (Figure 3.1F). Furthermore, only four of the nine breast cancer cell lines had more than 10% of the cells expressing HER4, but only three of these cell lines expressed enough HER4 per cell to be above baseline (Figure 3.1E and 3.1F). All three of the HER4 positive breast cancer cell lines also expressed high levels of HER3. MDA-MB-231 were about 20% positive for HER4, but with low HER4 levels per cell. This cell line was also odd in HER3 expression, where most of the cells expressed only a low level of HER3 receptors. Surprising, though very few 293 cells expressed HER4, the average number of receptors per cell was more than three-fold higher than any of the breast cancer cell lines. Due to the overall low levels of HER4 expression, the levels of HER4 were not used for subsequent cell classification. CHO cell lines were not tested for HER4 expression.

3.2.1.4 Expression of α_v-integrin

Finally, the panel of cell lines was tested for α_v-integrin expression. All cells expressed α_v-integrin on more than 90% of cells. However, there was some variability in the relative number of receptors per cell, within about a five-fold range. It is possible that cells with higher α_v-integrin expression per cell could take up both viruses more effectively, since both of these viruses retain integrin binding activity. For this study all cells were considered positive for α_v-integrin, and this receptor was not used for further cell categorization. CHO cell lines were not tested for α_v-integrin expression.

3.2.1.5 Cell line categories

The panel of cell lines was divided roughly into four categories, based on HER3 and CAR expression and summarized in Table 3.1: (i) cells which expressed moderate to high levels of CAR and HER3, (ii) cells which expressed low or undetectable levels of CAR and high levels of HER3, (iii) cells which expressed moderate to high CAR but low levels HER3, and (iv) cells which expressed very low or undetectable levels of both CAR and HER3.

Many of the cell lines outlined above were also assessed for receptor expression by Western blot analysis in a previous study carried out by M. Elgadi (McMaster University, Figure 3.2). Though there were many similarities in the cell line categorization, there were some differences (Table 3.1). Notable differences include MDA-MB-361 cells, which appeared to express less CAR in the Western blot analysis, and MDA-MBA-231, which appeared to express more CAR and less

Cell line	Category	CAR	HER3/4
(A) *Flow cytometry and Western blot*			
A549	(i)	+	++
MDA-MB-468	(i)	++	+++
T47D	(i)	++	+++
ZR-75-1	(ii)	(-)	+++
MCF7	(ii)	(-)	+++
MDA-MB-453	(ii)	(-)	+++
SK-BR-3	(ii)	(-)	+++
293	(iii)	++	(-)
(B) *Flow cytometry*			
MDA-MB-361*	(i)	++	+++
CHO-CAR/HER3	(i)	++++	++
MDA-MB-231*	(ii)	(-)	+
CHO-al 2/HER3	(ii)	(-)	++
CHO-CAR	(iii)	++++	(-)
BT549b	(iv)	(-)	(-)
CHO-al 2	(iv)	(-)	(-)
CHO-NT	(iv)	(-)	(-)
(C) *Western blot*			
SKOV-3	(i)	+++	(-)
HeLa	(i)	+	(-)
MDA-MB-361*	(ii)	(-)	+++
RD	(ii)	(-)	++
MDA-MB-231*	(iii)	+	(-)
BT549a	(iv)	++	(-)
U118MG	(iv)	(-)	(-)

Table 3.1: *Cell lines categorized by CAR and HER3 levels.*

Receptor levels were measured by (B) flow cytometry (Figure 3.1), (C) Western blot (Figure 3.2), or (A) both. In all cases the results were consistent between the two assays, except the cell lines MDA-MB-361 and MDA-MB-231, each marked by an asterisk.

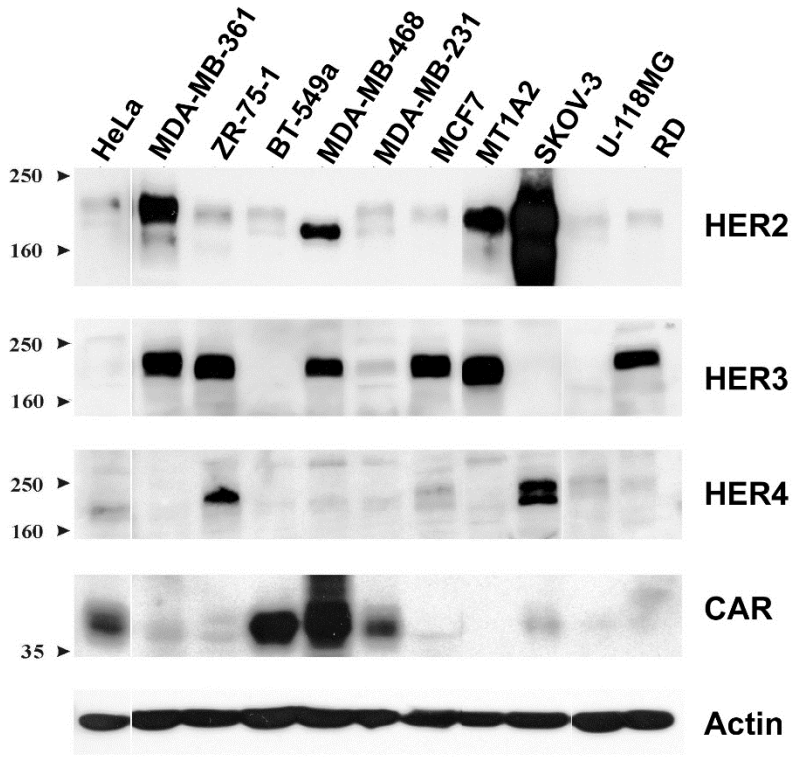
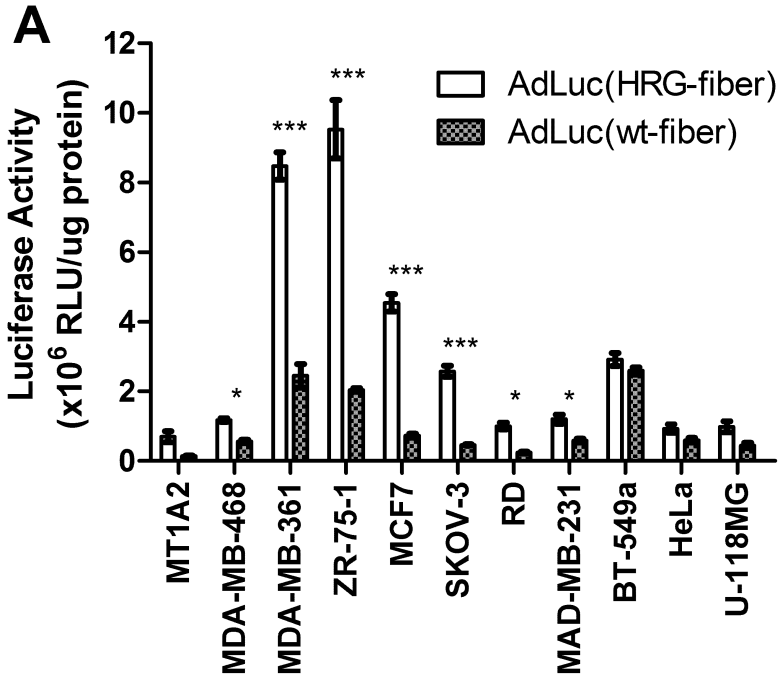


Figure 3.2: *Expression of cell surface receptors in various cell lines.*
Analysis of expression levels of HER2, HER3, HER4 and CAR in the indicated cell lines. Lysates were resolved by SDS-PAGE, blotted, and probed for the indicated proteins as described in Section 2.4.1. The position of molecular weight markers and their corresponding sizes (in kDa) are located to the left of each panel. The Western blot of β-actin as loading control is shown in the bottom panel. The data were generated, and this figure was created by M. Elgadi (McMaster University). This figure was also previously presented in MacLeod *et. al.* (2012) (3).

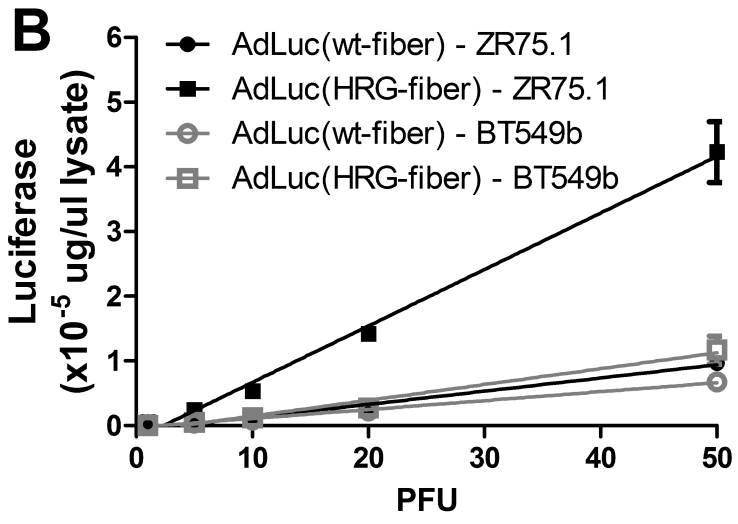
HER3 in the Western blot analysis. In addition, two separate isolates of BT549 cells (here designated BT549a (Figure 3.2) and BT549b (Figure 3.1) demonstrated different levels of CAR. These differences may be explained by the different methods used to detect the receptors (Western blot versus flow cytometry), or differences in inherent or conditional levels of expression by the two isolates, which were analyzed at two separate institutions more than two years apart. Because of the potential disparity, we used the receptor levels measured at the same institution, at the same time as the relevant reporter assay to categorize the cell lines. Additional cell lines tested by Western blot only include the following human cancer cell lines: SKOV-3 (ovarian), RD (rhabdomyosarcoma), HeLa (cervical) and U118MG (malignant glioma).

3.2.2 Increase in gene expression after infection with AdLuc(HRG-fiber) in breast cancer cells expressing receptors HER3

Previously, our lab compared reporter gene expression after infection with either AdLuc(HRG-fiber) or AdLuc(wt-fiber) in cells tested for gene expression by Western blot (Figure 3.3A). As expected, AdLuc(wt-fiber) infection of cell lines with very low levels of CAR (*e.g.*, MCF-7) resulted in very low absolute levels of reporter gene expression. However, some cell lines expressing high levels of CAR did not show high infectivity (*e.g.*, of cancer lines, MDA-MB-468 showed the highest level of CAR, but relatively low levels of reporter expression), which suggested that other factors involved in Ad entry (*e.g.*, internalization mechanism), nuclear localization, CMV promoter activity, and/or other processes could also



HER3/4	high	high	high	high	high	high	high	low	low	low	low
CAR	ND	+++	+/-	+/-	-	+/-	+/-	+	++	+	+/-



	ZR-75-1	BT-549b
HER3	high	low
CAR	-	-

Figure 3.3: *Luciferase expression in breast cancer cell lines 24 hours after infection with AdLuc(HRG-fiber) compared to AdLuc(wt-fiber).*

Luciferase expression in breast cancer cell lines 24 hours after infection with AdLuc(HRG-fiber) compared to AdLuc(wt-fiber).

(A) Comparison of viruses at a single MOI (1000 vp/cell). Luciferase activity is expressed as relative light units (RLU) per μg of total protein. *** significant difference between AdLuc(HRG-fiber) and AdLuc(wt-fiber) infections in the same cell line at $p < 0.001$, * significant at $p < 0.05$, by ANOVA. (B) Dose response of infection of HER3-high ZR75.1 cells compared to infection of HER3-low BT-549b cells. Luciferase activity is expressed as μg luciferase/ μl of cell lysate. Luciferase activity in infections with AdLuc(HRG-fiber) was significantly different from each of the other infections at each MOI ($p<0.01$, ANOVA). The tables below each figure indicate receptor levels as measured by (A) Western blot (see Figure 3.2) or (B) flow cytometry (see Figure 3.1) performed at the same time as the luciferase assay. Cells were infected in triplicate. Error bars are equal to one standard deviation. Data for Figure 3.3A was generated by Mabrouk Elgadi. This figure was previously presented in MacLeod et. al. (2012) (3).

have contributed to transgene activity. The influence of these variable factors on interpretation of results was minimized by directly comparing luciferase expression after AdLuc(HRG-fiber) infection to that after AdLuc(wt-fiber) infection for each cell line. An increase in luciferase expression was seen after AdLuc(HRG-fiber) infection relative to AdLuc(wt-fiber) infection in all cell lines that express high levels of the HRG receptors HER3/4 [categories (i) and (ii)]. This increase was statistically significant in all but the mouse breast cancer cell line MT1A2, which was poorly infected by both viruses. In contrast, for three of the four cell lines that have no or low HER3/4 [categories (iii) and (iv)], there was no statistical difference between reporter expression in infections with the two viruses. It is possible that the low but detectable level of HER3 expressed in the fourth cell line of this group (MDA-MB-231) was sufficient to increase infectivity by AdLuc(HRG-fiber). The level of HER2 in our panel of cells did not seem to have either a positive or negative impact on reporter gene expression. These results are consistent with the hypothesis that HER3 and/or HER4 mediate internalization of the HRG-modified fiber virus, at least in CAR-negative cell lines.

In order to further examine whether the increase in reporter gene expression observed after infection with AdLuc(HRG-fiber) is retained at a range of viral doses, two cell lines were examined in greater detail: ZR-75-1 [category (ii), high HER3 and low CAR] and BT549b [category (iv), low HER3 and low CAR]. A linear dose response with both viruses up to an MOI of 50 PFU/cell was observed (Figure 3.3B). There was very little variability in luciferase expression between triplicate samples. Luciferase expression in ZR-75-1 infected with AdLuc(HRG-

fiber) was significantly higher than in other infections at each MOI. Again, these results are consistent with the hypothesis that HER3 and/or HER4 mediate internalization of the HRG-modified fiber virus.

3.2.3 Increase in gene expression after infection with AdLuc(HRG-fiber) in CHO cell lines expressing receptors other than CAR

As noted in the previous section, infectivity can be dependent on many factors in addition to primary receptor expression. Thus, it is important to examine Ad-delivered reporter gene expression in isogenic cell lines that differ only in receptor expression. Previously, Bergelson *et al.* (1997) demonstrated that CAR expressing CHO cells are susceptible to infection by wild-type Ad5 infection, and control cells expressing the unrelated receptor human α2 integrin are refractory (57). We acquired both of these cell lines and used them to generate stable cell lines that express moderate levels of HER3 (CHO-al2/HER3 and CHO-CAR/HER3). CAR and HER3 levels were tested by flow cytometry (see Figure 3.1). Additionally, we acquired a CHO cell line that did not express any exogenous receptors, CHO-NT, from Dr. Zhixiang Wang. These cell lines were infected by both viruses in two separate experiments. CHO-al2, CHO-al2/HER3 and CHO-CAR were common to both experiments, whereas CHO-CAR/HER3 was used in the first experiment only (Figure 3.4A and 3.4B), and CHO-NT in the second experiment only (Figure 3.4C and 3.4D). The relative levels of luciferase generated after infection with the two viruses was in agreement in the two experiments (exceptions are noted below).

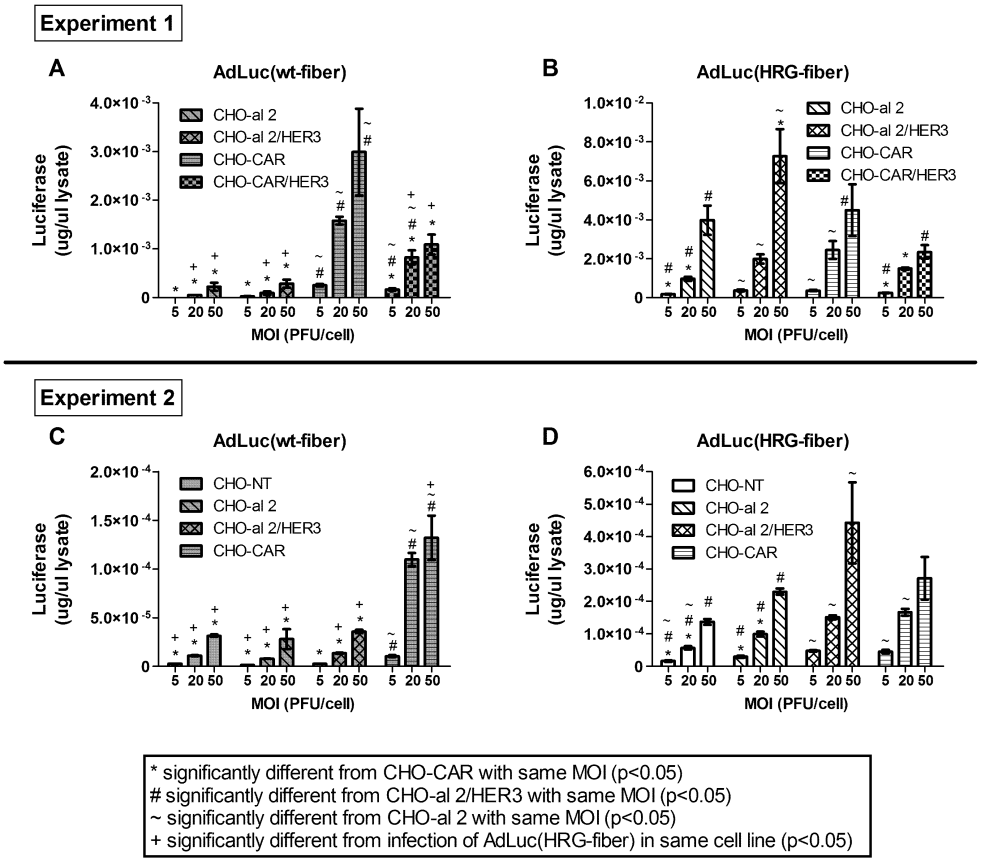


Figure 3.4: *AdLuc(HRG-fiber) and AdLuc(wt-fiber) infection of CHO cell lines stably transfected with CAR, HER3, and/or human α2 integrin (al 2) genes.*
NT= CHO cell line not transfected with any exogenous receptor gene. (A, C) Luciferase expression 48 hours after infection with AdLuc(wt-fiber) at multiple MOIs (PFU per cell, as indicated). Bar height represents the average of triplicates and error bars are one standard deviation. (B, D) AdLuc(HRG-fiber) was used to infect cells using methods and analysis as described for panel A. Both experiments were compared to a luciferase standard curve to generate measurements in μg/μl luciferase. Note differences in y-axis scales. Experiment 2 was previously presented in MacLeod *et. al.* (2012) (3).

3.2.3.1 Reporter gene transfer by AdLuc(wt-fiber)

High luciferase expression after infection with AdLuc(wt-fiber) was seen in CHO-CAR, compared to all other cell lines, in both experiments (Figure 3.4A and 3.4C). Luciferase levels in cell lines not expressing CAR were similarly low in all experiments. Surprisingly, infection of CHO-CAR/HER3 resulted in less luciferase expression than infection of CHO-CAR, but luciferase expression was still higher than cell lines not expressing CAR, particularly at lower MOIs. CAR levels cannot be used to explain the difference in CHO-CAR/HER3 and CHO-CAR, since CAR expression was actually higher on CHO-CAR/HER3 than on CHO-CAR. Additionally, there may be a plateau of gene expression in each cell line that is approached by the highest MOI in some of the cell lines.

3.2.3.2 Reporter gene transfer by AdLuc(HRG-fiber)

Luciferase expression after AdLuc(HRG-fiber) infection of CHO-CAR was similar to that after infection of CHO-al2/HER3 (Figure 3.4B and 3.4D). These two cell lines also showed significantly higher luciferase expression than the cell lines not expressing CAR or HER3 at most MOIs. Surprisingly, AdLuc(HRG-fiber) also infected CHO cell lines that expressed neither HER3 nor CAR. It is interesting that luciferase expression after AdLuc(HRG-fiber) infection of CHO-CAR/HER3 was reduced compared to infection of CHO-al2/HER3 or CHO-CAR (Figure 3.4B), which was similar to AdLuc(wt-fiber). These differences cannot be explained by receptor expression alone, but may be accounted for by receptor interference or that other pathways required for virus infection were altered in the CHO-CAR/HER3

cell line. Furthermore, a plateau of gene expression was seen in CHO cell lines transfected with CAR, but not necessarily in the other cell lines.

3.2.3.3 AdLuc(HRG-fiber) infection compared to AdLuc(wt-fiber) infection

AdLuc(HRG-fiber) infection generally resulted in significantly higher luciferase expression than AdLuc(wt-fiber) in most cell lines, except CHO-CAR. The lack of difference at low MOI seen in some cell lines, may not be real, since the RLU values obtained were very close to the limit of detection for the luminometer, especially for AdLuc(wt-fiber) in cell lines without exogenous CAR. We have shown here that the modifications to AdLuc(HRG-fiber) resulted in either similar or increased expression in some cell lines infected by wild-type binding Ad, and also expanded the tropism to cell lines not infected by AdLuc(wt-fiber).

3.2.4 Competition with soluble ligands decreases reporter gene expression in cell lines

In order to examine the interactions between the virus and the receptors CAR and HER3, we used soluble ligands to compete with virus for receptor binding. Soluble wild-type fiber knob (s-knob) was used to compete for CAR binding, and a recombinant peptide, the EGF-like domain of HRG-α1, was used to compete for HER3 binding.

3.2.4.1 Competition in cancer cell lines

In one experiment (M. Elgadi, McMaster), three cell lines differing in levels of receptor expression were infected with both viruses: MDA-MB-468 [category (i), high CAR and HER3/4], SKOV-3 [category (ii), low CAR, high HER3/4], and MDA-MB-231 [category (iii), high CAR, low HER3/4] (Figure 3.5). S-knob addition prior to infection with either virus significantly decreased luciferase expression in both cell lines expressing high CAR, but not in low CAR expressing SKOV-3. HRG addition significantly decreased expression of luciferase in both cell lines that expressed high HER3/4, but only after infection with AdLuc(HRG-fiber), as expected. There was not a large increase in inhibition of infection when the combination of s-knob and HRG was used. This is not surprising after infection with AdLuc(wt-fiber), since HRG alone did not inhibit luciferase expression after infection with this virus. However, since both s-knob and HRG inhibited luciferase expression significantly in MDA-MB-468 cells infected with AdLuc(HRG-fiber), one might have expected the combination to be better than either ligand alone. The fact that this was not the case may be linked to the thus far unidentified factors that limit viral infection in this cell line to below that expected by high receptor expression (see Figure 3.3A). Another notable result seen in this experiment is that s-knob decreased luciferase expression after AdLuc(wt-fiber) infection to almost undetectable levels in MDA-MB-468 cells, but no combination of inhibitors could decrease luciferase expression after AdLuc(HRG-fiber) to that level. This may demonstrate an increased ability of AdLuc(HRG-fiber) to circumvent the competition provided by s-knob, HRG and/or both.

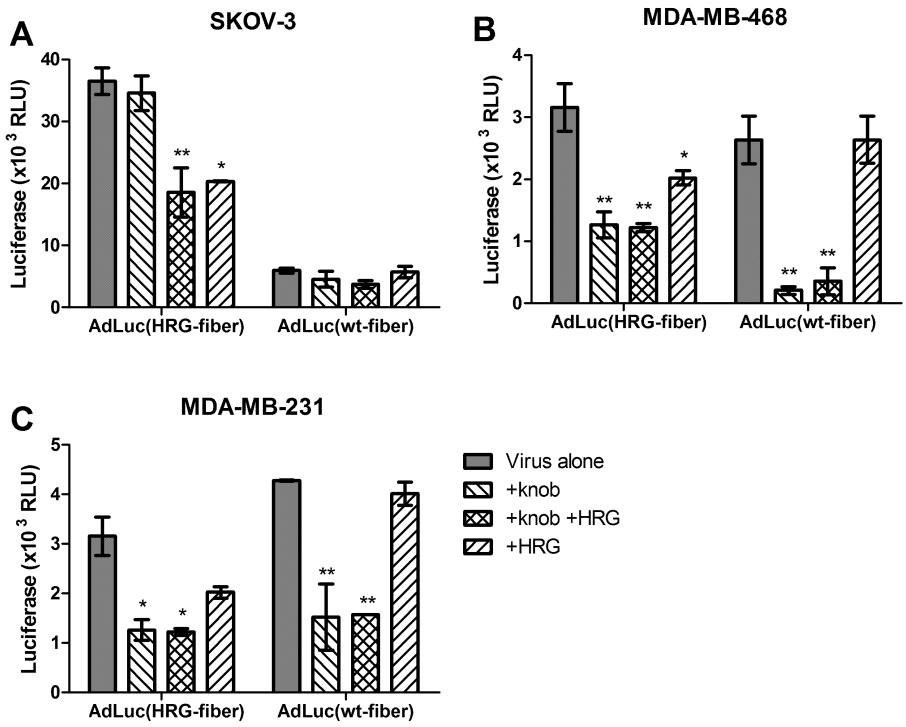


Figure 3.5: *Effect of recombinant soluble knob protein and/or EGF-like domain of HRG on reporter gene expression.*

(A) to (C) Cancer cell lines {(A) SKOV-3 [category (ii), low CAR, high HER3/4], (B) MDA-MB-468 [category (i), high CAR, high HER3/4] and (C) MDA-MB-231 [category (iii),moderate CAR, low HER3/4]} were untreated, treated with 2 μg of soluble knob protein, treated with 0.25 μg EGF-like domain of HRG-α peptide, or treated with both. Cells were then infected with either AdLuc(HRG-fiber) or AdLuc(wt-fiber) at an MOI of 100 vp/cell as outlined in Section 2.7.1. Luciferase activity (RLU) and total protein were determined 24 hour later. Each bar represents the average of duplicate infections and error bars represent one standard deviation. * significantly different from infection in the same cell line with no competitor added ($p<0.05$, ANOVA). ** $p<0.01$, ANOVA. The data were generated by M. Elgadi (McMaster University). This figure was previously presented in MacLeod *et. al.* (2012) (3).

3.2.4.2 Competition in CHO cell lines

We also used HRG in a competition study to determine whether AdLuc(HRG-fiber) infected CHO cell lines *via* the HER3/4 receptor (Figure 3.6). HRG significantly decreased luciferase expression in both CHO-al 2 and CHO-al 2/HER3, but not CHO-NT. This indicated that at least in these two cell lines, AdLuc(HRG-fiber) interacted with cell surface receptors that bind HRG. It is possible that the receptor that AdLuc(HRG-fiber) interacts with in HER3 negative CHO cell lines may be a hamster receptor that binds HRG. However, since we did not see significant inhibition of infection of CHO-NT cells by HRG, we hypothesize that AdLuc(HRG-fiber) may interact with CHO cell lines, albeit at a low level, in a manner that is not solely dependent on HRG-mediated interactions. This interaction appears to be specific to AdLuc(HRG-fiber), since AdLuc(wt-fiber) showed poor gene transfer to CAR-negative CHO cell lines (Figure 3.4A and 3.4B). Additionally, competition with HRG did not either increase or decrease reporter gene expression in CAR-negative cell lines after AdLuc(wt-fiber) infection (infection of CHO-al2/HER3 is shown in Figure 3.6).

3.2.5 Reporter gene expression after intratumoral injection of virus in vivo

All mouse experiments were carried out in accordance with CCAC guidelines at the Cross Cancer Institute Vivarium.

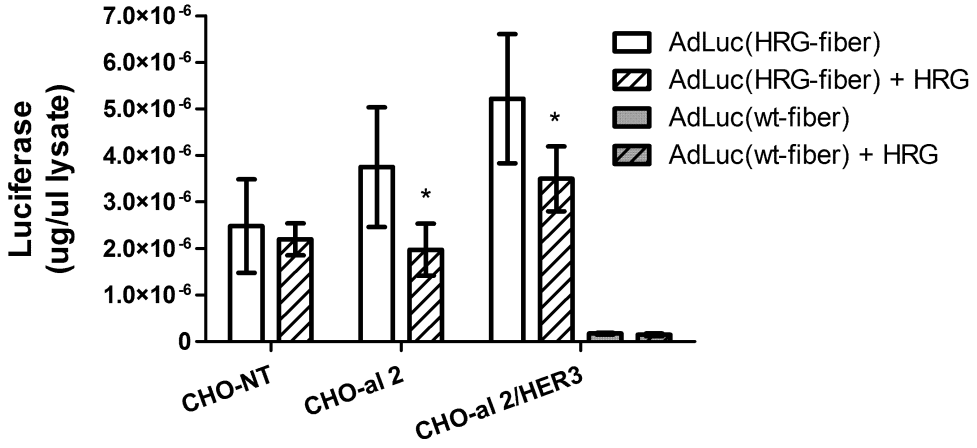


Figure 3.6: *HRG competition of infection of CHO cell lines by AdLuc(HRG-fiber) or AdLuc(wt-fiber).*

CHO cell lines stably transfected with indicated receptor genes (or no exogenous genes, CHO-NT), were untreated or treated with 0.25 μg EGF-like domain of HRG-α peptide then infected with either AdLuc(HRG fiber) or AdLuc(wt-fiber) at an MOI of 1 PFU/cell. Luciferase activity was determined 48 hour later. Each bar represents the average of quadruplicate infections with error bars representing one standard deviation. *significantly different from infection in the same cell line with no competitor added ($p<0.05$, t-test). This figure was previously presented in MacLeod *et. al.* (2012) (3).

3.2.5.1 Establishment of ZR75.1 tumor model

Our lab has expertise in the human breast cancer xenograft model using the cell line MDA-MB-231-luc, but this model was inadequate to test the infectivity of these viruses. First, this cell line does not express high levels of HER3, and the level of transgene expression after infection with either virus is not high. Furthermore, the cell line used to generate these tumors had been previously transfected with the reporter gene luciferase, which is also the reporter gene used in the viruses to be tested. As a result, it was necessary to choose a different breast cancer xenograft model. We chose the breast cancer cell line ZR75.1 because, in addition to expressing some of the highest luciferase activities after infection with both viruses *in vitro*, this cell line also demonstrated one of the largest differences between the two viruses in the experiment (Figure 3.3).

We first set up a pilot study to determine which of two cell doses might be better for future experiments. Ten mice were injected with either 5×10^5 or 2×10^6 cells in their abdominal mammary fat pads contralaterally. The tumors were measured every 3-6 days, and the volume was calculated using tumor measurements obtained with digital calipers (Figure 3.7). All mice developed two tumors, except for mouse 9, which had one tumor on the left side and two tumors on the right (9R, 9L, and 9R-A in Figure 3.7). Many tumors appeared to be formed by a single lobe, while others appeared to be multilobed. Most of the largest tumors appeared to be multilobed. Though the tumors varied in size, there was no discernible difference in tumors in mice injected with either cell dose.

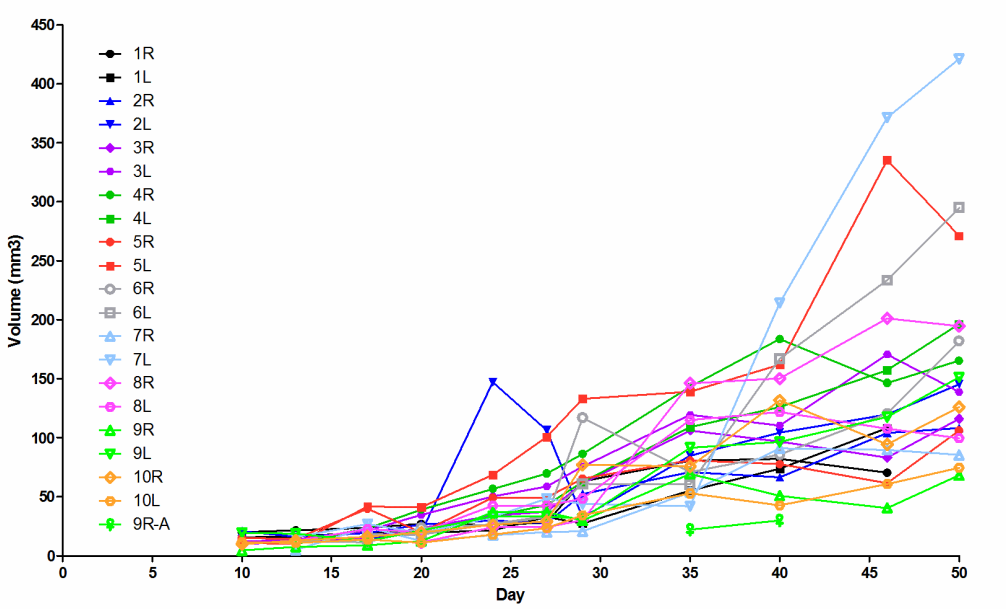


Figure 3.7: *Growth of ZR75.1 human breast tumors in a mouse xenograft model.*

The numbers in the figure legend represent the mouse number, while the letter represents either left (L) or right (R) tumor. Ten mice were implanted with estrogen pellets and injected with either 2 x 10^6 (solid symbols) or 5 x 10^5 (open symbols) ZR75.1 human breast cancer cells on day 0. Tumors were measured with digital calipers every 3-6 days beginning at day 10. On day 40 the average tumor size for 2 x 10^6 cells injected was 108 ± 39 mm^3 and 115 ± 53 mm^3 for 5 x 10^5 cells. Tumors were injected with 3 x 10^8 pfu each, on days 43, 46 and 48. Mice were euthanized on day 50.

3.2.5.2 Assessment of reporter gene transfer by AdLuc(wt-fiber) and AdLuc(HRG-fiber)

On day 40 post tumor cell injection, the mice were divided two groups to be injected with either of the two viruses (Table 3.2). We chose primarily single lobed tumors from the pilot study (one per mouse) to be injected, and sorted the mice so that the average tumor volume was similar between the groups. All mice were injected directly into a single tumor with either AdLuc(HRG-fiber) or AdLuc(wt-fiber) three times over a one week period (3×10^8 pfu per injection). Mice were euthanized 2 days after the last injection. Tumors and livers were harvested after euthanization, flash frozen in liquid nitrogen, and stored at -80°C. Tumors and livers were homogenized prior to testing for luciferase activity, protein concentration and receptor levels (as described in Section 2.8.3).

Reporter gene expression in injected tumors was much higher than in either the uninjected contralateral tumors or the livers (Figure 3.8C), as expected. Additionally, the reporter gene was under the control of the human cytomegalovirus immediate early promoter, which is not highly expressed in murine tissue (440). However, the two viruses were not statistically different in their abilities to mediate gene transfer to the xenografted tumors, and the ratio of gene transfer into tumors compared to livers was also similar (Figure 3.8A and 3.8B). This was in contrast to our observations with *in vitro* cultured breast cancer cells (Figure 3.3). We performed Western blots for HER3 and CAR in order to determine if receptor levels on the tumors were different than that observed *in vitro*, possibly contributing to the reporter gene expression levels seen here (Figure 3.9). HER3 levels were

	Tumor sizes (mm3, day 40)	
Mouse #	**injected**	**non-injected**
AdLuc(wt-fiber)		
1	82	74
3	110	97
7	91	214
9	51	96
10	132	43
mean ± SD	93 ± 30	
AdLuc(HRG-fiber)		
2	67	104
4	126	184
5	78	162
6	86	167
8	122	150
mean ± SD	96 ± 27	

Table 3.2: *Tumor sizes of ZR75.1 human breast tumors in a mouse xenograft model on day 40.*

Primarily single lobed tumors (one per mouse) were chosen to be injected. These tumors were divided into two groups to be injected with either AdLuc(wt-fiber) or AdLuc(HRG-fiber). The average size and standard deviation of the two groups are similar.

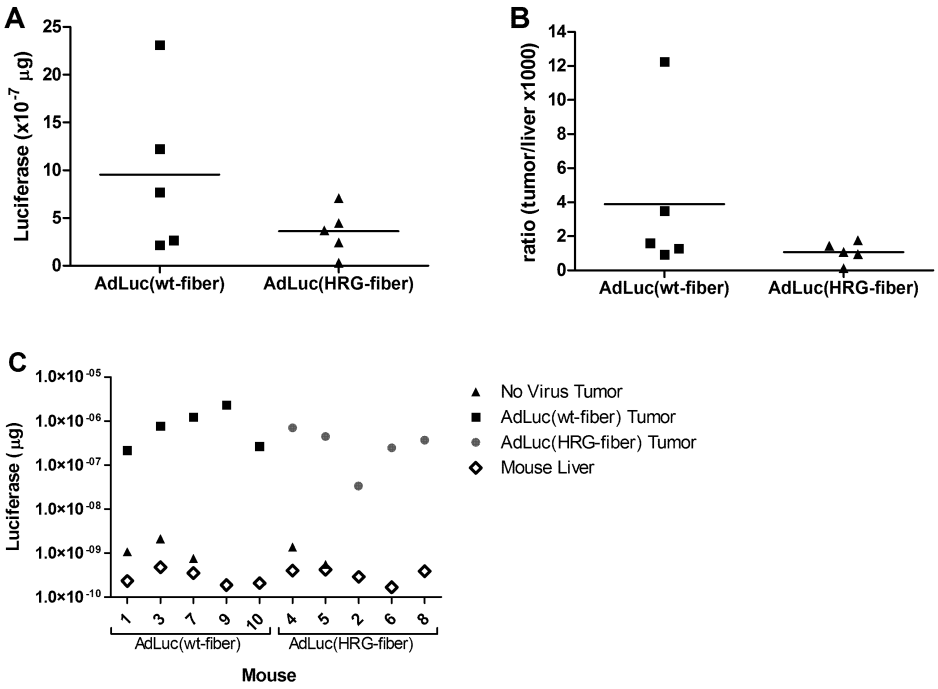


Figure 3.8: *AdLuc(HRG-fiber) and AdLuc(wt-fiber) reporter gene transfer after direct injection into ZR-75-1 human breast tumors established in NIH-III mice.* Tumors were injected with 3 x 10^8 pfu each, three times over a one week period. Tumors and livers were harvested two days after the final injection, flash frozen in liquid nitrogen, and stored at -80°C, before homogenization. (A) Luciferase expression in the whole tumor. (B) Ratio of luciferase expression in the whole tumor to luciferase expression in the liver of the same mouse. (A and B) Each point represents an individual animal and the bar represents the average. (C) Luciferase expression of the whole infected tumor, shown per individual mouse. As well as that in the uninjected contralateral tumor and/or liver. Luciferase expression was measured for each injected tumor and liver for each mouse, but was only measured in a subset of the uninjected tumor (n = 5 mice). Note: The y-axis in (A) and (B) are linear, while (C) is a log scale. Parts of this figure were previously presented in MacLeod *et. al.* (2012) (3).

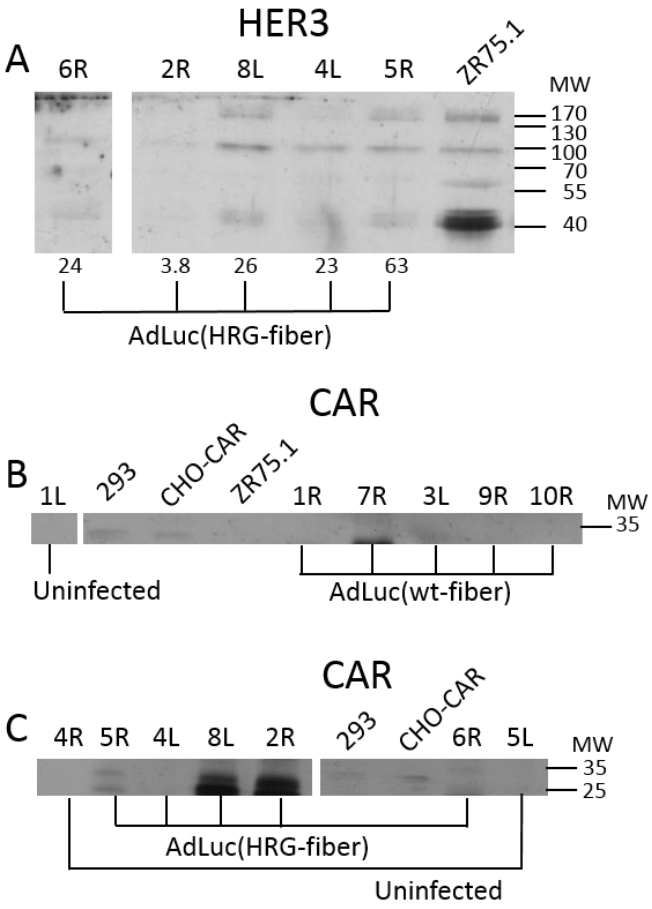


Figure 3.9: *Western blots of HER3 and CAR expression in mouse tumors.*
(A) HER3 expression in mouse tumors injected with AdLuc(HRG-fiber). The numbers above the lanes represent the mouse number and tumor side (L is left, R is right). The numbers below the blot represent the luciferase activity in 20 μg protein (the protein amount loaded per lane). Some HER3 bands likely represent degradation products. (B) CAR expression in mouse tumors injected with AdLuc(wt-fiber), except 1L is a an uninjected tumor. (C) CAR expression in mouse tumors injected with AdLuc(HRG-fiber), except 4R and 5L are uninjected tumors. 20 μg protein, as detected by a BCA assay was loaded into each lane. Other bands in (B) and (C) are likely a result of the reaction of the anti-mouse secondary antibody with mouse proteins.

slightly variable in the tumors, but this variability did not correlate with reporter gene expression levels (Figure 3.9A). CAR expression was not detected in the tumors (Figure 3.9B and 3.9C), which was as expected, since this cell line did not express CAR when tested *in vitro* (Figures 3.1A, 3.1B, and 3.2). Therefore, expression of HER3 or CAR does not appear to explain the lack of differential gene expression after infection with the two viruses. Possible reasons for the lack of differential gene expression will be discussed further in Chapter 5 (Section 5.4.8).

3.3 Discussion

A substantial effort has been invested in targeting Ad vectors by ablating the native tropism of Ad and providing alternate, tissue specific targeting ligands.(271)

In our studies we inserted a highly structured 68 aa residue insertion, consisting of the EGF-like domain of HRG and linker sequences, into the HI loop of fiber. To our knowledge, this insertion is the largest functional, structured ligand to be rescued into the HI loop thus far.

The choice of the EGF-like domain of HRG as the targeting ligand stems, in part, from its ability to target retroviral particles (438) and fused polypeptides (451) to cells expressing its cognate receptors, HER3 and HER4. In addition, because the HER3 and HER4 receptors are frequently over-represented in some cancers, (321; 407) an Ad with HRG-mediated expanded tropism may have potential therapeutic value.

Virions bearing HRG-modified fiber showed a significant receptor-dependent increase in transduction efficiency relative to wild-type fiber bearing virions.

Moreover, virions containing HRG-modified fiber are less sensitive to soluble knob protein competition, suggesting that, in addition to the native fiber/CAR entry pathway, the modified virus was able to utilize a novel CAR-independent pathway. The involvement of the HRG ligand in this novel pathway was confirmed by the observation that recombinant HRG protein significantly inhibited modified virus-mediated reporter gene expression while having no effect on that mediated by wild-type virus. Furthermore, the increase in reporter gene expression is unlikely to be due to effects mediated by the binding of HRG to HER3/4, such as promoter activation, as this effect was not seen with the addition of HRG to wild-type virus infection.

Unexpectedly, the higher *in vitro* infectivity of cells expressing high levels of HER3/4 seen with the fiber-modified virus, as compared with the wild-type binding virus, did not translate to the mouse xenograft model. This may be due, in part, to decreased dispersion of the virus after intratumoral injection due to the high pressure of the tumor. (452) This will be discussed in greater detail in Section 5.4.8. Taken together, the data presented here demonstrate the ability of the modification to the HI loop of fiber to result in increased gene expression in high HER3/4 cell lines, compared to wild-type binding Ad. Since we expect that this modification would affect the binding of AdLuc(HRG-fiber) to HER3+ cells, and potentially the internalization mechanism, we sought to examine this in greater detail in Chapter 4.

CHAPTER 4: Binding and internalization of HRG-targeted adenovirus occurs more slowly than wild-type adenovirus

S. MacLeod performed all experiments in this chapter, except as noted below.
M. Caindec performed the preliminary internalization shown in Figure 4.3.
H. Strickfaden assisted with all immunofluorescence experiments described in Sections 4.2.5 and 4.2.6, performed portions of the microscopy and analysed the experiment described in Section 4.2.6.

4.1 Introduction

In Chapter 3, we demonstrated increased gene expression in cell lines expressing high HER3/4, by AdLuc(HRG-fiber) compared to AdLuc(wt-fiber). The addition of HRG to the HI loop of fiber knob also expanded the viral tropism to cells not highly infected by AdLuc(wt-fiber). We expected that the increased gene expression seen in Chapter 3 was a result of increased binding of AdLuc(HRG-fiber) to HER3/4 receptors on the cell surface. To investigate receptor-specificity, we examined the binding of virus to CHO cells transfected with CAR and/or HER3 in several ways, including using fluorescently-labelled virus. Though gene expression studies have been performed with wild-type binding Ad in CHO-CAR cells, (57) to our knowledge no specific binding assays have been carried out using this system.

Because receptor binding may not be independent of internalization, we also examined the internalization of fluorescently-labelled AdLuc(HRG-fiber) and AdLuc(wt-fiber) into CHO cells transfected with CAR and/or HER3 . Like the binding assays, the results for internalization of Ad into CHO-CAR would also be novel.

We assessed binding and internalization of fluorescently-labelled virus both by flow cytometry and by microscopy. Surprisingly, our results differed from those predicted from experiments presented in Chapter 3. Differences between conditions of the gene expression assay described in Chapter 3 and conditions of the binding and internalization assays described here were examined as a potential cause of this disparity.

4.2 Results

4.2.1 Labeling of virus with fluorescent dye

In order to detect virus in binding or internalization assays by flow cytometry or fluorescent microscopy, we covalently labeled virus with either Cy2 or A488 (see Section 2.2.3). Prior to labeling both viruses, we tested labeling of AdLuc(wt-fiber) with various dye dilutions. The dye concentration was measured by NanoSpec, and the dye to capsomere ratio was calculated (Table 4.1). Additionally, the relative change in infectivity was tested using a limiting dilution assay. This demonstrated that though there was some loss of infectivity after covalent labeling with dye, the labeled virus was still highly infectious. As a result, all further labeling was done with a 1:1 ratio (v/v) of dye to virus, since this produced the highest dye to capsomere ratio. The dye to capsomere ratios of each subsequent batch of labeled virus was calculated, and each virus preparation was compared to mock-labeled controls by limiting dilution. All results were similar to those seen in Table 4.1.

4.2.2 Binding of retargeted virus to CHO cell lines

4.2.2.1 Fluorescent virus binding

The binding of virus to cells was tested using fluorescently labeled virus. In this experiment, Cy2 was covalently linked to the viral capsid and fluorescence was detected by flow cytometry. The results are shown both as proportion of cells with bound virus (% of cells positive for green fluorescence, Figure 4.1A), and relative number of virus particles bound per cell (mean fluorescence of the test samples after subtracting the mean fluorescence from the No Virus samples in the same cell

Dye	Dye dilution	Dye to capsomere ratio	ifu/ml	ifu/ml ratio to No dye
Cy2	0	0.52	1.28E+10	1.00
Cy2	1:4	2.97	1.41E+10	1.10
Cy2	1:2	7.06	4.20E+10	3.28
Cy2	1	11.66	1.94E+09	0.15
A488	0	11.05	2.40E+08	1.00
A488	1:16	18.08	3.20E+08	1.33
A488	1:8	17.32	2.40E+08	1.00
A488	1:4	11.18	1.44E+08	0.60
A488	1:2	16.19	1.00E+08	0.42
A488	1	40.03	1.66E+08	0.69

Table 4.1: *Dye to capsomere ratio for Cy2 and Alexa 488 labeling of AdLuc(wt-fiber).*

The dye was diluted as indicated and tested for infectivity (ifu/ml) using a limiting dilution assay. Infectivity of labeled virus was compared to infectivity of virus subjected to a mock labeling procedure (dye dilution = 0; or No Dye).

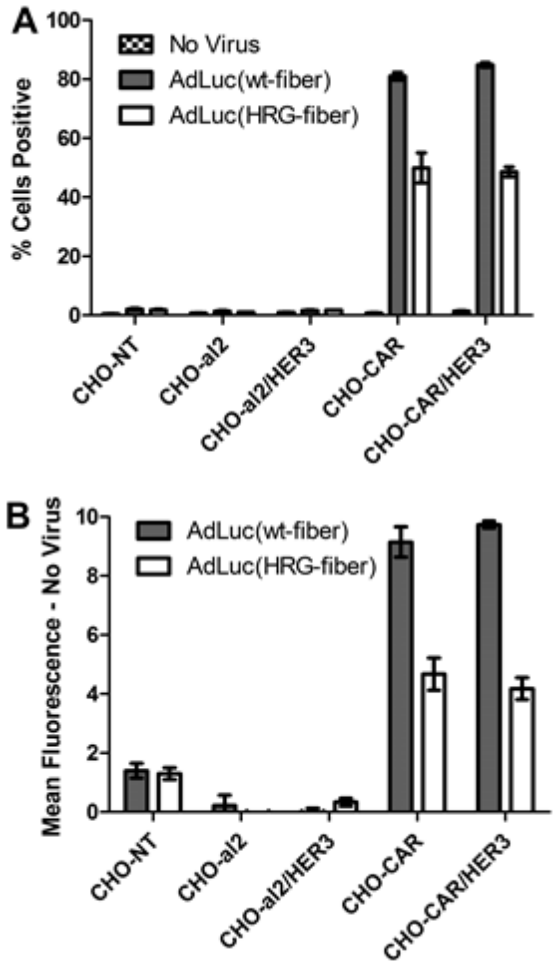


Figure 4.1: *Binding of Cy2 labeled virus to CHO cells.*

Cy2 labeled virus ($1x10^5$ virus particles per cell) was allowed to bind to cells for 30 min on ice, the cells were warmed to room temperature for 10min, and then washed in PBS. Green fluorescence (Cy2 level) was detected by flow cytometry after fixation. The results were graphed as (A) proportion of cells with bound virus (% of cells positive for green fluorescence) and (B) relative number of virus particles bound per cell (mean fluorescence – mean fluorescence of no virus sample in the same cell line).

line, Figure 4.1B). These two methods of measurement gave similar results. Comparing virus samples across all the cell lines, both AdLuc(HRG-fiber) and AdLuc(wt-fiber) binding to CHO cell lines transfected with CAR were significantly higher than all other cell lines ($p<0.001$). However, the binding of either of the viruses to CHO-CAR was generally not significantly different from its binding to CHO-CAR/HER3. The binding of either virus to CHO cell lines not expressing exogenous CAR were generally not significantly different from each other. Comparing the two viruses in each cell line, they were significantly different from each other only in CHO cell lines transfected with CAR, where AdLuc(wt-fiber) was significantly higher than AdLuc(HRG-fiber) ($p<0.01$).

Very little binding was detected to any CAR-negative CHO cell, even following treatment with AdLuc(HRG-fiber). This was surprising, since AdLuc(HRG-fiber) infection was shown for all CHO cell lines tested, and gene expression after infection in CHO-al 2/HER3 was equal or greater than that seen in CHO-CAR cells (Figure 3.4). Furthermore, luciferase expression in CHO-CAR/HER3 after infection with either virus was lower than CHO-CAR, while here the binding was similar. Additionally, the binding of AdLuc(HRG-fiber) was less than that of AdLuc(wt-fiber) in both CAR expressing cell lines, but not significantly different in any other cell line. It is possible that the modifications to fiber in AdLuc(HRG-fiber) may have interfered with virus binding, decreasing the binding in CAR expressing cells. It is also possible that the binding which resulted in the transgene expression after infection by AdLuc(HRG-fiber) into CAR- cell lines may require more time to occur. However, the fluorescent virus binding results were still not

entirely consistent with the reporter gene expression assay in Figure 3.4.

4.2.2.2 CHO cell lines binding to immobilized virus

In order to examine virus-cell binding in a different manner, we allowed virus to adhere to a 96-well plate and then determined the number of cells that bound to the immobilized virus (Figure 4.2). Similar to the results described in Section 4.2.2.1, we could detect few CAR^- cells bound to either virus, but significant binding of CAR^+ cell lines to both viruses. This is consistent with the luciferase expression analysis after infections with AdLuc(wt-fiber) (Figure 3.4A and 3.4C). However, this is not consistent with luciferase expression analysis after infection with AdLuc(HRG-fiber), which showed high levels of infection of all CHO cell lines, especially CHO-al 2/HER3 (Figure 3.4B and 3.4D).

The similarity between viruses in their ability to bind CAR^+ cells using this immobilized virus binding assay is in contrast to results of the fluorescent virus binding assay, where AdLuc(wt-fiber) binding to CAR expressing cells was significantly higher than AdLuc(HRG-fiber) (Figure 4.1). In addition, the immobilized virus binding assay did discriminate between binding of CHO-CAR/HER3 and CHO-CAR, with the former binding to both viruses at higher levels than the latter did. These differences may reflect differences in the method used to allow binding between virus and cells and the detection of binding in the two assays.

In summary, comparison of luciferase expression analysis and direct binding assay results suggests that either very little binding is required for gene expression in

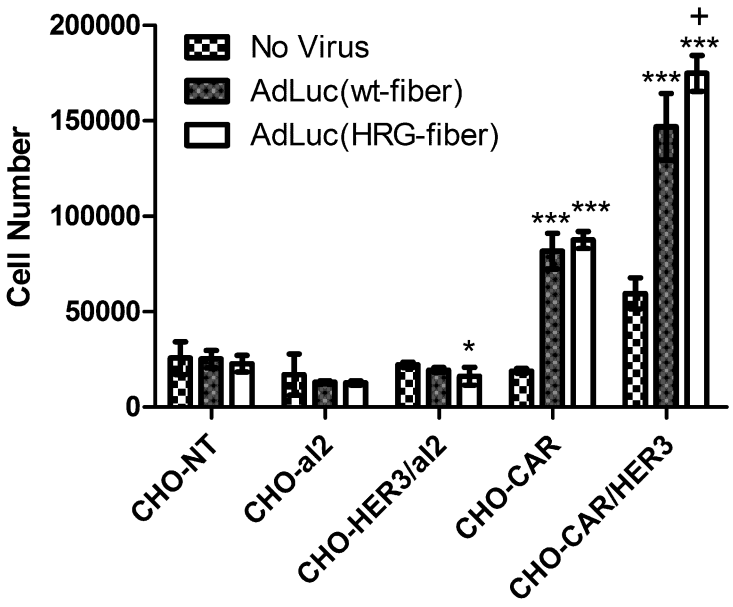


Figure 4.2: *Binding of cells to plate with no virus, AdLuc(wt-fiber) or AdLuc(HRG-fiber) on surface.*

Virus was bound to the plate overnight at 4°C and remaining binding surfaces were blocked with BSA. The plate was incubated on ice for 1 h, and warmed to 37°C for 10 min before washing in PBS. Cell number was determined by alamar blue staining and comparison to a standard curve of the respective cell line. *significantly different from No Virus in the same cell line ($p<0.05$) ***significantly different from No Virus in the same cell line ($p<0.001$) $^{+}$significantly different from AdLuc(wt-fiber) in the same cell line ($p<0.05$)

CAR$^-$ CHO cell lines, the rates of internalization are very different between the two viruses, or that the binding required for gene expression in these cell lines was not detected under conditions of the two binding assays. The final possibility is addressed in Section 4.2.6.

4.2.3 Clathrin-mediated internalization of retargeted virus into CHO cell lines

4.2.3.1 Timing of virus internalization into CHO-CAR cells

To examine the internalization of the viruses into CHO cell lines, we detected internalization of fluorescently labeled viruses by flow cytometry. To begin, we tested the timing of virus internalization in a preliminary assay using fluorescently labeled virus and CHO-CAR cells. Cy2-labeled AdLuc(wt-fiber) was incubated with the cells on ice for 30 min, followed by an incubation at 37°C for a time ranging from 0 to 40 min. As can be seen in Figure 4.3, the maximum % positive cells begins to plateau between 0 and 10 min incubation at 37°C, and does not increase drastically after the 10 min time point. This experiment was carried out by Matt Caindec, a summer student under my direct supervision. As a result, we chose to test virus internalization after 30 min on ice, followed by 10 min at 37°C.

4.2.3.2 Internalization of transferrin and inhibition by CPZ

Since transferrin is reported to be taken up by clathrin-mediated internalization, A488 labeled transferrin was used as a positive control (453). We tested a range of transferrin concentrations and determined the proportion of CHO-CAR cells that had taken up transferrin after 0 and 10 minutes at 37°C (Figure 4.4A). Additionally,

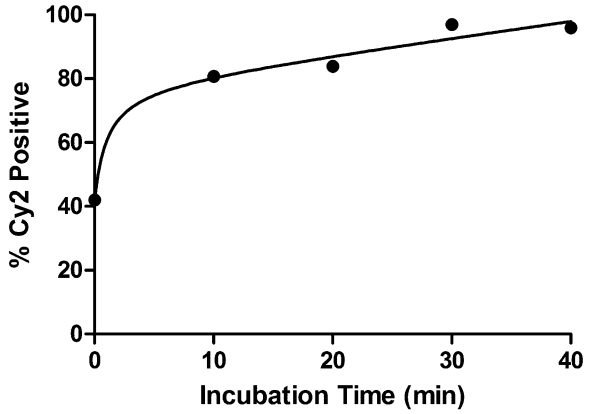


Figure 4.3: *Internalization of AdLuc(wt-fiber) into CHO-CAR cells.*
AdLuc(wt-fiber) was allowed to bind to cells for 30 min on ice. Cells were either washed immediately with PBS and trypsin, or incubated at 37°C for the time indicated before washing, to allow for viral internalization. The cells were fixed and the proportion of cells positive for virus (% green fluorescence, Cy2) was measured by flow cytometry. This experiment was carried out by Matt Caindec, a summer student under my direct supervision.

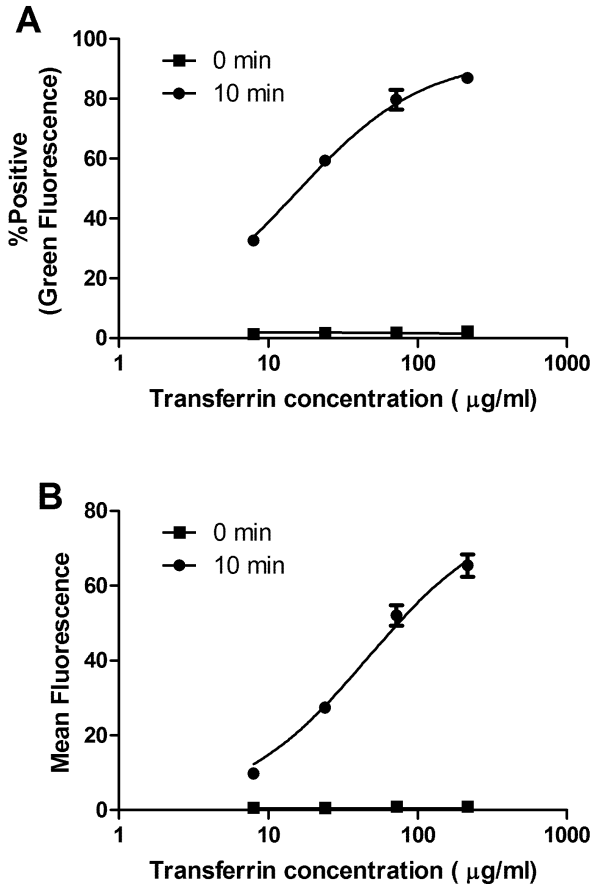
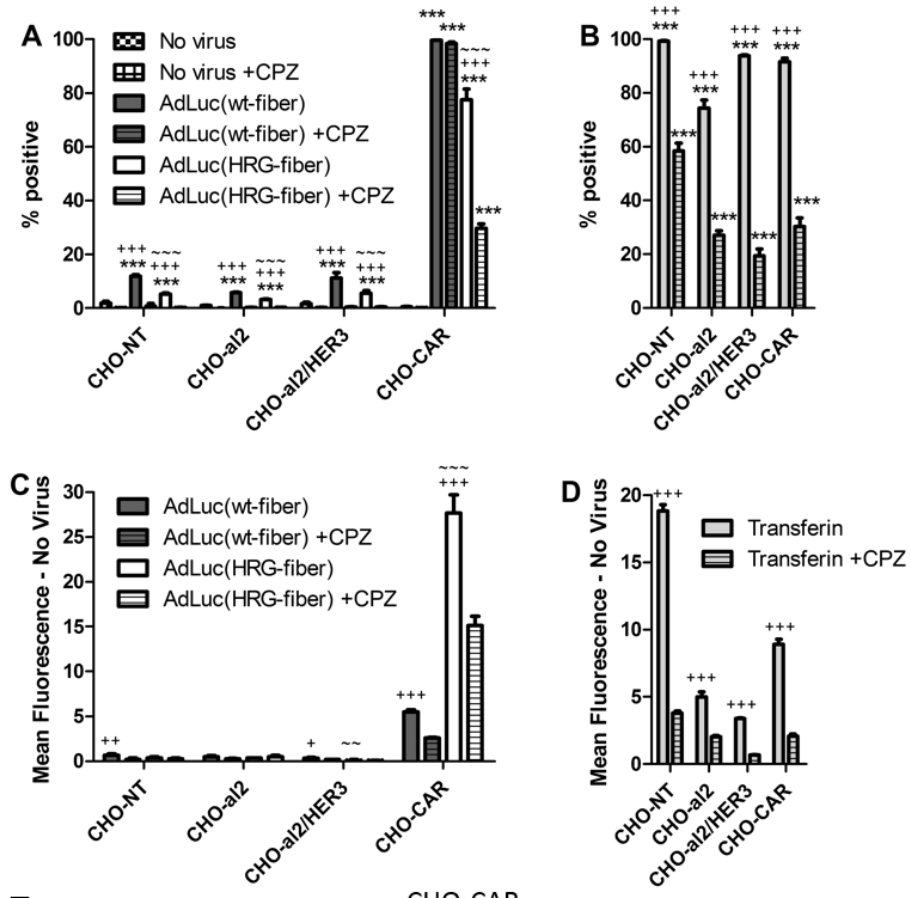


Figure 4.4: *Internalization of transferrin into CHO-CAR cells.*

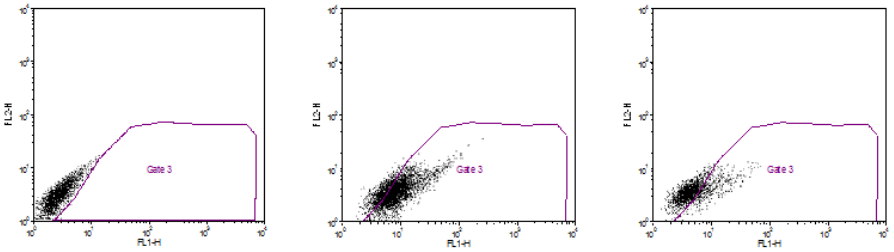
Transferrin was allowed to bind to cells for 30 min on ice. Cells were either washed immediately with PBS then trypsinized, or incubated at 37°C for 10 min before washing and trypsinization to allow for viral internalization. The tryspinized cells were fixed and (A) the proportion of cells that had taken up transferrin (% green fluorescence, A488), and (B) the relative amount of transferrin taken up (mean fluorescence) was measured by flow cytometry.

we determined the relative number of transferrin molecules taken up at these time points (Figure 4.4B). There was almost no transferrin taken up without incubation at 37°C, regardless of the transferrin concentration. However, after incubation for 10 min at 37°C, transferrin internalization was concentration-dependent both in terms of the proportion of cells which took up transferrin, and the relative number of transferrin molecules per cell. We chose to use 100 μg/μl transferrin as a positive control for internalization in each cell line, at the same time as virus internalization assays (Figure 4.5B and 4.5D). Though the cell lines varied in the relative number of transferrin molecules taken up per cell (Figure 4.5D), high proportions of all tested cell lines internalized transferrin (Figure 4.5B).

In order to determine if internalization occurred by clathrin-mediated endocytosis, we used the inhibitor CPZ (207). Though the concentration of CPZ used is within the commonly used range for clathrin-mediated endocytosis inhibition (208), CPZ did not completely inhibit internalization of transferrin in any cell line tested. The CPZ concentration was not increased due to the potential for cellular toxicity. However, we confirmed a significant decrease both in the proportion of cells positive for transferrin and the number of transferrin molecules per cell in all cell lines tested, indicating clathrin-mediated endocytosis is responsible for a large portion of transferrin internalization in the CHO cell lines tested (Figure 4.5B and 4.5D).



E CHO-CAR



No Virus
No Drug
0.7% positive

AdLuc(HRG-fiber)
No Drug
79.6% positive

AdLuc(HRG-fiber)
+CPZ
27.8% positive

Figure 4.5: *Internalization of fluorescently labeled virus and inhibition of internalization by chlorpromazine.*

CHO cell lines were trypsinized and incubated in suspension with or without chlorpromazine (CPZ, clathrin-mediated internalization inhibitor) for 30 min, then virus was added. The cells were incubated for 30 min on ice, then warmed to 37°C for 10 min, then washed with PBS, trypsinized, and fixed. (A) Proportion of cells positive for viral uptake (% of cells positive for Alexa 488, green fluorescence) was measured by flow cytometry. n=3 (B) Proportion of cells positive for A488-labeled transferrin uptake, used as a positive control for uptake, and for inhibition of uptake by CPZ. (C and D) Relative number of particles ((C) virus or (D) transferrin) taken up per cell (mean fluorescence – mean fluorescence of no virus sample in the same cell line). (E) An example of the flow cytometry dot plots used in the analysis of (A). Green fluorescence (X-axis) was graphed against unrelated fluorescence (red fluorescence, Y-axis). The gate was set so that about 1% of No Virus--No Drug samples were positive. *significantly different from No Virus in the same cell line ($p<0.05$) $^{+}$significantly different from virus + CPZ in the same cell line ($p<0.05$) $^{\sim}$significantly different from AdLuc(wt-fiber) in the same cell line ($p<0.05$). Duplicated symbols refer to $p<0.01$ and triplicate symbols refer to $p<0.001$.

4.2.3.3 Virus internalization by CHO cell lines

We compared fluorescent virus internalization by CHO-NT, CHO-al 2, CHO-al 2/HER3, and CHO-CAR cell lines, both by proportion of cells which have internalized virus (% positive, Figure 4.5A) and relative number of viral particles internalized per cell (mean fluorescence, Figure 4.5C). High levels of virus internalization were detected in CHO-CAR cells, similar to the results of the virus binding assays (Figures 4.1 and 4.2). Despite the low levels of virus internalization in the other cell lines, the proportion of cells positive for either virus internalization was significantly different from the No Virus sample in the same cell line. This indicates that all cells were permissible for at least some virus internalization, though with very few viral particles per cell taken up in some cases.

AdLuc(wt-fiber) infection resulted in almost 100% of CHO-CAR cells positive for virus internalization and AdLuc(HRG-fiber) infection resulted in almost 80% positive cells. Contrarily, the relative number of viral particles taken up per cell for AdLuc(HRG-fiber) was much higher than for AdLuc(wt-fiber). Thus it appears that more AdLuc(HRG-fiber) virus particles were taken up into fewer CHO-CAR cells than AdLuc(wt-fiber). The levels of internalization were very low for both viruses in the other cell lines tested, with internalization of AdLuc(HRG-fiber) in most cases lower than that of AdLuc(wt-fiber).

This assay is consistent with the luciferase assay for AdLuc(wt-fiber) infection (Figure 3.4A and 3.4C), but not for AdLuc(HRG-fiber) infection. It is possible that AdLuc(HRG-fiber) internalization into CAR- cells occurs at a later time point than that tested here. The results are also not entirely consistent with the two binding

assays (Figure 4.1 and 4.2). A possibility that might explain the latter (*i.e.* differences seen in number of virus particles per cell, Figure 4.1B versus 4.5C) may be that there was more AdLuc(wt-fiber) bound to cells that was removed by the trypsinization (Figure 4.5C) than removed by the PBS wash (Figure 4.1B). If this were the case, it is possible that AdLuc(HRG-fiber) bound to cells more strongly than AdLuc(wt-fiber), or was more efficiently internalized, at least when bound to CHO-CAR cells. In any case, there may be differences in virus binding that may not be elucidated by either of the binding experiments (Figures 4.1 and 4.2). Some of these differences are apparent in this internalization assay.

4.2.3.4 Inhibition of virus internalization into CHO cell lines by CPZ

Internalization of AdLuc(HRG-fiber) by CHO-CAR cells was significantly inhibited by CPZ, in terms of both proportion of cells with detectable virus internalization and number of viral particles per cell (Figure 4.5A and 4.5C). However, CPZ only partially inhibited internalization in this case. Surprisingly, CPZ only inhibited AdLuc(wt-fiber) internalization by CHO-CAR cells in terms of the number of viral particles taken up, and not the proportion of cells positive for virus internalization, suggesting that other mechanism(s) of entry into CHO-CAR cells may also be utilized by the wild-type capsid virus. Furthermore, although internalization in all other cell lines besides CHO-CAR was low, CPZ significantly decreased the proportion of cells positive for internalization of both viruses in these other lines to an almost undetectable level.

Thus, although clathrin-mediated endocytosis, which is sensitive to CPZ, has been reported as an adenovirus internalization mechanism in many human cell lines (56; 64; 67; 68; 165; 203; 212; 217-219; 449; 450), these data suggest that additional mechanism(s) may also be important in these CHO-derived cell lines.

4.2.4 Internalization of retargeted virus into ZR-75-1 breast cancer cell line

4.2.4.1Rationale and internalization of transferrin into ZR-75-1 cells

We next examined virus internalization by a breast cancer cell line because the $HER3^+$ CHO cell lines have a lower density of HER3 receptors per cell than many breast cancer cell lines (Figure 3.1D). We chose ZR-75-1 cells because this cell line expresses a high level of HER3, low level of CAR and had one of the highest levels of reporter gene expression after AdLuc(HRG-fiber) infection (Figure 3.3A). Furthermore the ratio of reporter gene expression after AdLuc(HRG-fiber) infection to that following AdLuc(wt-fiber) infection was one of the highest detected (about 5 fold, Figure 3.3A). We examined virus internalization in a similar manner to Section 4.2.3.

Transferrin was taken up into ZR-75-1 cells, with about 80% of cells positive for transferrin internalization, and with the relative levels of internalized transferrin similar to some of the CHO cell lines (compare Figure 4.6B and 4.6D to Figure 4.5B and 4.5D), demonstrating that ZR-75-1 cells are permissive for transferrin internalization, which likely occurs by clathrin-mediated internalization.

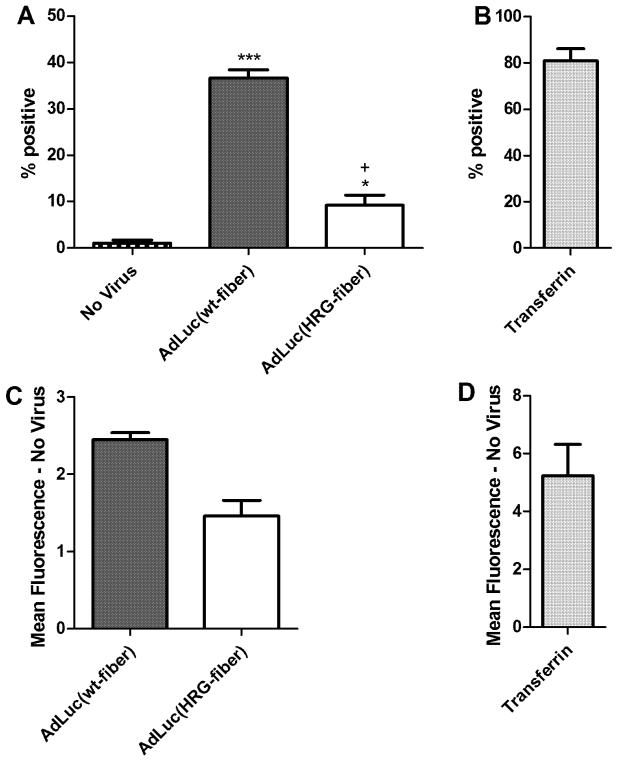


Figure 4.6: *Internalization of fluorescently labeled virus by ZR75.1 breast cancer cells.*

Fluorescently labeled virus was added to cells and incubated for 30min on ice, then warmed to 37°C for 10 min, before washing with PBS, trypsinizing, and fixing. (A) Proportion of cells positive for viral uptake (% of cells positive for Alexa 488, green fluorescence) was measured by flow cytometry to determine uptake of the two viruses. n=3 (B) Proportion of cells positive for A488-labeled transferrin uptake, used as a positive control. (C and D) Relative number of particles ((C) virus or (D) transferrin) taken up per cell (mean fluorescence – mean fluorescence of no virus sample in the same cell line). *significantly different from No Virus in the same cell line ($p<0.05$) ***significantly different from No Virus in the same cell line ($p<0.001$) $^{+}$significantly different from AdLuc(wt-fiber) in the same cell line ($p<0.001$)

4.2.4.2 Internalization of virus by ZR-75-1 cells

Both viruses were taken up by ZR-75-1 cells (Figure 4.6A and 4.6C), but the proportion of cells that internalized AdLuc(HRG-fiber), and the relative number of virus particles internalized per cell, was much lower than expected and similar to that with CAR^- CHO cell lines (Figure 4.5A and 4.5C). Thus, the internalization of AdLuc (HRG-fiber) did not appear to correlate with the level of HER3 expression, since there was similar internalization in cells that expressed no HER3 (CHO-NT and CHO-al 2), mid-level HER3 (CHO-al2/HER3) or high levels of HER3 (ZR-75-1). Also surprising, is that the proportion of ZR-75-1 cells positive for AdLuc(wt-fiber) internalization was above 35%, which was considerably higher than internalization in all CHO cell lines except CHO-CAR (Figure 4.6A and Figure 4.5A). Since ZR-75-1 cells do not express CAR, and did not express high levels of reporter gene after AdLuc(wt-fiber) infection, we expected the internalization of this virus to be significantly lower than AdLuc(HRG-fiber), rather than the reverse. There was less difference in internalization between the two viruses when comparing the relative number of virus particles taken up per cell (Figure 4.6C). The disconnect in some cases between the number of cells that take up virus, and the amount of virus taken up per cell was also noted in Section 4.2.3.3.

Like the internalization into CAR- CHO cell lines, it was possible that AdLuc(HRG-fiber) internalization occurs at a time after 10 min at 37°C. Additionally, the internalization of virus into ZR-75-1 cells matched the results from the *in vivo* assay. Thus, one possible explanation for the lack of difference in

the *in vivo* assay is that the virus may be washed out of the tumor at a time point that is too early to allow for virus internalization.

Unfortunately, we were unable to determine whether virus internalization was dependent on clathrin-mediated endocytosis in ZR-75-1 cells because CPZ treatment resulted in recovery of too few live cells to be accurate for detection by flow cytometry. CPZ likely either killed most of the cells and/or prevented the cells from remaining in the pellet during the washes in the flow cytometry procedure. There was no problem with the procedure without the addition of CPZ, and this occurred in every instance of CPZ addition to ZR-75-1 cells. Therefore, we cannot directly conclude that internalization in these cells was clathrin-mediated. However, since transferrin internalization into ZR-75-1 cells was similar to CHO-al2 and CHO-al2/HER3, we expect that this cell line has a functional clathrin-mediated endocytosis pathway.

In summary, binding and internalization of the two viruses, as measured by assays described in this section as well as the previous section, did not appear to predict reporter gene expression.

4.2.5 Microscopic analysis of virus entry into cells

Some discrepancy was observed in binding studies particularly between number of virus particles taken up per cell and number of cells that have taken up virus. Therefore, we reasoned that direct visualization of virus internalization at the single cell level might be informative.

4.2.5.1 Time course for virus internalization

Virus internalization by HER3- or CAR-labeled CHO-al2/HER3 or CHO-CAR cells, respectively, was analysed at 10 min and 30 min following addition of virus. Representative pictures of infected cells at each timepoint can be seen in Figure 4.7. A visualization of a single virus infected cell (AdLuc(HRG-fiber) infected, 30 min at 37ºC) at different angles can be seen in Figure 4.8.

We used the immunofluorescence of HER3 or CAR receptors (Imaris 7.5 analysis) to generate a surface (Figure 4.8F) that allowed us to count the virus signal (Figure 4.8G, modeled as spheres) that were inside the cell, on the edge of the cell, or outside the cell. The average total number of viruses per cell and the percentages of viruses in each category are outlined in Table 4.2. More than 90% of the viral particles detected were inside the cell. It is possible that the wash steps in the IF staining procedure removed the virus from outside the cell. The average number of viral particles inside the cell can be seen in Figure 4.9. There were detectable viral particles when either virus was added, though the only relationship which was significantly different from the No Virus sample in the same cell line was AdLuc(HRG-fiber) internalization into CHO-CAR cells, at both 10 min and 30 min. The variation in number of viruses per cell was relatively high in most samples, and this likely contributed to the lack of significant difference in many relationships seen here. At 10 min, AdLuc(HRG-fiber) internalization into CHO-CAR cells was also significantly higher than AdLuc(HRG-fiber) internalization into CHO-al2/HER3 cells and AdLuc(wt-fiber) internalization into CHO-CAR

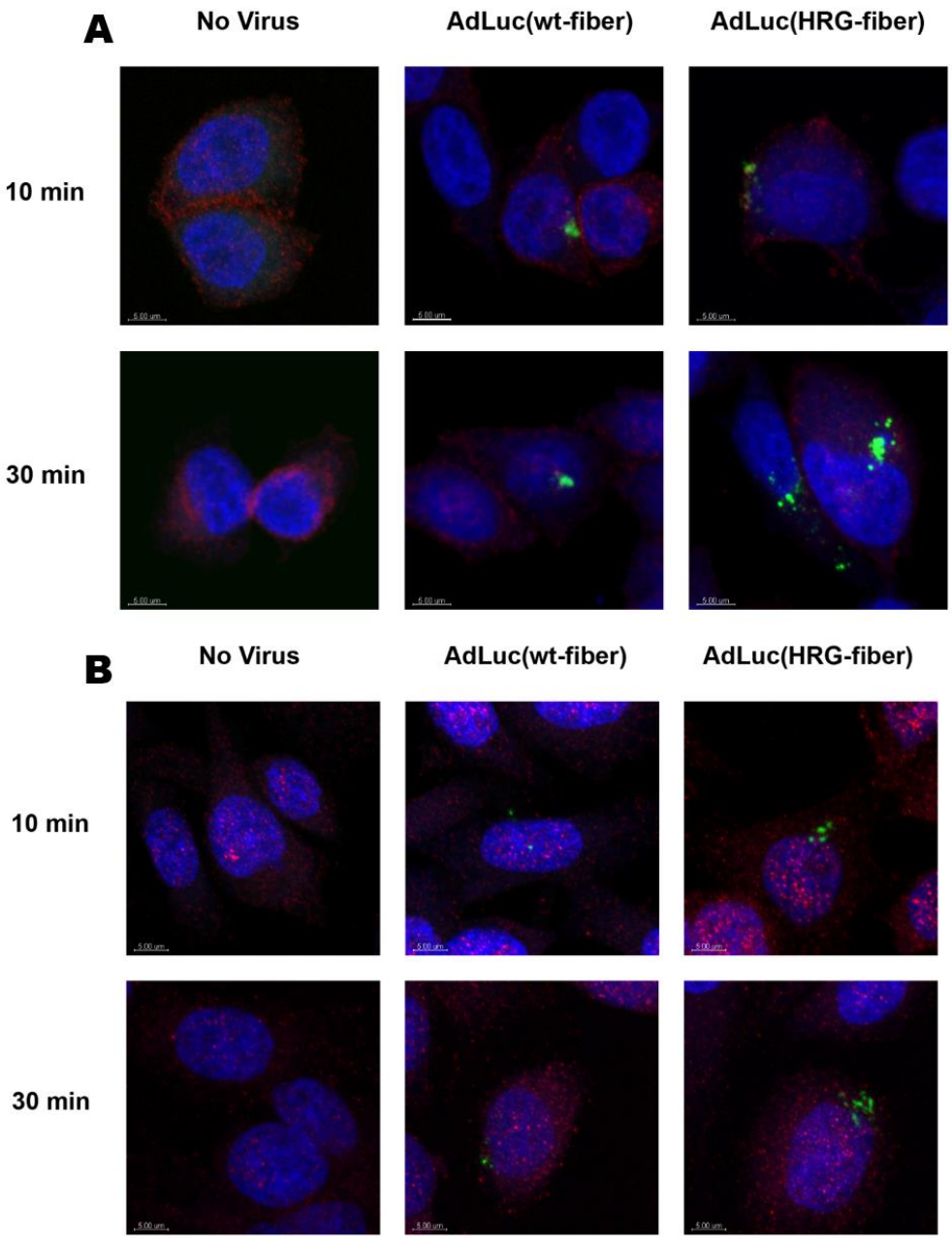

A
No Virus
AdLuc(wt-fiber)
AdLuc(HRG-fiber)
10 min
30 min
B
No Virus
AdLuc(wt-fiber)
AdLuc(HRG-fiber)
10 min
30 min

Figure 4.7: *Fluorescent virus uptake into CHO-CAR and CHO-HER3 cells.* Viruses were incubated with either (A) CHO-CAR cells or (B) CHO-HER3 on coverslips for 30 min on ice, before incubation at 37°C for either 10 min or 30 min. The cells were then washed three times with PBS before fixation and immunofluorescent staining for receptor expression. Pictures were taken of three to six representative virus infected cells per coverslip, and the virus location relative to a surface formed by receptor expression was quantified. Representative 2D images of z-stack series are shown. Green is virus, blue is DNA, and red is CAR (A) or HER3 (B).

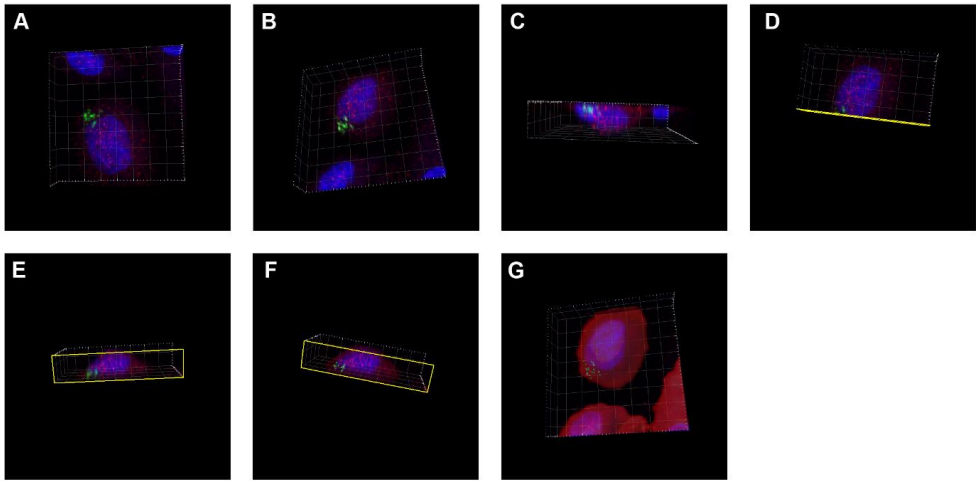


Figure 4.8: *A range of views of a fluorescent virus infected cell.*

Pictures were taken from the 3D confocal image from Figure 4.7B, AdLuc(HRG-fiber) infection of CHO-al2/HER3 cells at 30 min. (A) Top down view of the cell with green virus, red receptors and blue DNA. (B) A slightly angled view, where in addition to the three colors in (A), the colocalization of green and red signal is shown in white. (C) A side view of the cell, including all four colors. (D) A top down view, where the image was cleaved on the xz plane. (E) A side view of (D), which allows for easier visualization of the virus inside the cell. (F) A similar view to (E), except the surface representing the outside of the cell (red) and the green spheres representing the virus have been added. (G) A top down view of the cell where the surface used to represent the outside of the cell and the spheres which represent the virus particles are visible. Images similar to (G) were used to determine if the virus was inside, outside or on the edge of the cell.

Cell Line	Virus	Time at 37°C	Average total virus per cell	% of total virus average		
				inside	on edge	outside
CHO-HER3	none	10	0.0	0.0	0.0	0.0
CHO-HER3	Ad-Luc(wt-fiber)	10	1.5	94.4	5.6	0.0
CHO-HER3	Ad-Luc(HRG - fiber)	10	4.6	95.0	5.0	0.0
CHO-CAR	none	10	0.0	0.0	0.0	0.0
CHO-CAR	Ad-Luc(wt-fiber)	10	3.8	100.0	0.0	0.0
CHO-CAR	Ad-Luc(HRG-fiber)	10	11.8	100.0	0.0	0.0
CHO-HER3	none	30	0.0	0.0	0.0	0.0
CHO-HER3	Ad-Luc(wt-fiber)	30	3.2	100.0	0.0	0.0
CHO-HER3	Ad-Luc(HRG - fiber)	30	17.2	90.0	4.3	5.7
CHO-CAR	none	30	0.0	0.0	0.0	0.0
CHO-CAR	Ad-Luc(wt-fiber)	30	8.0	100.0	0.0	0.0
CHO-CAR	Ad-Luc(HRG - fiber)	30	25.0	91.5	8.0	0.5

Table 4.2: *Total number of fluorescent viruses per cell, and percentage of viruses inside, on edge, or outside the cell.*

Viruses were incubated with cells on coverslips for 30 min on ice, before incubation at 37°C for either 10 min or 30 min. The cells were then washed three times before fixation and immunofluorescent staining for receptor expression. Pictures were taken of three to six virus infected cells per coverslip, and the virus location relative to a surface formed by receptor expression was quantified. The total number of viruses per cell was significantly different from No Virus samples for both viruses in both cell lines at both time points.

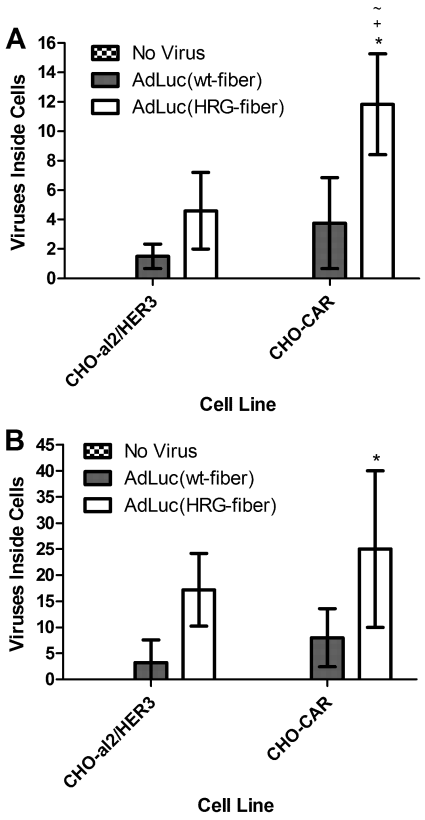


Figure 4.9: *Number of fluorescent viruses inside CHO-al2/HER3 or CHO-CAR cells.*

Viruses were incubated with CHO-CAR or CHO-al2/HER3 cells on coverslips for 30 min on ice, before incubation at 37°C for either (A) 10 min or (B) 30 min. The cells were then washed three times with PBS before fixation and immunofluorescent staining for receptor expression. Pictures were taken of three to six representative virus infected cells per coverslip, and the virus location relative to a surface formed by receptor expression was quantified (Imaris 7.5 software). The average number of viruses determined to be inside cells is shown. *significantly different from no virus in the same cell line at the same timepoint ($p<0.01$). + significantly different from AdLuc(wt-fiber) in the same cell line at the same timepoint ($p<0.001$). ~ significantly different from CHO-al2/HER3 with infected with the same virus at the same timepoint ($p<0.001$).

cells. Furthermore, internalization of AdLuc(HRG-fiber) into CHO-al2/HER3 cells increased from 10 min to 30 min, while that of AdLuc(wt-fiber) did not.

Though the relationship was not always significant, more AdLuc(HRG-fiber) appeared to be taken up into cells than AdLuc(wt-fiber). This was also seen in the internalization assay in Figure 4.5C and the luciferase assay in Figure 4.11. Furthermore, there appeared to be more virus internalization in CHO-CAR cells with both viruses, compared to CHO-al2/HER3 cells. This was consistent with the previous binding and internalization assays.

4.2.5.2 Colocalization of virus with receptor

Using the pictures taken for Section 4.2.5.1, we sought to determine if virus colocalized with the appropriate receptors. We looked at virus colocalization with HER3 in CHO-al 2/HER3 cells and with CAR in CHO-CAR cells (Figure 4.10). Not surprisingly, there was very little colocalization of AdLuc(wt-fiber) with HER3. As predicted, AdLuc(HRG-fiber) colocalized with HER3 by 10 min. The colocalization of AdLuc(HRG-fiber) with HER3 actually decreased by 30 min, possibly because of dissociation of the virus from the receptor in an endosome. There were not many receptors present inside the cell, relative to the cell surface. Additionally, the receptors formed granular structures, which may have affected the colocalization with virus.

Both viruses colocalized with CAR, and this colocalization was significantly higher than the respective virus colocalization with HER3 at 10 min. This may possibly be explained by tighter binding to CAR or by the fact that CAR is highly

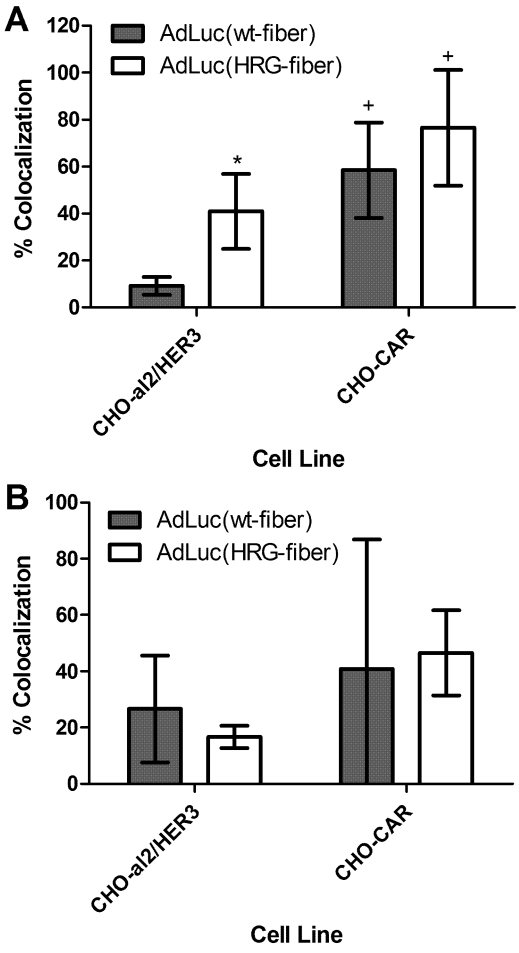


Figure 4.10: *Percent of virus colocalized with receptor in CHO-al 2/HER3 or CHO-CAR cell lines.*

The percent of virus colocalized with either HER3 in CHO-al 2/HER3 cells or CAR in CHO-CAR cells after incubation at 37°C for either (A) 10 min or (B) 30 min. *significantly different from AdLuc(wt-fiber) in the same cell line at the same timepoint; + significantly different from the same virus in the other cell line at the same timepoint

overexpressed in the CHO-CAR cell line (Figure 3.1). There was no significant difference between the two viruses in their colocalization with CAR.

In general, the colocalization of virus with receptor decreased at the 30 min timepoint, which could indicate virus dissociation from the receptor after endocytosis. Additionally, the detection of less colocalization of AdLuc(HRG-fiber) with HER3 than with CAR, could support the existence of another undetected receptor on CHO-al2/HER3 cells to which AdLuc(HRG-fiber) can bind. This would help explain the lack of complete internalization inhibition by HRG competition (Figure 3.6). Though these results are similar to the binding assays in Figures 4.1 and 4.2, there was more binding to HER3 detected in the colocalization assay. It is possible that this assay was more sensitive than the previous binding assays, allowing us to detect some binding here that was not detected in Figures 4.1 and 4.2.

In summary, the virus colocalized with the expected receptors at the 10 min timepoint, but colocalization generally decreased by the 30 min timepoint. Colocalization with CAR was also generally higher than with HER3.

4.2.6 Luciferase expression analysis under conditions similar to internalization assays.

The binding and internalization assays described in Sections 4.2.2 through 4.2.5 generated results that were not predicted by the previous luciferase assay results described in Section 3.2.3 (Figure 3.4). To minimize differences due to procedures, we repeated the luciferase assay in CHO cell lines, using a procedure similar to the

internalization assay. The relevant sections in Chapter 2 have these methods in greater detail (Section 2.5 compared to Section 2.10). Briefly, the main differences in this case were the infection for this experiment was performed with the cells in a single cell suspension, rather than in a monolayer; and after binding on ice (30 min) and internalization at 37°C (10 min) the cells were trypsinized and PBS before plating.

The levels of luciferase were much lower in this experiment than in the previous luciferase assay (Figure 4.11 compared to Figure 3.4). This was expected, since the additional wash steps were expected to remove virus which previously may have been taken up at a later timepoint. Similar to the internalization assay, luciferase expression was not much higher than controls, except in CHO-CAR cells. In addition, luciferase expression after AdLuc(HRG-fiber) infection of CHO-CAR cells was significantly higher than after AdLuc(wt-fiber) infection, which was consistent with the relative number of viruses taken up per cell in the internalization assay (Figure 4.5C), and the microscopic analysis (Section 4.2.5). In this experiment, reporter gene expression was measured for the population of cells in an entire well, and therefore, was expected to be a combination of both proportion of cells infected and number of viruses per cell. Another commonality with the internalization assay is that CPZ appeared to inhibit luciferase expression after AdLuc(HRG-fiber) infection in all cell lines, and this relationship was significant in CHO-al 2/HER3 cells. Similarly, CPZ did not appear to inhibit luciferase expression after AdLuc(wt-fiber) infection of CHO-CAR.

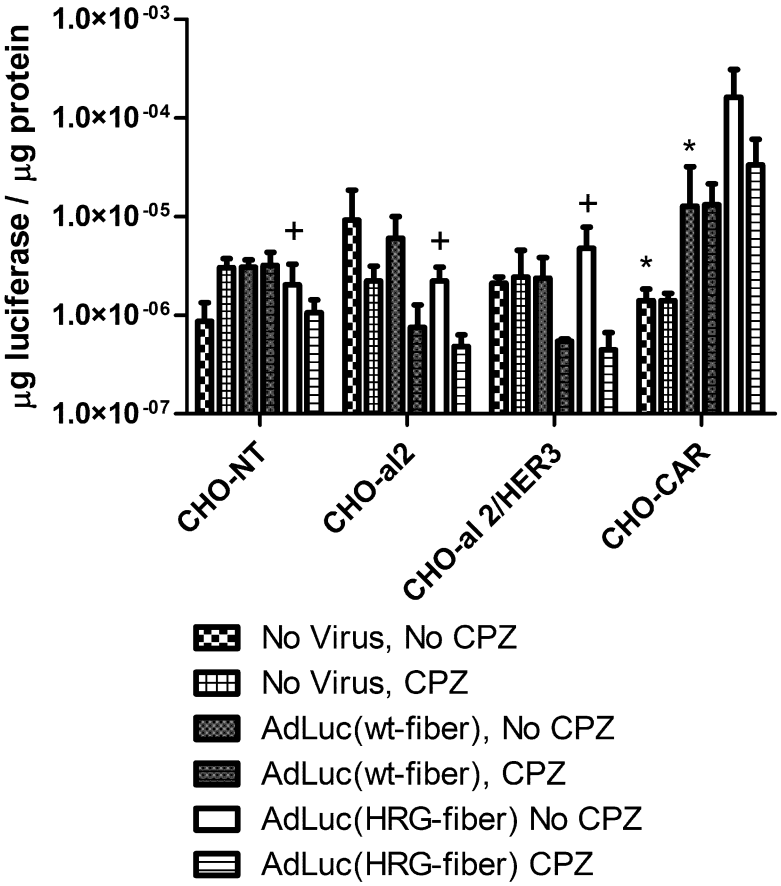


Figure 4.11: *Luciferase assay after AdLuc(HRG-fiber) and AdLuc(wt-fiber) infection of CHO cells using a similar procedure to Figure 4.5.*

Cells were trypsinized, incubated with or without CPZ for 30 min before addition of virus. The cells were next incubated for 30 min on ice, then warmed to 37°C for 10 min, before washing with PBS and trypsinizing, and finally plating into a 24-well plate. Luciferase expression was measured after 48 hours, and normalized to µg protein in the sample. * is significantly different from AdLuc(HRG-fiber) without CPZ treatment, in the same cell line ($p<0.05$). + is significantly different from AdLuc(HRG-fiber) infection in CHO-CAR cells ($p<0.05$). n=3

The results from the luciferase assay in Figure 4.11 are generally consistent with the percent positive results from the internalization assay. This may mean that the transgene expression shown in Figure 3.4 was as a result of binding and internalization events that were not detected in the assays performed here. It is possible that the different virus-receptor complexes were internalized at different rates. If, for example, the virus-HER3 complexes remain outside the cell for longer periods, they may be more sensitive to trypsin cleavage, and thus may not remain complexed in most of the experiments performed here.

4.3 Discussion

This chapter examined the binding and internalization of an adenoviral vector retargeted to HER3, compared to that of a wild-type binding virus. As expected, we showed much higher AdLuc(wt-fiber) binding to CAR+ cell lines than CAR- cell lines. The internalization results were similar. Furthermore, the gene expression assay performed under stringent binding and internalization conditions resulted in detectable transgene expression only in CHO-CAR cells. This is consistent with expected results for a wild-type binding Ad, and also with the results in Chapter 3. For AdLuc(HRG-fiber), binding and internalization were generally similar to AdLuc(wt-fiber) for both CAR+ and CAR- cells. This was surprising because the gene expression assays in Chapter 3 suggested high gene transfer to HER3+ CHO cell lines, as well as to CAR+ CHO cell lines. A repeat of the gene expression assay, but this time under stringent conditions mimicking the binding and internalization assays, resulted in uptake only by CHO-CAR+ cells, consistent with most of the

binding and internalization assays in this chapter. In contrast, fluorescence microscopy, which may be a more sensitive method of detecting virus binding, showed some AdLuc(HRG-fiber) binding and internalization into HER3+ cells even at early time points.

One of the primary differences between the transgene expression assays in Chapter 3 and most of the assays in Chapter 4 is the time at either room temperature or 37°C. Most assays in Chapter 3 were performed after 30 min at 37°C, and those in Chapter 4, after 10 min at 37°C (except for the microscopy, which was performed after both 10 and 30 min at 37°C). As a result, we hypothesize that AdLuc(HRG-fiber) is internalized at a slower rate in CAR- cells than either virus in CAR+ cells. Table 5.1 summarizes the results of most of the assays in this thesis. A detailed discussion of the results of Chapters 3 and 4 can be found in Chapter 5 (Sections 5.2 and 5.3)

CHAPTER 5: Summary, Discussion, and Future Directions

5.1 Summary of thesis

In this thesis, we have examined the binding, internalization and transgene expression of an adenovirus vector retargeted to HER3/4, receptors overexpressed in breast cancer. A summary of the results can be found in Table 5.1. We have shown that the control virus, AdLuc(wt-fiber), bound to CAR and was taken up within 10 min at 37°C. This resulted in a higher level of transgene expression after AdLuc(wt-fiber) infection in CAR+ cells than in CAR- cells. The modified virus, AdLuc(HRG-fiber), retained CAR binding and showed similar results after 10 min at 37°C in CAR+ cells. However, in CAR- cells, a 30 min infection with AdLuc(HRG-fiber) resulted in higher transgene expression and apparently higher internalization by microscopic analysis than a 30 min infection with AdLuc(wt-fiber). Thus it is likely that AdLuc(HRG-fiber) internalization into HER3 expressing cells was highest between 10 and 30 min at 37°C, accounting for the observed high level of transgene expression.

5.2 AdLuc(wt-fiber) binding, internalization, and transgene expression

The context of the inserted gene, including proximity to Ad sequences, can have a profound effect on transgene expression levels and timing (454). Furthermore, in first generation Ads, such as AdLuc(HRG-fiber), there is leaky expression of other viral genes, which could potentially affect reporter gene expression (455). Therefore, it was important to compare two viruses of similar construction in these experiments. Both AdLuc(wt-fiber) and AdLuc(HRG-fiber) contain the same gene expression cassette in the same locations. AdLuc(wt-fiber) binding, internalization

					10 min at 37°C					10 to 20 min RT		30 min at 37°C			
					Binding		Internalization		Gene expression	Gene expression inhibition by competition		Binding	Internalization	Gene expression	*In vivo*
Category (CAR/ HER3)	**CAR**	**HER 3/4**	**Cell line**	**Virus**	**Fig 4.1/4.2**	**Fig 4.10: Colocalization**	**Fig 4.5/4.6: Flow cytometry**	**Fig 4.9: Microscope**	**Fig 4.11: Stringent binding**	**Fig 3.5*: s-knob**	**Fig 3.5*/ 3.6: HRG**	**Fig: 4.10: Colocalization**	**Fig 4.9: Microscope**	**Fig 3.3/3.4**: BrCa Panel or MOI 20**	**Fig 3.8**
-/-	-	-	CHO-NT, CHO-al2	AdLuc(wt-fiber)	-/+		-/+		-					-/+	
				AdLuc(HRG-fiber)	-/+		-/+		-		-/++			+/++	
-/-	-	-	U-118MG	AdLuc(wt-fiber)										-/+	
				AdLuc(HRG-fiber)										-/+	
-/+	-	++	CHO-al2/HER3	AdLuc(wt-fiber)	-/+	+	-/+	+	-		-	++	+	-/+	
				AdLuc(HRG-fiber)	-/+	+++	-/+	++	-		++	+	+++	+++/ ++++	
-/+	-	+++	ZR75.1	AdLuc(wt-fiber)			+++							++	+++
				AdLuc(HRG-fiber)			+							+++++	++
-/+	-	++/+++	MDA-MB-361, MCF7, SKOV-3, RD	AdLuc(wt-fiber)						-	-			-/+/++	
				AdLuc(HRG-fiber)						-	++			+/++/ +++/ +++++	
+/-	+++++	-	CHO-CAR	AdLuc(wt-fiber)	++/++++	++++	++/ +++++	++	++			+++	++	++/+++	
				AdLuc(HRG-fiber)	++/+++	+++++	++++/ +++++	++++	++			+++	+++	++++/ +++++	
+/-	+/++	-	MDA-MB-231, BT549a, HeLa	AdLuc(wt-fiber)						+++	-			-/+/++	
				AdLuc(HRG-fiber)						++	+			-/+/++	
+/+	+++++	++	CHO-CAR/HER3	AdLuc(wt-fiber)	++++									++	
				AdLuc(HRG-fiber)	+++/ +++++									+++	
+/+	++	+++	MDA-MB-468	AdLuc(wt-fiber)						+++	-			-/+	
				AdLuc(HRG-fiber)						+++	++			+	

Table 5.1: *Summary of results*

The results are summarized based on cell line receptor expression (CAR and HER3/4), time at room temperature or 37°C, and type of assay (binding, internalization, or gene expression).

*Figure 3.5 (Gene expression inhibition by competition): The only cell lines used were SKOV-3 (-/+), MDA-MB-231 (+/-), and MDA-MB-468 (+/+).

**Figure 3.4 (Gene expression, CHO cells): MOI 5, 20, and 50 were used in the experiment, but only MOI 20 was summarized here.

and transgene expression were as expected, and thus AdLuc(wt-fiber) served as an effective control for AdLuc(HRG-fiber) in our experiments.

5.2.1 AdLuc(wt-fiber) binds to CAR+ cells

As expected, higher AdLuc(wt-fiber) binding to CAR+ CHO cells was seen than to CAR- cells, in both binding assays. Colocalization of fluorescent AdLuc(wt-fiber) with CAR was seen inside the cells. It is possible that the degree of colocalization may have even been higher if we had looked at an earlier time point, since dissociation of the virus from the receptor may have already started even at the 10 min time point. However, this was not feasible in this experiment. There was also some decrease in colocalization at 30 min which may have been dissociation of virus from the receptor. This is consistent with Ad in the vicinity of the nucleus and likely outside of the endosome within 20-30 min of infection (260).

Thus, using multiple methods we showed increased AdLuc(wt-fiber) binding to CAR+ cells, compared to CAR- cells within 10 min at room temperature or 37°C.

5.2.2 AdLuc(wt-fiber) is taken up into CAR+ cells

AdLuc(wt-fiber) internalization into CAR+ was very high after 10 min at 37°C. Furthermore, we demonstrated that internalization of AdLuc(wt-fiber) into CHO-CAR cells began to plateau before 10 min at 37°C. This is consistent with other studies which have shown Ad5 in the cytoplasm within 10 to 15 min and in the vicinity of the nucleus within 20 to 30 min (260). Though there was detectable

internalization of AdLuc(wt-fiber) into CAR- cells, this was much lower than CAR+ cells.

Wild-type Ad is generally endocytosed by a clathrin-mediated mechanism (64; 67; 68; 449; 450). Surprisingly, AdLuc(wt-fiber) internalization was only inhibited by chlorpromazine (CPZ) in the measure of relative number of viruses per cell, and not in the proportion of cells positive for virus internalization. The latter was consistent with the modified transgene expression assay, where CPZ failed to inhibit luciferase expression (Figure 4.11). Thus, it would appear that AdLuc(wt-fiber) was either not taken up by clathrin-mediated endocytosis in CHO-CAR cells, or that internalization by other mechanisms was able to compensate for the decrease in clathrin-mediated internalization, resulting in very little change in internalization or transgene expression. Alternative mechanisms of virus internalization, such as caveolin-mediated internalization or macropinocytosis, were not examined here. Though Ad entry and gene expression have been shown in CHO-CAR cells, to date there appear to have been no prior studies directly looking at the Ad internalization mechanism in CHO-CAR cells (57; 456).

Contrary to the results in CHO-CAR, all detectable internalization in CAR- cell lines appears to be mediated by clathrin, since it is inhibited by CPZ.

5.2.3 AdLuc(wt-fiber) transgene expression

Wild-type binding Ads, such as AdLuc(wt-fiber), are generally expected to infect most cells expressing CAR (40; 57). This is consistent with our experiments where

we saw higher transgene expression after AdLuc(wt-fiber) infection in CAR+ CHO cell lines, compared to CAR- cell lines, after either 10 min or 30 min at 37°C.

The relationship between CAR expression and AdLuc(wt-fiber) binding is not as clear with the breast cancer cell lines. Though some of the variation may be explained by CAR levels, not all cell lines which expressed high CAR also showed high luciferase expression after Ad infection. However, when soluble fiber knob (s-knob) was used to compete for AdLuc(wt-fiber) binding of CAR, luciferase expression was decreased in cells with moderate or high CAR levels (Figure 3.4), but not low CAR levels. Thus in the breast cancer cell lines, CAR expression is only one factor that is important in transgene expression. This is one of the reasons we performed many of the other experiments with the isogenic CHO cell lines.

In conclusion, as expected with AdLuc(wt-fiber), there was more binding, internalization and transgene expression in CAR+ cells than CAR- cells, and this generally occurred by 10 min at 37°C. Surprisingly, though the limited internalization in CAR- cells appeared to be clathrin mediated, internalization in CHO-CAR cells was not completely inhibited by CPZ.

5.3 AdLuc(HRG-fiber) binding, internalization, and transgene expression

5.3.1 AdLuc(HRG-fiber) construction

Because of the overexpression of HER3 and HER4 and the importance in breast cancer, we constructed an adenovirus (Ad) gene therapy vector targeted to these two receptors. The coding sequence for the EGF-like domain of the HER3/4 ligand heregulin-α (HRG) was inserted into the HI loop of the Ad fiber protein. Other than

this insertion, AdLuc(HRG-fiber) and AdLuc(wt-fiber) are the same. Importantly, we have shown here that insertion of HRG did not impede fiber trimer formation or viral replication in HEK293 cells (Figure 1.4 and 1.5) (3).

5.3.2 AdLuc(HRG-fiber) binding, internalization, and transgene expression is similar to AdLuc(wt-fiber) in CAR+ cells

In CAR+ CHO cells, AdLuc(HRG-fiber) binding, internalization and transgene expression were generally similar to AdLuc(wt-fiber). It appears that when CAR was present AdLuc(HRG-fiber) could utilize this receptor in a similar manner to AdLuc(wt-fiber).

AdLuc(HRG-fiber) binding and internalization into CAR+ cells was much higher than into CAR- cells after 10 min at room temperature or 37°C, similar to AdLuc(wt-fiber). The modified transgene assay was consistent with the binding and internalization assays. However, when comparing AdLuc(HRG-fiber) binding and internalization to that of AdLuc(wt-fiber), the results were more variable. This likely reflects differences in the assays used to detect binding. One assay detected fluorescent virus binding to cells in solution, while the other detected cells binding to virus fixed to a plate. However, most binding, internalization and transgene expression assays resulted in similar or higher AdLuc(HRG-fiber) than AdLuc(wt-fiber). It is possible that the results from the first binding assay (Figure 4.1), which have lower AdLuc(HRG-fiber) binding to CAR+ CHO cells than AdLuc(wt-fiber), might be an anomaly.

AdLuc(HRG-fiber) internalization and transgene expression after 30 min at 37°C was similar to or higher than AdLuc(wt-fiber). However, as observed with AdLuc(wt-fiber), transgene expression in AdLuc(HRG-fiber) infected cells did not appear to strictly correlate with CAR expression (Figure 3.3). Also like AdLuc(wt-fiber), competition by s-knob was able to decrease transgene expression after AdLuc(HRG-fiber) infection. It is interesting to note that though s-knob competition was able to decrease transgene expression after AdLuc(wt-fiber) infection down to very low levels in the cell line MDA-MB-468, this was not the case with AdLuc(HRG-fiber). It is possible that the high levels of HER3/4 in this cell line allowed AdLuc(HRG-fiber) to partially circumvent this competition.

Unlike AdLuc(wt-fiber) in CAR+ cells, AdLuc(HRG-fiber) internalization in these cell lines appeared to be clathrin mediated. It is possible other mechanisms of internalization were not active enough to compensate for the CPZ inhibition with AdLuc(HRG-fiber) infection. This internalization may have also occurred slower than with AdLuc(wt-fiber), and thus could have been more susceptible to inhibition. It is possible that the modification to AdLuc(HRG-fiber) may have changed the internalization of this virus, even in CAR+ cell lines. It is also possible that the addition of HRG to the fiber knob may affect the fiber shedding from the virus capsid, which in turn could have affected virus internalization, even in CAR+ cell lines. The reasons for the differences in internalization between AdLuc(HRG-fiber) and AdLuc(wt-fiber) were not fully examined in this thesis.

As expected, the modifications to the fiber protein did not appear to have decreased the infectivity of AdLuc(HRG-fiber) in cells which were highly infected by

AdLuc(wt-fiber). Of course, the purpose of these modifications is to expand the virus tropism to cells not highly infected by AdLuc(wt-fiber). This is discussed in the next section (Section 5.3.3).

5.3.3 AdLuc(HRG-fiber) internalization in CAR- cells likely occurs between 10 min and 30 min at 37°C

In CAR- cells, for the most part, there was no detectable binding by 10 min at room temperature, with either virus. One exception was colocalization, where AdLuc(HRG-fiber) colocalized with HER3, and was higher than AdLuc(wt-fiber). However, the AdLuc(HRG-fiber) colocalization with HER3 was lower than that with CAR.

Internalization of AdLuc(HRG-fiber) into CAR- cells by 10 min at 37°C was always lower than that of AdLuc(wt-fiber), except in number of viruses inside cells in the microscope assay. Even in the breast cancer cell line ZR-75-1, which expressed high levels of HER3, the internalization of AdLuc(HRG-fiber) remained below that of AdLuc(wt-fiber). Like AdLuc(wt-fiber), internalization into CHO cell lines appeared to be clathrin-mediated, since this internalization was inhibited by CPZ. This is consistent with internalization by HER3, which has been shown to be slower than that of EGFR, and may not be ligand dependent (346; 353; 374-376).

In the modified transgene assay, there was no detectable expression of AdLuc(HRG-fiber) to CAR- cells by 10 min at 37°C. However, there was some transgene expression in the competition assays. The washing in the latter assays did

not involve trypsinization and was less extensive, which likely contributed to the low but detectable transgene expression. Surprisingly, the combination of s-knob and soluble HRG was not better at decreasing luciferase expression after AdLuc(HRG-fiber) infection than was s-knob alone. Furthermore, no combination of competitive inhibitors was able to eliminate transgene expression after AdLuc(HRG-fiber) infection.

After 30 min at 37°C, however, AdLuc(HRG-fiber) transgene expression was high in all cell lines expressing HER3/4, and internalization and transgene expression was either similar or higher than AdLuc(wt-fiber), in all CHO and breast cancer cell lines. In the microscope assay the amount of AdLuc(HRG-fiber) inside cells increased from 10 min to 30 min at 37 °C. This is consistent with an assay showing 80% of cells positive for internalization of the ligand HRG by 30 min (375). In the breast cancer cell lines, transgene expression after AdLuc(HRG-fiber) infection appeared to be dependent primarily on HER3/4, much more so than AdLuc(wt-fiber) was dependent on CAR. Further supporting the relationship between HER3/4 and infection with AdLuc(HRG-fiber) is the observation that transgene expression after AdLuc(HRG-fiber) infection in breast cancer and CHO-al2/HER3 cells was significantly decreased by soluble HRG competition.

Since there appeared to be a significant difference between internalization and transgene expression assays performed at 10 min and 30 min at 37°C, we can conclude that internalization of AdLuc(HRG-fiber) likely occurs between 10 and 30 min, and that this internalization ultimately results in transgene expression. This transgene expression is higher than after AdLuc(wt-fiber) infection in CHO cell

lines, and all breast cancer cell lines expressing HER3/4. The infection of CAR- cells appears to be dependent on the addition of the HRG EGF-like domain and/or linkers to the fiber protein in AdLuc(HRG-fiber). It is possible that this modification has created a new binding domain between the Ad fiber gene and the HRG EGF-like domain. There is no indication of what receptor may be mediating this binding other than HER3/4 or possibly other family members by comparison to a protein sequence database.

It is also important to note that the addition of soluble HRG did not increase transgene expression in infections with AdLuc(wt-fiber), as might be expected if the activation of HER3/4 were increasing transgene expression in some other manner, like, for example, increasing activity of the CMV promoter which controls luciferase. This suggests that the addition of the HRG EGF-like domain to AdLuc(HRG-fiber) was required in *cis* to affect transgene activity. This has been shown for other Ad retargeting strategies, where the addition of an Ad retargeted to FGF2 does not increase the transgene expression of another wild-type-binding Ad (235). These results were consistent in both the breast cancer cell lines and CAR- CHO cell lines.

Thus, it appears that the addition of the HRG EGF-like domain had specifically expanded viral tropism to include HER3+ cells that were not highly infected by AdLuc(wt-fiber), as long as there was enough time to allow internalization of AdLuc(HRG-fiber) into the cells.

5.4 AdLuc(HRG-fiber) infectivity in a wider context

5.4.1 Breast cancer and HER3/4 targeted therapy

Despite many years of study into the causes and treatment of breast cancer, this disease remains a leading cause of cancer death in Canadian women (6). Treatment of metastatic breast cancer remains especially difficult. Breast cancer treatment in general relies heavily on surgery, chemotherapy and radiation; though there have been significant advances since the origins of these therapies. There are also newer targeted therapies available, such as endocrine therapy for ER positive tumors, and trastuzumab for HER2 positive tumors.

Thus, expression of tumor markers such as ER/PR and HER2 are important characteristics in treatment decisions (8). Furthermore, more recent studies have increased the number of breast cancer subtypes to five or six, which are only partially stratified based on traditional tumor markers (7; 9). Thus, tumors expressing ER or HER2 do not exclusively fall into a single subtype (7). However, these more recent six breast cancer subtypes may more closely match tumor biology, and thus may ultimately result in a better compatability of treatment options.

At this time, there remains a need for new treatments for many of the different subtypes of breast cancer. Additionally, there is a need for more specific breast cancer treatments, targeted to particular tumor markers or characteristics. Targeted gene therapy vectors can help fill this niche.

A potentially useful target for breast cancer treatment is the growth factor receptor HER3. HER3 is overexpressed in many tumors, including those expressing HER2

(321; 326). Though HER3 is not thought to drive cancer alone, it is thought to be required by HER2 to drive breast cancer (358; 407). There have not been many studies linking HER3 mRNA or protein expression to the various breast cancer subtypes, although some studies have linked it to other important breast cancer markers. HER3 has been shown to be frequently co-expressed with HER2, though whether it is primarily expressed in one of the HER2+ subtypes is unclear (408; 415; 416; 457). The relationship between HER3 and ER expression is also unclear, as some studies have associated HER3 mRNA or protein with estrogen receptor positive status (457-459), and others have shown no significant correlation (415). Some of these variation may be related to different breast cancer subtypes or expression of a soluble instead of membrane bound isoform of HER3 (321; 416). As the importance of HER3 in tumor progression and drug resistance becomes more prominent, more studies are likely to measure this protein in relation to the subtypes.

5.4.2 Receptor expression levels

5.4.2.1 Receptor expression in breast cancer cell lines

We divided our cell lines into groups based on receptor levels (Table 3.1). Most breast cancer cell lines tested were positive for HER3 and/or HER4 expression, and several were also positive for CAR expression. All cell lines tested were positive for αv-integrin. In general, our breast cancer cell line data agree with other reports of receptor expression detected by RT-PCR, northern or Western blot, or flow cytometry (305; 311; 447; 460-467).

Most breast cancer cell lines with a high proportion of cells positive for HER3 also express a relatively high number of receptors per cell. However, we found by flow cytometry that in a high proportion of MDA-MB-231 cells, the actual level of HER3 per cell was low, but detectable. As a result, the classification of MDA-MB-231 cells as either positive or negative for HER3 was not entirely straightforward. HER3/4 levels in this cell line were low by Western blot, likely because a Western blot measures the total receptor level of a population of cells, unlike flow cytometry. Other studies have considered MDA-MB-231 cells low or negative for HER3 and HER4 by RT-PCR, Western blot, or Northern blot, all methods that measure the total receptor level of a population of cells (438; 451; 468-470).

The relatively low HER3/4 expression on MDA-MB-231 cells could be relevant for different experiments, since the receptor number and concentration on the cells might be expected to affect virus binding and internalization, or transgene expression. Since relatively few receptors are expressed on the surface of the cell, this might mean that there would be less virus infectivity than would be expected based on the proportion of positive cells. We did see an increase in transgene expression after AdLuc(HRG-fiber) infection compared to AdLuc(wt-fiber) in MDA-MB-231 cells, however this difference was not as large as some of the other HER3/4 positive breast cancer cell lines, consistent with the measured receptor levels on the surface. Soluble HRG was able to compete with AdLuc(HRG-fiber) in this cell line, though the result was not significant, which may be because of the relatively low number of HER3/4 receptors on the cell surface.

5.4.2.2 Receptor expression in CHO cell lines

Chinese hamster ovary (CHO) cells, previously shown to be non-permissive for Ad infection (57), were chosen as the isogenic background strain for testing virus interactions with cell surface receptors. The CHO cell lines stably transfected with exogenous human HER3 had much lower numbers of receptors on the cell surface than the breast cancer cell lines. Contrarily, CHO cell lines transfected with CAR (57) had much higher levels of CAR than any breast cancer cell line. The CHO cell line transfected with HER3 also expressed the human receptor α2-intergrin. Though this receptor was not thought to be important in wild-type virus infection (57), as controls we tested two additional CHO cell lines: one transfected with only human α2-intergrin (CHO-al2) and another that had not been transfected with any exogenous receptor (CHO-NT).

It is also important to note that these cells are Chinese hamster ovary cells, and thus are expected to express hamster receptors, potentially including hamster versions of HER3, HER4 or CAR. We did not test specifically for hamster receptors. At the time of publication, there were no antibodies to detect the hamster version of any of the receptors described here. Though it is not known whether our antibodies to human receptors would cross-react with the hamster receptors, neither of the antibodies used (CAR and HER3) detected endogenous receptor on untransfected CHO cells. It remains possible that the CHO cells may endogenously express one or more of the receptors relevant to this study. However, since all of the CHO cell lines can be expected to contain a similar background of receptor expression, we expect that any effect seen would be relatively consistent across cell lines.

Thus, we do not know the nature of the receptor that mediates AdLuc(HRG-fiber) binding and internalization into the CHO cell lines CHO-NT and CHO-al2. Competition with soluble HRG appeared to indicate that the binding of AdLuc(HRG-fiber) to a HRG binding receptor was important for transgene expression in CHO-al2, but not CHO-NT (Figure 3.6). The primary difference between the two cell lines is the expression of exogenous human α2-integrin in CHO-al2. However, since these cell lines came from different sources, it is possible that there are other unknown differences.

5.4.3 Receptor expression levels relative to virus infection

5.4.3.1 HER2 expression and virus infection

HER2 was originally hypothesized to be the receptor for HRG, and is now thought to comprise a high affinity receptor for HRG in combination with HER3 (335; 337). HER2 is also an important factor in breast cancer (358; 407). In our study, some HER2 expression was seen in all the cell lines tested. High HER2 levels were seen in some cell lines that supported high transgene expression after AdLuc(HRG-fiber) infection, however, since all of these cell lines also express high levels of HER3/4, any increase resulting from HER2 expression could not be fully separated from that of HER3/4. Therefore, this study does not address the potential of HER2 involvement in HER3-targeted Ad internalization.

Ad has been previously targeted successfully to HER2 with a bispecific antibody (311). However, like our results, the expression of HER2 was not the only factor required for successful Ad infection. In fact, our transgene expression ratios of

targeted to untargeted virus were similar to their results for the cell lines we tested in common (MDA-MB-468, MCF7, and SKOV-3).

5.4.3.2 Integrin and virus internalization

Both viruses tested retain CAR and αv-integrin binding sites. These are known to mediate viral binding and internalization into cells (57; 64). The modified virus was expected to bind to HER3/4, but also retain CAR and αv-integrin binding. This resulted in a virus with expanded tropism, but not a virus specific to HER3/4 expressing cells. Thus, some of the challenges in using wild-type binding Ad are retained with the modified virus used here, for example, inadvertent infection of liver cells that normally express CAR and αv-integrin (122-124).

We did not include integrin in most of our analyses, since αv-integrin was expressed on almost all cells in all cell lines of this study, although at variable levels. Since αv-integrin is important for Ad internalization into cells, the relative levels of αv-integrin in each cell line may affect some of the results of the experiments. Nonetheless, we found no correlation of integrin levels with reporter gene activity in infected cells. For example, BT549b cells had high αv-integrin levels but supported much lower luciferase expression following infection than ZR75.1 cells, which displayed a relatively modest number of integrin receptors on their surface.

A recent study has shown that CHO cells lack β-integrin and fail to express αvβ3 and αvβ5 at the surface (260). We tested for αv-integrin in other cell lines, but not in CHO cells. However, we and others have not seen any problems with Ad transgene expression in Ad-infected CHO-CAR cells (Figure 3.4) (57). In fact, even

untransfected CHO cells showed high levels of luciferase expression after AdLuc(HRG-fiber) infection. It is possible that Ad exploited another mechanism of internalization in these cells, or that very low levels of integrin detected in the Corjon 2011 paper were enough to mediate Ad internalization (260). We showed that at least some of the internalization was clathrin-mediated, which is the expected mechanism of Ad internalization mediated by integrins. However, clathrin-mediated internalization is a common mechanism of internalization, and is not limited to integrin binding. Furthermore, we were expecting that HER3 may mediate Ad endocytosis in a clathrin-dependent manner. However, since integrin binding is retained in our modified virus, it is possible that integrin binding, and not HER3 binding, is mediating the internalization of AdLuc(HRG-fiber) in HER3+ cells.

Though our assays generally resulted in relatively low virus internalization in CHO cell lines other than CHO-CAR, we do not expect that this is a result of a lack of integrin expression. The binding assays showed similar results, and again, there is no reason to think that there would be differential αv-integrin expression in the different CHO cell lines tested here.

5.4.3.3 Infection of CHO-CAR/HER3 cells by both viruses

Interactions between receptors at the surface of the cell could influence virus binding and internalization in several ways. It is possible that cooperation between two or more receptors of one type, or even receptors of different types, may enhance or be required for viral binding or internalization. Additionally, it is possible that

multiple receptors on the cell surface could compete with each other for viral binding, or inhibit viral binding in some other manner, and thus decrease detected virus binding or internalization. This may explain the results seen with CHO cells expressing both CAR and HER3.

Infection of the CHO-CAR/HER3 cell line with either virus resulted in reduced reporter gene expression (by standard assay) relative to that in CHO-CAR cells. This was surprising, particularly for the AdLuc(wt-fiber) virus which is unlikely to interact with HER3 directly. One explanation could be competition or inhibition between receptors, though this is unlikely with AdLuc(wt-fiber). It would be interesting to examine further how the receptors may interact on the surface, what HER3 might do to CAR to change binding to wt, and whether the inhibition lies in virus internalization or a downstream in the virus infection pathway. Furthermore, since binding of wt capsid virus was not inhibited by co-expression of CAR and HER3, it seems more likely that inhibition is a downstream event.

The binding in the two CAR+ cell lines was similar in Figure 4.1, however, in Figure 4.2, the binding in CHO-CAR/HER3 cells was higher than in CHO-CAR cells. These differences may relate more to the differences in binding assays. However, they do indicate that it is unlikely that differences in binding result in the lower transgene expression in CHO-CAR/HER3 cells. AdLuc(HRG-fiber) binding and transgene expression in these cell lines was similar to that of AdLuc(wt-fiber).

5.4.3.4 Infection of MDA-MB-468 cells by both viruses

The only cell line in our carcinoma panel which expressed high levels of CAR, HER3/4 and integrin was MDA-MB-468, and this cell line was poorly infected by both viruses. This demonstrates that in addition to receptor expression, other factors could affect transgene expression. Any number of steps between virus binding and transgene expression could be inhibited, including virus internalization into cells, virus escape from the endosome and virus entry into the nucleus. Alternatively, the hCMV promoter could be less active in this particular cell line. This highlights one of the reasons for performing many of our experiments in isogenic CHO cell lines, namely that a diverse spectrum of activities characterize each of the breast cancer cell lines, complicating comparisons between them.

5.4.4 Microscope observation of fluorescent virus and receptors

We examined internalization of fluorescently labeled virus into CHO cells to attempt to observe virus bound to cells and taken up into cells. Surprisingly, in this assay we, unlike others (68), did not detect many virus particles bound to the outside of the cell,. Since microscopic analysis required additional manipulations to stain cells for receptor expression, it is possible that virus bound to the outside of the cell was washed off, accounting for the discrepancies between the assays. In contrast to the binding and internalization assays, less than 1% of the cells in the microscope assay were positive for virus internalization. It is likely that these differences are as a result of the additional manipulations required to stain for receptor expression on cells fixed to slides.

We have observed multiple viruses taken up into a single cell, which is consistent with another study (74). Furthermore, their study showed that Ad escape from the endosome occurs quickly after endocytosis because different preparations of a single virus that are labeled with different fluorescent dyes have very little colocalization by 10 min of internalization (74). This also means that the viruses endocytosed into the cell can be separated from each other far enough to not be colocalized, likely in separate endosomes, which do not merge cargoes before virus escape from the endosome.

We also examined colocalization of virus with the receptors HER3 and CAR. The colocalization of virus with receptor was generally what we expected, however, there was some increase in AdLuc(wt-fiber) colocalization with HER3 from 10 min to 30 min after incubation at 37°C. It is possible that this is nonspecific colocalization of virus and receptor that are close to each other inside the cell, but not necessarily bound. The virus particle size and receptor size are approximately three-fold and 25-fold smaller, respectively, than the resolution of the light microscope used (271; 471). As a result, the location of the virus or receptor signal cannot be determined with precision. Thus, while colocalization can tell us that the virus and receptor are close together, determining actual binding is not possible with these methods. Thus, though we expect colocalization represents virus binding to receptor, it remains possible that the two molecules are simply close together inside the cell.

It would be interesting to examine virus colocalization with other endocytic factors to determine the importance of various stages of internalization. A recent study was

unable to detect colocalization of Ad5 with an early endosomal protein (Rab5A-RFP), even at early time points (260). This is consistent with previous studies which show that internalized Ad likely either does not pass through the early endosome before endosomal escape, or is only in the early endosome briefly (74).

5.4.5 Inhibition of clathrin-mediated endocytosis by CPZ

Inhibition of clathrin-dependent endocytosis was accomplished in these assays by the inhibitor CPZ. Like many endocytosis inhibitors, CPZ is not strictly specific to clathrin-mediated endocytosis (see review (208)). CPZ treatment of cells results in the redistribution of clathrin and AP-2 from the plasma membrane to the endosomal membrane (207; 210). However, CPZ also appears to prevent the generation of large intracellular vesicles by either increasing membrane fluidity or inhibition of phospholipase C (472; 473). Inhibition of fluid phase internalization, such as macropinocytosis, is actually a common result of most non-genetic clathrin inhibitors (208). As a result, CPZ inhibition of clathrin-mediated endocytosis is an effective first step in the examination of retargeted Ad internalization, but it would be valuable to examine other mechanisms of internalization and/or other mechanisms of clathrin inhibition.

5.4.6 Measuring virus infection

5.4.6.1 Methods of measuring virus infection

Lytic viruses pass through many stages before the culmination of the viral life cycle: lysis of the infected cell and release of progeny virus. Viral infection can be

measured at many points in this life cycle, and the point of measurement can change what the experiment can describe. Various studies have utilized different methods to measure virus replication, such as plaque assay, late virus gene expression or virus DNA levels. Both viral genome replication and viral late gene expression occur after virus entry into the nucleus, but these steps are not simultaneous. Furthermore, a modification to the virus or the infected cell may prevent late gene expression, for example, without affecting viral genome replication. An additional consideration in measuring first generation vector infection is the lack of virus replication in most infected cells. As a result, detection of infection cannot rely on viral genome replication or any later stage life cycle process in most cells. It is common to measure virus infection in terms of reporter gene expression, and this is a valid method of measurement, especially when the goal of vector applications is to deliver a transgene to be expressed in the target cell. However, in order to examine earlier events in the virus life cycle, other methods must be employed. This is due to the many events that transpire before gene expression occurs, for example, but not limited to: virus binding, entry into the cell, escape from the endosome, travel to the nucleus, and nuclear entry.

Several methods have been employed to measure specific viral particles which are participating in early infection events, such as receptor or cellular binding and entry into the cell. Radioactively labeled virus has been used, and was shown to yield results different from gene expression (235). The use of radioactive labeling has disadvantages depending on the radioactive isotope used, and the safety precautions required are relatively extensive. The use of fluorescently labeled virus capsids is

advantageous because they are much safer to use, and fluorescence can be used to examine single infected cells with flow cytometry or fluorescence microscopy. To this end, some experiments have used genetically modified capsid proteins fused to fluorescent molecules, such as GFP, while others have covalently labeled the Ad capsid (68; 236). The advantage in using a genetic fusion of a fluorescent molecule is that the capsid is evenly labelled, and there should not be any unlabelled capsid, as long as only fusion proteins are available for capsid assembly. However, there is the potential disadvantage in that there can be difficulty in assembling the labeled viral capsid, and a new virus must be made for each virus to be fluorescently detected. Chemically labelling the virus capsid, whether by radioactive or fluorescent tags, has the advantage of being able to label any virus in a relatively simple manner. The disadvantage is that some capsids could be much more heavily labelled than others. This may affect the detection of labeled virus capsids in some methods more than others. Additionally, some labelling mechanisms can be unstable, and cleavage can result in free label. Furthermore, the label may also inhibit receptor binding or virus internalization. We have confirmed that with our method there was very little loss in infectivity of the labeled virus, compared to an unlabeled virus which underwent a similar procedure. It is also important to note that both infectious and non-infectious virus particles are likely labeled with the fluorochromes. This is relevant to the binding and internalization assays using fluorescently labeled virus since the majority of the signal detected in these assays is actually comprised of non-infectious virus. The potential caveats of any labelling

mechanism must be taken into account when evaluating experiments with labeled virus.

An important consideration in a luciferase assay is that one is measuring the amount of luciferase produced in a population of cells. High luciferase expression in a small fraction of the cells will give the same result as low expression in a high proportion of cells. However, luciferase was chosen as a reporter gene because the sensitivity of luciferase detection *in vitro* is relatively high (474; 475). Furthermore, the light produced by luciferase can be measured directly in live animals (474). Thus, this reporter gene remains useful for both *in vitro* and *in vivo* assays, and is particularly well suited for *in vitro* analysis performed here.

5.4.6.2 Particle to pfu ratio for virus

A potential issue with labeled virus detection is that infectious virus (here measured as pfu or ifu) cannot be separated from non-infectious virus. Production of virus results in a range of virus concentrations, and a range of ratios of total virus particles (vp) to infectious virus, depending on the virus. Two methods were used to titer infectious virus: plaque assay, where a virus produces a plaque, or cleared area of cells, on a monolayer of HEK293 cells; and staining for expression of the late viral protein, hexon in infected HEK293 cells. Both methods of measurement require late gene production, but the second method does not require repeated rounds of infection. The particle to infectious virus ratios of the virus preparations used here can be found in Table 2.1.

The method of fluorescent labelling used here would tag both infectious and non-infectious virus particles. Thus, there can be some difficulty in distinguishing the real infectious virus signal from "biological noise" of non-infectious particles, especially when the particle to pfu ratio is high (237). The nature of these non-infectious particles is not known, nor is their effect on infectious viral gene expression. These particles could be defective and/or containing small deletions in the virus genome, which may allow them to bind and enter cells, but not always express virus transgenes. Non-infectious particles may also be unable to complete all the lifecycle steps prior to gene expression if the capsid is lacking essential components, such as the viral protease. This is an additional reason to investigate the early steps in viral infection in particular, as this may show that these "non-infectious" particles can have some effect on infection. For example, they could bind and enter cells. Activation of signaling pathways by non-infectious particles may either increase or decrease the likelihood of further virus infection. For example, macropinocytosis may be triggered, which could possibly result in internalization of infectious virus.

Though there are limitations to the investigation of early infection events, such as binding and cellular internalization, the examination of these steps is important in the understanding of the more complex infection environment found *in vivo*.

5.4.7 Other HRG-targeted therapies

5.4.7.1 HRG bound polypeptides

HRG is a natural ligand for HER3, and has been used previously to target fused polypeptides (451; 469; 470; 476-478). These polypeptides include diphtheria toxin (DT) and Pseudomonas exotoxin (PEx), which both mediate cell death by the inactivation of eukaryotic elongation factor 2 (451; 469; 470; 476-478). Endosomal escape mediated by a translocation domain can be important for the function (451). In general, the HRG-fused polypeptides were shown to activate HER2/3/4 receptors and resulted in receptor phosphorylation (451; 469; 470; 478). There is some disagreement as to whether HER4 or the HER2/3 dimer are the most efficient at either binding or mediating cell death by HRG-fused polypeptides (451; 469; 470; 476; 478). This may be due to different methods of receptor detection, different cell line isolates, or differential binding by different isoforms of HRG.

Some of the cell lines which were found to be susceptible to the HRG-fused polypeptides were also susceptible to AdLuc(HRG-fiber) infection, and some were not. Yang et. al. saw high efficacy in the MDA-MB-361 cell line, mid-level in the MCF-7 cell line and low level efficacy in the MDA-MB-231 cell line, which were all similar to the relative luciferase activity after AdLuc(HRG-fiber) infection (3; 469). Several other studies had similar results for the MCF-7 and MDA-MB-231 cell lines (451; 470; 476; 478). In contrast, we saw the highest luciferase activity in the ZR75-1 cell line, while the efficacy of the polypeptides was relatively low in the study by Jeschke et. al. (470). Also differently, we saw relatively low levels of infection in the MDA-MB-468 cell line, while Yang et. al. saw mid-level efficacy

(469). Our results for the MDA-MB-468 cell line were closer to that seen in the study by Kihara et. al. (478). Many different studies looked at SKOV-3 killing or inhibition of SKOV-3 tumor xenograft growth, and saw little to no efficacy, while we saw more mid-range levels of luciferase expression after infection (469; 470; 476; 478). It is possible that the events following binding, including internalization, may differ between our virus and the HRG-targeted peptides, leading to some difference in results with similar cell lines.

Interestingly, it is thought that the DT fused to HRG requires internalization into an acidic endosome for efficacy (451; 479). Furthermore, the sensitivity of the cell lines to DT-HRG was better correlated to rate of internalization than to number of binding sites (451). Thus it appears that HRG is able to mediate DT-HRG entry into cells. This supports the supposition that binding to HER3/4 alone can mediate endocytosis to an acidic endosome, which is one of the requirements for the wild-type Ad entry and endosomal escape. HER3 internalization, though slower than that of EGFR, has been demonstrated in several studies (346; 353; 374; 376). Thus, we hypothesize that HER3/4 may be able to mediate retargeted Ad internalization, replacing the function of integrin for wt Ad. This is further supported by the evidence of low integrin expression on CHO cells (260), and that this internalization may occur between 10 min and 30 min, and is slower than integrin mediated internalization. However, similar to our results, internalization was shown to be only one factor important for DT-HRG toxicity, and that other downstream events also play an important role (451).

In vitro results with these fused polypeptides did not always correlate with efficacy in tumor xenograft models. Intratumoral injection of HRG-fused PE resulted in tumor shrinkage in N87 tumor xenografts (human gastric cancer), but not SKOV-3 or MDA-MB-231 (469).

Though the HRG-fused polypeptides generally result in activation of HER family receptors and phosphorylation, this may not be necessary for either the polypeptide activity or Ad targeted gene therapy (451; 469; 470; 478). An Ad was successfully retargeted to EGFR by using cetuximab fused to a shielding polymer (poly hydroxypropylmethacrylamide, pHPMA) (314). Since cetuximab is not a ligand for EGFR, it is likely that receptor activation was not required for transgene expression.

5.4.7.2 HRG-targeted retrovirus

HRG has also been used to target a retroviral vector (the Moloney murine leukemia virus), by the fusion of HRG to the viral envelope glycoprotein (438). This virus does not normally infect human cells, but this retargeting rendered it able to infect human cells expressing the receptors HER2 and HER4 (438). This paper did not quantify the level of HER3 on the cell lines studied. We infected some of the same cell lines as reported for the HRG-targeted retrovirus (438), and saw some similarities. Both viruses successfully infected MDA-MB-361 (high HER3/4, low CAR) and neither virus highly infected MDA-MB-231 (low HER3/4, mid CAR).

There was a difference in the results of infection of the MCF-7 cell line (high HER3/4, low CAR), where our virus infected and the targeted retrovirus did not. This could reflect differences in susceptibility to the different types of virus or to

genetic/epigenetic drift in the cell lines in their lab compared to ours. They hypothesized that the receptor levels on the surface may have been too low, though they did quantify the amount of HER4 (438). We detected high HER3 on the MCF-7 cell line.

5.4.8 Mouse xenograft tumor infection

5.4.8.1 Mouse receptor expression

Previous studies have detected high amounts of mCAR mRNA in the mouse heart, lung, liver and kidney, with the highest amount in the liver (60; 130). Similar to hCAR, expression of mCAR renders CHO cells permissible to Ad infection (130). Furthermore, Ad5 knob has been shown to bind mCAR (130). While human Ad does not replicate well in mouse tissue, transgene transduction is possible (480-482). In our *in vivo* assay, we did not detect high levels of reporter gene expression in mouse liver after intratumoral injection of either virus, although liver was a possible site for infection *via* mCAR, and a significant portion of Ad would have traveled to the liver from the injection site (483) (60; 130).

Murine HER3 expression has been studied and manipulated extensively (reviewed in (321)). In general, expression of murine HER3 appears to be similar to humans. We did not test directly for murine HER3 in our experiments. Furthermore, we are not certain if the human HER specific antibody used in our study (Figure 3.1) was also able to detect murine HER3, though there is homology between the two receptors. It is not clear that detection of murine HER3 would be relevant in our

studies, since our focus was on transgene expression in tumor xenografts of a human HER3+ cell line.

5.4.8.2 Virus spread after intratumoral injection

In our experiments, virus was injected intratumorally to allow the virus maximal access to the tumor, and as a model for future cancer therapy studies with an HRG-modified Ad. Injection of both viruses resulted in similar transgene expression in infected tumors. Intratumoral pressure and density made it difficult to inject the tumors in three separate locations, as we had intended. Thus more of the virus remained localized in a small area in the tumor, and likely was not able to spread as effectively throughout the tumor due to pressure. High intratumoral pressure has previously been advanced as a limiting factor in viral dispersion (452). High localized concentrations may lead to a maximization of transgene expression, but we have no evidence of this here. We found no correlation between luciferase levels and either CAR or HER3 receptor expression. There are reports of other *in vitro* studies not translating to *in vivo*, including an Ad5 vector targeted to Ephrin A which did not increase targeting to a subcutaneous pancreatic cancer xenograft in spite of promising cell culture results (484).

If viral spread within the tumor is the problem, this could be improved by multiple methods to enhance viral dispersion replacing luciferase with the Ad E1 region to convert it to an oncolytic virus would allow viral spread through multiple rounds of infection. Though, if the expression plateau hypothesis is correct, untargeted virus would be expected to infect cells as efficiently as targeted virus during the

first round of infection. As the virus spreads throughout the tumor, virus concentrations at the periphery would be lower, so, later rounds of infection and replication may favor targeted virus.

If the expression plateau hypothesis is correct, another option would be targeting a tumor which is naturally more dispersed. For example, many ovarian tumors spread within the peritoneal cavity (485; 486), and also express high levels of HER3/4 (417; 487-490). Intraperitoneal injection would allow viral dispersion and decrease concentration of the virus at the point of infection. There are mouse models of ovarian cancer available for testing this hypothesis, and these have been used for other Ad studies (491; 492). In fact, one of the potential reasons for difficulty in infecting ovarian tumors has been linked to poor viral transduction due to low or variable CAR and αv-integrin on ovarian tumors in animal models and in the clinic (486; 491; 493-496). We propose that the expanded tropism of AdLuc(HRG-fiber) may permit effective infection of low CAR, high HER3/4 ovarian tumors.

5.5 Significance and future directions

We have modified an Ad to target breast cancer cells expressing HER3/4 by the addition of the EGF-like domain of HRG into the fiber coding region of the Ad genome. These studies have shown expanded tropism of this virus, AdLuc(HRG-fiber), to cells not effectively infected by the wild-type binding virus, AdLuc(wt-fiber). In particular we report better transduction of breast cancer and CHO cells expressing HER3/4. AdLuc(HRG-fiber) infection appears to occur either by a CAR-dependent or CAR-independent pathway as predicted. However,

AdLuc(HRG-fiber) did not show improved gene transfer in a breast cancer xenograft model in mouse relative to AdLuc(wt-fiber).

Though virus binding and internalization of AdLuc(wt-fiber) was generally consistent with expectations, AdLuc(HRG-fiber) did not bind to or get taken up as efficiently as expected in CAR- cell lines. It is possible that the kinetics of virus internalization of AdLuc(HRG-fiber) in CAR- cell lines was slower than that of either AdLuc(wt-fiber) or AdLuc(HRG-fiber) in CAR+ cell lines. Additionally, we have established that AdLuc(HRG-fiber) was endocytosed by a clathrin-mediated mechanism, at least partially, in all cell lines tested.

The results of our binding and internalization studies raise questions about the specific internalization pathway used by modified Ad, and how this may affect transgene expression, the goal of gene therapy. It would be interesting to examine AdLuc(HRG-fiber) binding and internalization at later time points, including multiple mechanisms of internalization.

In the following sections we highlight some changes that could be made to the vector to improve its utility as a cancer gene therapy delivery vehicle.

5.5.1 Virus Construction

5.5.1.1 Genetic Ad targeting

In the construction of the fiber-modified virus, the HRG EGF-like domain flanked by linkers were inserted into the virus fiber protein coding sequence (Figure 2.3). These linker sequences encode primarily glycine and were added to ensure flexibility flanking the inserted binding domain. There is no indication that the

linker sequences will recognize a receptor on the cell surface and mediate either binding or internalization of the modified virus. In addition, the inserted sequence has no sequence similarity to any known receptor ligand, except heregulin. However, with the experiments performed here, receptor binding to the linker or to the HRG EGF-like domain or a fusion of the two cannot be fully differentiated. Thus binding and internalization, though likely mediated by the known HER3/4 ligand, the HRG EGF-like domain, could also be mediated in part by the linker. It could be useful to know whether the linker participated in binding or internalization into CAR- cells, which could be tested by the replacement of the linkers with others. We used soluble HRG to compete with virus binding in our transgene assays (Figures 3.5 and 3.6). It would have been useful to use soluble HRG to compete for binding and internalization directly, rather than measuring indirectly by transgene expression. However, it may be difficult to see inhibition of the already low values for binding and internalization for CAR- cell lines. This may be remedied by a longer incubation for virus binding and internalization for these and other assays.

Since the modified virus contains a domain known to activate the HER3 receptor, the HRG EGF-like domain, it is expected that this virus could result in activation of the receptor and perhaps downstream signaling. Other HRG targeted moieties have been shown to activate HER receptors, though this is not always related to their mechanism of action (451; 469; 470; 478). However, receptor activation may be important in virus internalization, and may be important to test for in future experiments. Phosphorylation and activation of HER3 can be detected with antibodies, and downstream activation of the PI3K pathway could also be

determined in a similar manner. Furthermore, the requirement for kinase activity of other EGFR family members in virus internalization or transgene expression could be determined using a kinase inhibitor or kinase dead receptor mutants.

5.5.1.2 Reporter gene

In the construct used here, the reporter gene and promoter were inserted in place of the E3 region, rather than E1, as is common for first generation vectors. There may be some benefit to inserting this gene in the deleted E1 region, as this is a more common construct in our lab, and would facilitate comparisons with other previously constructed vectors. It may also be useful to replace the luciferase reporter gene with a trifusion gene encoding a fusion of modified firefly luciferase, red fluorescent protein and HSV-TK (497). This would allow us to take advantage of multiple imaging modalities, depending on which was best for a particular experiment.

5.5.2 Therapeutic HRG-targeted adenovirus

Ultimately, this targeted virus would be used to treat cancers that express the receptors HER3 and HER4. Though we have primarily mentioned breast and ovarian cancers in this discussion, there are other cancers, such as gastric cancer, melanoma, and head and neck cancer which may be useful to treat with a therapeutic version of this vector (reviewed in (321; 411)). In order to render the virus therapeutic there are several changes which would be beneficial and sometimes necessary, as outlined below.

5.5.2.1 Transcriptional control of transgene expression

The promoter controlling reporter gene expression in the viruses in this study was the human cytomegalovirus (CMV) immediate early promoter. This promoter was chosen as a highly active promoter in most cell lines (498), with the expectation that this would help to simplify the detection of luciferase and the comparison of the two viruses. A disadvantage of this promoter is that it is not highly active in mouse cells (499). However, since the animal experiments described here were designed to compare the two viruses directly in a human tumor, this was not anticipated to be a problem. Another promoter may be needed if future experiments were to be done in an immunocompetent mouse tumor model. For example, the murine CMV promoter has been shown to be highly active in both human and mouse tissues (499).

In order to increase selectivity for cancer, it may be useful to use a tumor or tissue specific promoter to drive transgene expression in the virus. A potentially useful promoter currently being studied in our lab is the mammaglobin promoter, for which we have shown selectivity for breast cancer cell lines (500). Alternatively, we could use a promoter from a gene such as human telomerase reverse transcriptase (hTERT), which has been shown to be highly active in many tumors, and has been used to target other Ad vectors (501).

5.5.2.2 Improving Ad delivery in vivo

Ad is known to be highly immunogenic, which can result in neutralization by antibodies, thereby decreasing viral efficacy (245). Though this was not directly

addressed in this study, there are two common ways of meeting this challenge. The most immunogenic sites can be removed or modified to render the virus less immunogenic (245; 246). For example, modification of the hexon hypervariable region has been shown to decrease Ad immunogenicity (245).

In our study we injected virus directly into the tumor to decrease exposure of the virus to the circulation, thus reducing exposure to non-target cells including the liver. For disseminated disease, intravenous delivery would likely be required. Therefore a strategy to block the liver tropism of Ad would be beneficial. Hexon is the primary binding site of Factor X, the blood coagulation factor thought to mediate liver internalization (183). Modification of hexon to prevent liver binding would decrease liver tropism (183; 193).

Further control over infection of non-target cells may be achieved by the ablation of the CAR and integrin binding sites on the virus capsid. This could be achieved in multiple ways. One way would be to delete or modify the binding sites in the genome of AdLuc(HRG-fiber). What may be easier would be to insert the EGF-like domain of HRG into the HI loop of a virus genome which already contains wild-type binding site ablations, such as the vector constructed by the Mizuguchi lab (272). These modifications would help to limit the infection of non-target cells.

5.5.2.3 Transgenes

To make the virus effective as a breast or ovarian therapeutic, the reporter gene must be substituted for a therapeutic gene. Potential genes include suicide genes, tumor suppressor genes, or immunomodulatory genes (34). Our lab has studied the

suicide gene herpes simplex virus (HSV) thymidine kinase (TK) (502). This gene converts the pro-drug ganciclovir into a more toxic form that is retained primarily inside the cell, limiting toxicity to cells containing both drug and transgene (503). Adp53, encoding the tumor suppressor p53, has been used in the clinic, however, it has failed in ovarian clinical trials (504). It is possible that retargeting by HRG might improve efficacy by mediating internalization into CAR- tumor cells.

5.5.2.4 Targeted oncolytic Ad

Another option for a targeted Ad therapy is an Ad engineer to replicate conditionally in tumor cells, an oncolytic Ad (34). Capsid targeting, in combination with transcriptional control, could yield a virus which would specifically replicate in tumor cells (107; 108). One mechanism would be to replace the transgene in a targeted virus similar to AdLuc(HRG-fiber) with the E1A coding region, under control of a tumor specific promoter, such as those discussed in Section 5.3.2.1. For example, an hTERT targeted oncolytic Ad is currently in phase I clinical trials (501). Expanding tropism of oncolytic Ads could be very important for efficient spread in low CAR tumors (34).

5.5.2.5 Combination with other therapies

Ultimately, it may be more effective to use an HRG-targeted Ad in combination with other treatments to help control tumors expressing high HER3/4. Previous studies have shown augmentation of oncolytic Ads by chemotherapy (505; 506). Treatments that rationally combine the HER3/4 targeted vector with another

therapeutic would deserve special consideration. For example, since HER3 is often important in HER2+ breast cancer, the combination of a therapeutic version of AdLuc(HRG-fiber) and HER2 targeted therapies should be examined. One potential mechanism of resistance to HER2 targeted therapies such as trastuzumab is increased signaling by HER3 (327). Therefore, sequential treatment with trastuzumab followed by HER3-targeted virus may be especially effective.

One current avenue of treatment targeting HER3 is monoclonal antibodies, some of which have reached phase II clinical trials (reviewed in (507)). Though these treatments help support the validity of our target, it would likely not be beneficial to treat a patient with HER3 antibodies at the same time as an Ad vector targeted to HER3.

5.5.3 Conclusion

We have shown expanded tropism of a HER3/4 targeted Ad to cells not highly infected by a wild-type binding Ad, including those expressing HER3/4. Though AdLuc(HRG-fiber) binding and internalization in short term assays were not higher than that of AdLuc(wt-fiber) in many cases, we propose that this may be due to a slower internalization mechanism in CAR- cells. Ultimately, we demonstrated that this targeting mechanism is useful *in vitro*, and may have potential uses *in vivo*. In combination with other vector modifications this targeted Ad vector could form an integral part of HER3-positive cancer therapy.

CHAPTER 6: References

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