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CONTROLLING TRANSDUCTION AND REPLICATION OF ONCOLYTIC ADENOVIRUSES

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ACADEMIC DISSERTATION

Helsinki University Biomedical Dissertations No. 139

To be publicly discussed with the permission
of the Faculty of Medicine of the University of Helsinki,
In Haartman Institute lecture hall 2, Haartmaninkatu 3, Helsinki
on November 19th 2010 at 12.00 noon.

Helsinki 2010

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ISBN 978-952-92-7953-1 (paperback)
ISBN 978-952-10-6462-3 (PDF)
http://ethesis.helsinki.fi/

Helsinki 2010
Yliopistopaino

To my family.

I have not failed. I´ve just found 10 000 ways that won´t work.
Thomas Alva Edison (1847-1931)

ABSTRACT

Despite progress in conventional cancer treatment regimes such as chemotherapy and radiation therapy, metastatic disease essentially remains incurable and new treatment alternatives are desperately needed. Virotherapy is a relatively novel approach in cancer treatment. It harnesses the natural ability of oncolytic viruses to kill the cells they proliferate in and to spread to neighboring cells, thereby amplifying the therapeutic effect of the initial input dose. The use of replicating, oncolytic viruses for cancer treatment necessitates introduction of various genetic modifications to the viral genome, thereby restraining replication exclusively to tumor cells and eventually obtaining selective eradication of the tumor without side effects to healthy tissue. Furthermore, various modifications can be applied to the viral capsid in hope of gaining effective transduction of target tissue. In other words, the entry of viruses into tumor tissue can be augmented by allowing the virus to utilize non-native receptors for entry. Genetic capsid modifications may also help to avoid some major hurdles in systemic delivery that ultimately lead to the rapid clearance of the virus from the blood and virus induced toxicity.

We evaluated the transductional efficacy of various capsid modified serotype 5 adenoviruses *in vitro* in breast cancer cell lines and in fresh patient samples. An orthotopic breast cancer model was used to evaluate the biodistribution of capsid modified viruses *in vivo* in mice. Based on the transductional potency data of the viruses, we created a novel oncolytic adenovirus featuring a polylysine (pK) motif in the fiber C-terminus to allow binding to heparin sulphate proteoglycans in addition to primary coxsackie-adenovirus receptor and evaluated its oncolytic potency *in vitro* alongside existing replicating agents featuring the other previously tested capsid modifications. We concluded that capsid modifications result in transductional enhancement, and that enhanced transduction translates into more potent oncolysis *in vitro*.

The efficacy of the pK modified virus was evaluated in an orthotopic breast cancer model with green fluorescent protein (GFP) expressing breast cancer tumors treated with intratumoral injections. We developed a mouse model with imageable breast cancer tumors growing in the left lung to test the efficacy of capsid modified viruses in a systemic treatment model of disseminated breast cancer. With systemic delivery of the viruses, we saw prolonged survival of tumor bearing mice treated with the capsid modified agents. Furthermore, we were able to follow the tumor growth without invasive procedures in both breast cancer mouse models.

Kupffer cells (KC) are responsible for the majority of viral clearance after systemic viral delivery and they play a major role in adenovirus induced acute toxicity. The therapeutic window could possibly be widened by transiently depleting KCs, which might allow smaller viral input doses and diminish KC related toxicity. KC depleting agents were used *in vivo* prior to viral injections to further enhance transductional efficacy of the viruses. Enhanced tumor transduction was seen, but this effect was not translated into enhanced antitumor activity.

Only a small proportion of cells within a tumor may possess the capacity to proliferate indefinately and form new tumors. These cancer initiating cells, or cancer stem cells, would drive tumor formation and be responsible for posttreatment relapses and metastasis.

Therefore, we evaluated whether transcriptionally controlled replicating viruses would be effective in eradicating putative breast cancer stem cells, postulated to reside in the subset of $CD44^{+}/CD24^{-/LOW}$ cells. Effective cell killing was seen *in vitro* and more importantly, significant growth reduction of tumors derived from the $CD44^{+}/CD24^{-/LOW}$ cell population was seen *in vivo*.

Genetically modified viruses feature enhanced oncolytic potency, which unfortunately can increase the possibility of side effects. Therefore, it would be beneficial if the replication of adenoviruses could be reduced with pharmacologic intervention. We studied the ability of chlorpromazine and apigenin to reduce adenoviral replication and replication associated toxicity *in vitro* in normal cells, ovarian cancer cells, and patient liver samples. Antitumor efficacy, viral replication, and liver toxicity *in vivo* in an ovarian cancer model were also evaluated. We concluded that these agents reduced adenoviral replication and might be useful as safety switches in case viral replication related side effects are encountered.

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ABBREVIATIONS

5-FU	5-fluorouracil
Ad2	adenovirus serotype 2
Ad3	Adenovirus serotype 3
Ad5	Adenovirus serotype 5
Ad7	Adenovirus serotype 7
ADP	adenovirus death protein
ATCC	American Type Culture Collection
bp	base pair
BT	biologic therapy
CAR	coxsackie-adenovirus receptor
CEA	carcinoembryonic antigen
CCNDI	cyclin D1
CIC	cancer initiating cell
CMV	cytomegalovirus
CPE	cytopathic effect
CR1	constant region 1
CR2	constant region 2
CSC	cancer stem cell
CTL	cytotoxic T-cell
DC	dendritic cell
DHEA	dehydroepiandrostene
EA	epiandrostene
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EGR	early growth response gene 1
ER	estrogen receptor or endoplasmic reticulum
ET	endocrine therapy
FCS	fetal calf serum
FIV	coagulation factor IV
FX	coagulation factor X
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HER2	Human epidermal growth factor 2
HSC	hematopoietic stem cell
HSPG	heparin sulphate proteoglycan
hTERT	human telomerase reverse transcriptase
IFN	interferon
i.ha.	intrahepatic artery
i.p.	intraperitoneal
i.t.	intratumoral
i.v.	intravenous
kb	kilobase

KC	Kupffer cell
kD	kilodalton
KKTK	lysine-lysine-threonine-lysine
luc	luciferase
mdr	multidrug resistance
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MOI	multiplicity of infection
MoMuLV	Moloney murine leukemia virus
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium
NAb	neutralizing antibody
NK	natural killer cell
NMRI	Naval Medical Research Institute
PAMP	pathogen associated molecular pattern
pfu	plaque formin unit
PR	progesterone receptor
pK	polylysine
pRb	retinoblastoma protein
PSA	prostate-specific antigen
RGD	arginine-glycine-aspartic acid
s.c.	subcutaneous
SCCHN	squamous cell carcinoma of the head and neck
SCID	severe combined immunodeficiency
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSG	tumor suppressor gene
TSP	tissue/tumor specific promoter
VP	viral particle

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Ranki T**, Kanerva A, Ristimäki A, Hakkarainen T, Särkioja M, Kangasniemi L, Raki M, Laakkonen P, Goodison S, Hemminki A: A heparan sulfate-targeted conditionally replicative adenovirus, Ad5.pK7-Δ24, for the treatment of advanced breast cancer. 2007 Gene Ther 14:58-67.

II **Ranki T**, Särkioja M, Hakkarainen T, von Smitten K, Kanerva A, Hemminki A: Systemic efficacy of oncolytic adenoviruses in imagable orthotopic models of hormone refractory metastatic breast cancer. 2007 Int J Cancer 121:165-174.

III Bauerschmitz G*, **Ranki T***, Kangasniemi L*, Ribacka C, Eriksson M, Porten M, Herrmann I, Ristimäki A, Virkkunen P, Tarkkanen M, Hakkarainen T, Kanerva A, Rein D, Pesonen S, Hemminki A: Tissue specific promoters active in $CD44^{+}CD24^{-/LOW}$ breast cancer cells. 2008 Cancer Res 68:5533-5539.*= equal contribution.

IV Kanerva A, Raki M, **Ranki T**, Särkioja M, Koponen J, Desmond RA, Helin A, Stenman U-H, Isoniemi H, Höckerstedt K, Ristimäki A, Hemminki A: Chlorpromazine and apigenin reduce adenoviral replication and decrease replication associated toxicity. 2007 J Gene Med 9:3-9.

The publications are referred to in the text by their roman numerals.

REVIEW OF THE LITERATURE

1 Introduction

Cancer is a major public health problem accounting for more mortalities than heart disease in people under 85 years of age (Jemal et al. 2009). Despite progress made in reducing incidence and mortality due to improvements in cancer prevention, early diagnosis and conventional treatment methods, cancer still accounts for approximately 6.7 million deaths annually worldwide (Parkin et al. 2005). Approximately 10 000 cancer deaths occur annually in Finland (www.cancerregistry.fi). Metastatic disease remains essentially incurable.

Typically carcinogenesis is a multistep process representing alterations in proto-oncogenes and tumor suppressor genes (TSG) that ultimately lead to tumor promotion. In short, cancer is a disease of the genes, which has led to the logical conclusion of correcting genetic defects underlying carcinogenesis or utilizing the altered phenotype of cancer cells to gain specific antitumor effects. Traditionally, gene therapy aims at replacing dysfunctional genes with functional ones or delivering genes for the expression of therapeutic proteins within the cell. Since a cancer cell with a malignant metastazising phenotype may display up to a few hundred genes with altered functions or expression (Kenemans et al. 2004), traditional gene therapy approaches may not be sufficient to gain therapeutic effects.

Instead of correcting genetic defects, oncolytic virotherapy takes advantage of the similarities between requirements of carcinogenesis and DNA virus replication to direct the antitumor effect of an oncolytic virus to tumor. Adenoviruses are widely studied and used as oncolytic agents in cancer virotherapy approaches. Adenoviruses possess an inherent potential to lyse the cells they replicate in, but to avoid side effects in normal tissue their replication has to be rigorously controlled. Oncolytic virotherapy relies on the delivery of the viral genome to the target tissue, and in the context of disseminated disease, systemic delivery is a prerequisite for successful treatment. Therefore, the cancer cell transduction and viral replication must be controlled by genetic engineering of the viral genome to gain effective and safe tumor eradication. During the last decade, the interests of the cancer gene therapy field have focused on identifying the most effective tumor targeting approaches and means to tightly control replication of the oncolytic agents to avoid replication related toxicity to normal tissue. Furthermore, the balance between evading viral neutralizing immunity and enhancing the immune responses against cancer cells containing replicating virus within is a major focus of interest.

2 Cancer

Cancer is thought to be the result of sporadic and/or inheritable genetic mutations in somatic or germline cells (Edler and Kopp-Schneider 2005). Cancer cells are

characteristically different from normal cells. They display uncontrolled growth, loss of contact inhibition, loss of differentiation and increased invasiveness (Leber and Efferth 2009). They are also capable of inducing neoangiogenesis, *i.e.* the formation of small capillaries for constant supply of nutrients and oxygen as well as removal of metabolic waste. Importantly, cancer cells evade the host immune system and normal apoptotic restraints, and are essentially immortalized through chromosome telomere-lengthening (Bodnar et al. 1998). All these characteristics play a role in carcinogenesis and the differentiation of a normal cell to a malignant cell.

In sporadic cancer, expression of active oncogenes leads to uncontrolled cell proliferation (Kenemans et al. 2004). Other genetic mutations, mainly in TSGs, are then postulated to lead to malignancy. Oncogenes represent genetic alterations in proto-oncogenes, *i.e.* genes normally involved in cellular pathways regulating cell growth and differentiation (Edler and Kopp-Schneider 2005). These alterations are called gain-in-function mutations that ultimately send the cell from resting stage into cell division, and are comparable to stepping on the accelerator of a car. Oncogenes mediate their impact through either excess amounts of the protein product, due to amplification of the gene or overexpression of the protein, or through functional enhancement of the protein (Osborne et al. 2004).

While oncogenes essentially promote cell growth and proliferation, TSGs inhibit them (Kenemans et al. 2004). Their normal function is equivalent to stepping on the brakes. TSGs are called gatekeeper or caretaker genes, as they are responsible for restraining cell division or ensuring that DNA is not damaged (Osborne et al. 2004). Mutations in these genes are called loss-of-function mutations, and terminate the cell quiescence or cause resistance to apoptosis, thereby allowing cell proliferation.

A tumor that has not yet reached an invasive and metastasizing phenotype is often referred to as carcinoma *in situ*. Invasiveness refers to the ability to actively invade and, thus, destroy adjacent tissues (Leber and Efferth 2009). Metastasis refers to the ability to leave the primary tumor, circulate to a distant site, and form a secondary tumor. The metastatic cascade consists of five steps:

1) Invasion and migration: cells detach from the primary tumor and invade adjacent tissue with the help of secreted lytic enzymes that degrade the extracellular matrix (ECM).
2) Intravasation: cancer cells secrete proteolytic enzymes and thereby intrude blood and lymphatic vessels.
3) Circulation: cancer cells travel via the blood stream and undergo selection for particularly resistant and aggressive phenotypes with the ability to withstand the high oxygen concentration and evade cytotoxic lymphocytes present in blood.
4) Extravasation: cancer cells leave the circulation with the help of proteolytic enzymes and invade into tissue.
5) Colonization, proliferation and angiogenesis: a cancer cell settles at a distant site, forms a secondary tumor by proliferation, and ensures vascularization by neoangiogenesis.

2.1 Cancer stem cells

Normal stem cell characteristics include the capability of self-renewal, strict control over stem cell numbers and the ability to form phenotypically diverse tissues (Morrison et al. 1997). Similarily to normal stem cells, cancer initiating cells (CIC) have extensive proliferative potential and the ability to form new tissues, but they seem to lack control over cell numbers (Sagar et al. 2007). Most tumors have a clonal origin, which implies that CICs must give rise to phenotypically diverse cell populations with varying proliferative potential and different stages of differentiation (Reya et al. 2001), suggesting that referring to CICs as cancer stem cells (CSC) is warranted. CSCs undergo a poorly regulated process of organogenesis, resulting in the formation of phenotypically diverse tissues. As a result, tumors can be viewed as aberrant organs initiated by a CSC.

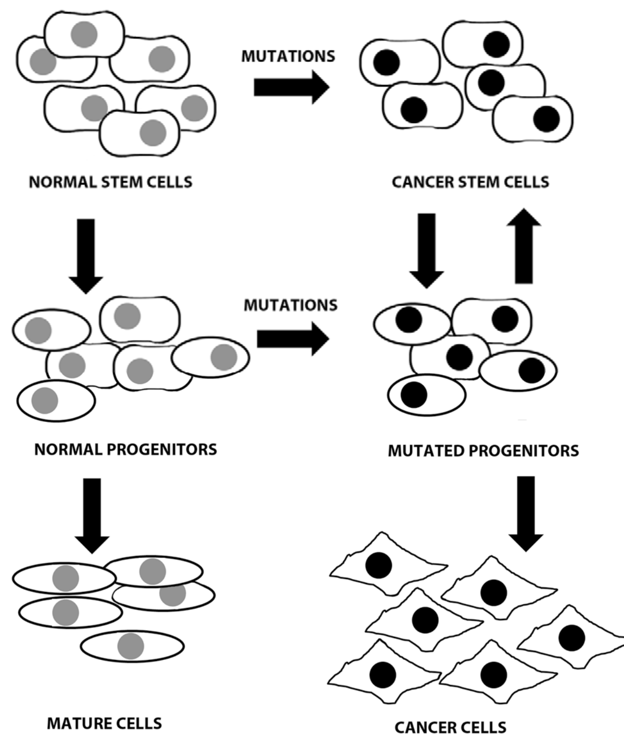

Figure 1. Hypotheses of cancer stem cell origin. Cancer stem cells may be derived from normal stem cells that have acquired mutations and have altered proliferative pathways. They may also develop from normal progenitor cells that go through multiple oncogenic mutations and acquire the ability to self-renew.

The origin of CSCs is debatable, but they are postulated to be derived from either the self-renewing normal stem cells that have transformed via altered proliferative pathways, or from progenitor cells that have acquired the ability for self-renewal through oncogenic mutations (Al-Hajj et al. 2004) (figure 1). The cancer stem cell model for cancer adds the silencing of the genes controlling stem cell self-renewal and proliferation as one key event to tumorigenesis in addition to the role of oncogenes and TSGs.

CSCs can be distinguished from nontumorigenic cells by specific cell surface markers. The presence of a transmembrane glycoprotein CD133 has been reported as a CSC marker for breast, colon and liver tumors to name a few (Boman and Wicha 2008; Vermeulen et al. 2008; Wright et al. 2008). Due to the CSC heterogenicity within a tumor and between different patients, a single marker may be a poor distinguishment and the use of multiple markers is considered more reliable, though less specific (Saini and Shoemaker). Putative breast CSCs presumably reside in the population positive for CD44 expression and negative or scarce for CD24 expression (Al-Hajj et al. 2003). This subset of $CD44^{+}/CD24^{-/LOW}$ breast cancer cells was enough to form tumors with an inoculation of as few as 100 cells in NOD/SCID mice, while tens of thousands of cells of the other populations failed.

The existence of putative CSCs has major implications for cancer treatment. Relapses that are often observed after chemotherapy are thought to result from the existence of drug-resistant CSCs (Li et al. 2008; Gillette and Nielsen-Preiss 2009). The drug-resistant phenotype may be partially explained by the presence of APC transporters that have a well-defined role in drug efflux (Gottesman et al. 2002). Furthermore, it has been postulated that in a given time most CSCs are not active in the cell cycle, which explains why CSCs are able to evade the therapeutic effects of most current treatment methods that kill proliferative cells (Sneddon and Werb 2007; Saini and Shoemaker). CSCs might also have enhanced DNA repair machinery and a higher tolerance for mutations due to disrupted apoptosis machinery (Johannessen et al. 2008).

2.2 Breast cancer

Over 1,1 million women are diagnosed annually with breast cancer (Parkin et al. 2005). Breast cancer treatment has advanced significantly over the last few decades with biological treatment modalities emerging as treatment options to be used alongside more conventional methods such as surgery, chemotherapy and radiation therapy (Di Cosimo and Baselga 2008; Tanaka et al. 2009). Mammographic screening has led to earlier diagnosis of smaller tumors with no lymph node metastasis and the benefit of adjuvant (post-operative) chemotherapy in combination with surgery is evident. Albeit extraordinary progress in understanding the molecular mechanisms underlying carcinogenesis, breast cancer remains the leading cause of cancer related mortality in women globally (Parkin et al. 2005). In 2008, more than 800 women died of breast cancer in Finland (www.cancerregistry.fi). Despite recent advances, metastatic breast cancer remains an incurable disease by current treatment methods. Metastatic breast cancer features a median survival of approximately 2 years, with a 5-year survival rate of 19 % (Gloeckler Ries 2007). Current therapies are limited by the emergence of therapy-resistant cancer cells (Al-Hajj et al. 2003).

Malignant breast cancer is a complex disease and the progression of normal to malignant breast tissue is not completely understood. A multistep carcinogenesis model has been proposed to describe the progression of breast cancer most accurately (Beckmann et al. 1997). The normal breast epithelium is thought to evolve via hyperplasia and

carcinoma *in situ*, into invasive cancer. Precursor lesions, such as atypical hyperplasias, are benign lesions of the breast and carcinoma *in situ* a malignant transformation without stromal invasion across the basement membrane (Kenemans et al. 2004). When such cells detach from the basement membrane and invade the stroma, they become invasive and can thereafter disseminate via the blood and lymph vessels into lymph nodes or distant organs, forming metastases. Breast cancer metastasis is a complex biological process and its risk can be predicted by the gene expression profile of the primary breast cancer tumor (de Snoo et al. 2009), though clinicopathological data and the extent of the disease still remain the most important predictive factors.

2.2.1 Molecular mechanisms of breast cancer

Sporadic disease is the predominant form of breast cancer. Oncogene amplification is a common mechanism in the development of sporadic breast cancer, and it has been estimated that after progression to a malignant, metastasizing phenotype, a single breast cancer cell may have a few hundred genes with altered expression patterns (Kenemans et al. 2004). Yet, only a few oncogenes seem to be essential for breast cancer development. These include the Human epidermal growth factor receptor tyrosine kinase gene (*HER2/neu*), *c-Myc,* and *cyclin D1* (*CCNDI*) (Beckmann et al. 1997). These genes share a common denominator, *i.e.* having central roles in fundamental cellular events such as growth, differentiation, proliferation, and apoptosis. HER2 is a transmembrane receptor that acts as an activator in intracellular signaling pathways (Slamon et al. 1989). It is amplified or the HER2 protein is overexpressed in approximately 25 % of breast cancers (Pauletti et al. 2000; Suter and Marcum 2007). Women with *HER2* overexpressing breast cancers have an aggressive disease with a significantly shorter disease-free and overall survival. *HER2* amplification is considered an independent adverse prognostic factor (Slamon et al. 1987). C-Myc is a ubiquitously expressed transcription factor belonging to the family of *myc* proto-oncogenes (Verykokakis et al. 2007; Hynes and Stoelzle 2009). Its expression is influenced by numerous signaling pathways that are frequently deregulated in cancer, and thereby its normal functions are likely to be altered by some mechanism in most cancers. In breast cancer, amplification is the most common alteration of *c-Myc*. The cyklin D1 protein plays an important role in the G1/S check point of the cell cycle, and its overexpression correlates with progression and poor prognosis (Takano et al. 1999).

A much more infrequent form of breast cancer, hereditary breast cancer, is the result of inheritable mutations in susceptibility genes, or TSGs, and accounts for 5–10% of all breast cancers (Osborne et al. 2004; Carroll et al. 2008). Both alleles of TSGs must be lost for the malignant phenotype to occur. *BRCA-1* or *2* are mutated in only 0.12% of the general population, but are associated with a lifetime risk of approximately 50-90% in defect carriers (Rahman and Stratton 1998). In addition to inherited abnormalities in susceptibility genes, TSGs play a role in sporadic cancer and are considered as genes whose loss of function results in the promotion of malignancy. Perhaps the most studied TSG is *p53*, which is estimated to be mutated in approximately 20-30% of breast cancers

(Hollstein et al. 1991). p53 regulates cell division, and it is rapidly increased when DNA damage occurs. Its expression leads to cell cycle arrest and enables DNA repair machinery functions, or, in case of extensive DNA damage, p53 can trigger apoptosis by interacting with other cellular proteins (Lane et al. 1994; Levine 1997).

2.2.2 Current treatment options of breast cancer

Surgery and radiation therapy may be adequate treatment for patients with local breast cancer. In case of locally advanced disease, however, surgery and radiation merely offer local control and additional therapy is needed due to possible distant metastasis (Liu et al. 2010). Currently, three systemic treatment modalitites are available for treating breast cancer: chemotherapy (CT), endocrine therapy (ET), and biologic (or targeted) therapy (BT). A combination of these is referred to as neoadjuvant (pre-operative) or adjuvant (post-operative) therapy, which is the standard of care for patients with locally advanced breast cancer, as well as for patients with inoperable inflammatory breast cancer. Conventional cancer therapies are associated with serious side effects that necessitate the development of novel therapies with less adverse effects.

Anthracyclins, taxanes and alkylating agents such as cyclophosphamide represent some of the chemotherapeutics commonly used as adjuvant therapy for early breast cancer (Liu et al. 2010). Chemotherapeutics are often associated with adverse side effects, some of which are even life threatning, such as neutropenia (Tanaka et al. 2009). Available cytotoxic agents are not selective in their activity, and therefore they nonspecifically damage normal replicating cells in the bone marrow, gastrointestinal epithelia, and hair follicles. For example, acute toxicities associated with doxorubicin, a common chemotherapeutic agent used in the treatment of breast cancer, include myelosuppression, nausea, vomiting, mucositis and alopecia.

Endocrine therapy is used when a tumor has a positive estrogen receptor (ER) and/or progesterone receptor (PR) status, *i.e.* it is hormone responsive. The first systemic endocrine therapy agent approved for the treatment of advanced disease was tamoxifen, an antagonist of the estrogen receptor (Jordan and Koerner 1975). Tamoxifen inhibits the growth promoting effects of estrogen. It is also associated, however, with side effects including a 2.4 fold increased risk for endometrial cancer (Fisher et al. 2005) and a nearly 2 fold increase in risk for thromboembolic disease (Cuzick et al. 2003). A more novel approach for endocrine therapy is the use of aromatase inhibitors which inhibit the synthesis of estrogens in the adrenal glands and adipose tissue, thus lowering the level of estrogen and thereby inhibiting tumor growth (Liu et al. 2010).

A *HER2* positive breast cancer can be treated with trastuzumab, a recombinant humanized monoclonal antibody against the *HER2* receptor (Hudis 2007). Trastuzumab represents targeted biologic therapy, and it was first tested in clinical trials in the 1990´s and was found to inhibit tumor growth when used as a single agent and to have synergistic effects when used in combination with chemotherapeutics (Greenberg et al. 1996). Trastuzumab is associated, however, with serious heart problems such as ventricular dysfunction and congestive heart failure (Tanaka et al. 2009).

A a possibility exists of a curative, multidisciplinary therapeutic approach for a small fraction of patients with metastatic breast cancer, when “cure” is perceived as rendering the disease harmless for prolonged periods rather than destroying every cancer cell(Pagani et al. 2010). When treated with a combination of therapies, and even surgery of the metastatic lesions, 1-3% of patients with solitary or a few metastatic lesions may remain free of overt disease for prolonged periods, even beyond 20 years (Tomiak et al. 1996; O'Shaughnessy 2005). Unfortunately, despite an initial response to endocrine, cytotoxic, targeted, or combination therapy, most patients with metastatic disease develop progressive disease within 12-24 months with less than five percent of patients surviving 5 years. Often metastatic disease is treated with palliative care, but perhaps a more balanced treatment philosophy could be a quality of life oriented approach with more personalized treatment that has reasonable risk to benefit ratio (Pagani et al. 2010). Nevertheless, novel and potent methods for the treatment of disseminated disease are called for.

2.3 Ovarian cancer

Ovarian cancer is the leading cause of mortality due to gynecological cancers in developed countries, causing annually an estimated 125 000 deaths globally (Parkin et al. 2005). Ovarian tumors are classified as serous, mucinous, endometroid, clear cell, undifferentiated and unclassified (Heintz et al. 2001). Ovarian cancer presents at an early stage in approximately 25% of patients with a good prognosis (Pomel et al. 2007). Due to mild symptoms in the early stages and lack of screening methods, however, ovarian cancer is often diagnosed at an advanced stage (FIGO stages III and IV), resulting in poor prognosis. Ovarian carcinoma commonly metastasizes to the peritoneum with frequent diaphragmatic, liver-surface, pleural, and pulmonary involvements. A 5-year survival rate of patients with metastatic disease is only approximately 20-30%(Gloeckler Ries 2007).

2.3.2 Current treatment options for ovarian cancer

Often ovarian carcinoma is diagnosed at an advanced stage of the disease, which makes curative treatment virtually impossible. Ovarian carcinoma is commonly treated with cytoreductive surgery and adjuvant platinum-based chemotherapy (Pomel et al. 2007). Novel therapies for ovarian carcinoma are under development, but no biologically targeted drugs have been approved yet. Ovarian carcinoma has been shown to upregulate HER2, and therefore trastuzumab therapy was studied as a novel targeted treatment method, unfortunately with disappointing results (Bookman et al. 2003). Recently, interest in trastuzumab therapy has risen again, as it apparently sensitizes ovarian cancer cells to EGFR-targeted drugs (Wilken et al. 2010). Nevertheless, novel therapeutics are desperately needed for the treatment of ovarian carcinomas. Oncolytic adenoviruses may prove to be efficient in this context, as ovarian carcinoma most often disseminates within the peritoneal cavity, allowing direct locoregional delivery that would circumvent possible side-effects related to systemic delivery.

3 Adenoviral Cancer Gene Therapy

Originally, the use of viruses in the treatment of cancer originated from the observation that cancer patients who contracted an infectious disease went into brief periods of clinical remissions (Kelly and Russell 2007). Adenoviruses were first isolated from adenoids during adenoidectomy in 1953 (Rowe et al. 1953). The 1950s and 1960s were periods of intense virotherapy research as the development of cell and tissue culture systems allowed *ex vivo* virus propagation (Kelly and Russell 2007). Adenovirus was first used in a clinical trial for cervical carcinoma as early as 1956. In retrospect it is not surprising, that no treatment associated benefit was seen, as the viral characteristics were not really known and therefore dosage, delivery route, replication potential, and safety of the virus were suboptimal. Even though adenoviral inoculations did not cause serious side effects, virotherapy was abandoned as a cancer treatment due to the fact that only a few clinical responses were reported, the effects of viruses were unpredictable, and development of effective chemotherapeutics more or less supplanted it (Wildner 2001). Methods for large-scale production of high-titer purified adenovirus and quantitation of its biological activity were also insufficient at the time.

Even before recombinant DNA technology became available, it was suggested that altering the viral genome would improve the targeting of oncolytic viruses (Southam 1960). In the 1990s this idea became reality, as recombinant DNA techniques allowed genetic engineering of viruses. Ever since adenoviruses have been extensively studied and developed for gene delivery purposes, as well as for oncolytic virotherapy. Adenoviruses have various advantageous characteristics for genetic manipulation and gene therapy. The genome of most widely used strains, serotype 5 (Ad5) and serotype 2 (Ad2), are well characterized and can be manipulated fairly easily, produced and purified to high titers, their genomes rarely integrate into the host genome, which is suitable for virotherapy that does not require stable gene expression, and they have a good safety profile in humans (Danthinne and Imperiale 2000).

The first adenoviral cancer gene therapy approaches with genetically engineered viruses utilized non-replicating agents that aimed at transferring a gene for correction of the disease phenotype or expression of therapeutic molecules inside or near the target cell (Brody and Crystal 1994; Clayman et al. 1995). Virotherapy is a novel approach that relies on the natural replication potential of Ads and the ability of the virus to kill the cancer cells (*i.e.* oncolysis) that are permissive for viral replication. Moreover, progeny virions infect neighboring cells and thereby display potential to amplify the therapeutic effect of the initial viral dose (figure 2). Relentless research efforts have provided various means for control over replication and target tissue transduction to improve safety and efficacy of oncolytic vectors.

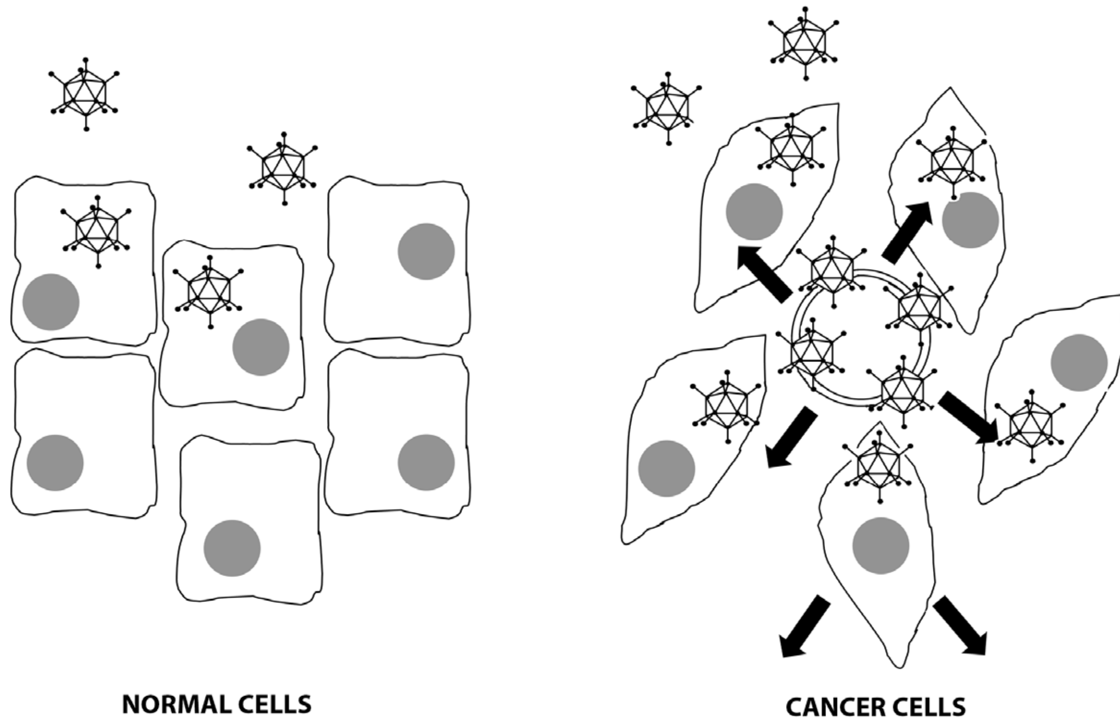

Figure 2. Oncolytic adenoviruses infect both normal and cancer cells, but are only able to replicate in cancer cells. Progeny virions are relased from dying cells and the therapeutic effect of the initial viral dose is amplified.

3.1 Adenovirus

Adenoviruses are common opportunistic pathogens rarely associated with severe clinical symptoms in healthy adults (Lenaerts et al. 2008). Adenoviruses are known to infect humans via the respiratory, the fecal-oral, or the ocular conjunctival routes (Kojaoghlanian et al. 2003). Adenoviral infections are endemic and frequent in humans and are associated with a broad range of symptoms, such as upper and lower respiratory tract disease, conjunctivitis and gastroenteritis (Lenaerts et al. 2008). Diseases are usually mild in immunocompetent humans, but can lead to disseminated and potentially life-threatening disease in patients with compromised immunity, such as AIDS patients and transplant recipients (Kojaoghlanian et al. 2003).

Adenoviruses belong to the family of *Adenoviridae,* which is subdivided into four genera (*Siadenovirus*, *Aviadenovirus*, *Atadenovirus,* and *Mastadenovirus*) (Davison et al. 2003). All human adenoviruses belong to the genera *Mastadenovirus*. There are 51 different serotypes of adenoviruses that were originally classified depending on the ability of different animal sera to neutralize them (Russell 2009). They can be further divided into six different subgroups of A-F based on their ability to agglutinate erythrocytes of different species and their oncogenicity on rodents. Ad5 from subgroup C is the most widely studied adenovirus, and is the serotype mainly discussed in this thesis.

3.1.1 Adenovirus structure and life cycle

Adenoviruses are non-enveloped viruses 70-90 nm in diameter with an icosahedral capsid. Their genome is linear, double stranded DNA varying between 25-45 kilobases in size with inverted terminal repeats (ITRs) at both termini and a terminal protein attached to the 5´ends (Rekosh et al. 1977; Russell 2000).

The icosahedral capsid is formed by three major proteins, of which the hexon trimers are most abundant (figure 3) (Nemerow et al. 2009). Each of the twelve vertices of the capsid also contains a pentameric protein, a penton base that is covalently attached to the fiber. The fiber is a trimeric protein that protrudes from the penton base and is a knobbed rod-like structure (Nicklin et al. 2005). Other viral proteins such as IIIa, VIII, and IX are also associated with the viral capsid (Vellinga et al. 2005).

All human adenoviruses have similarities in their fiber architecture. Each has an N-terminal tail, a shaft with repeating sequences, and a C-terminal knob domain with a globular structure (Nicklin et al. 2005). The knob domain is principally responsible for binding the target cellular receptor and its globular structure presents a large surface for lateral and apical binding. The fiber proteins of adenoviruses from different subgroups most distinctively differ in length and ability to bend.

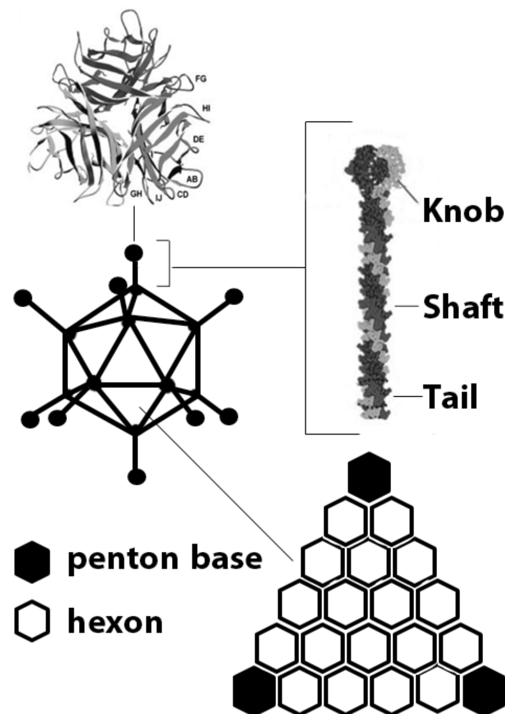

Figure 3. Adenovirus structure. The adenovirus capsid is icosahedral, comprising of 20 facets and 12 vertices with a penton structure. Each facet is comprised of 20 hexons. The penton capsomere is comprised of the homopentameric penton base and the homotrimeric fiber. The fiber has three distinct regions, the tail, shaft, and knob.

Adenovirus trafficking can be characterized by five stages: binding, entry, escape, translocation, and nuclear transport (Leopold and Crystal 2007). Initially the fiber knob binds a primary receptor with high affinity and once the virus is tethered on the cell surface, low affinity binding to secondary receptors leads to internalization (Wickham et al. 1993). Adenoviruses enter cells via dynamin dependent chlatrin-mediated endocytosis (Wang et al. 1998). Escape from the endosome into the cytosol occurs within minutes and is dependent on the acidification of the endosome (Leopold and Crystal 2007). Acidification triggers changes in the adenoviral capsid resulting in the lysis of the endosome membrane. Once in the cytosol, adenoviral capsids translocate towards the nucleus along the microtubules by interacting with cellular molecular motors, such as cytoplasmic dynein (Leopold et al. 2000). Thereafter, the adenoviral genome is transported inside the nucleus via nuclear pores (Saphire et al. 2000; Trotman et al. 2001).

3.1.2 Adenoviral transcription and replication

Adenoviruses are dependent on the cellular machinery to replicate the viral genome (Moran 1993). They can infect quiescent cells and induce them into a cell cycle S-phase-like state enabling viral DNA replication. The adenoviral genome can be divided into immediate early (*E1A*), early (*E1B*, *E2*, *E3*, *E4*), intermediate (*IX*, *Iva*), and late (*L1-L5*) genes (figure 4) (Russell 2000).

Adenoviral transcription can be described as a two-phase-event, early and late, characterized by the expression of different viral genes and separated by the onset of viral DNA replication (Russell 2009). The first transcription unit to be expressed is the *E1A*. The E1A proteins stimulate the transcription of other early genes and modulate the expression of cellular genes involved in the transition into S-phase, making the cell more susceptible to viral DNA replication (Berk 1986a; Berk 1986b; Volpers and Kochanek 2004). The E1B proteins suppress cell death elicited in response to unregulated cell proliferation signals, including those mediated by E1A (Moran 1993). The *E2* gene products provide the replication machinery for viral gene products.

E3 gene products are not essential for virus replication *in vitro*, but are dedicated to the control of various host immune responses (Horwitz 2004). E3-gp19K inhibits the transport of the class 1 major histocompatibility complex (MHC) from the endoplasmic reticulum (ER) to the plasma membrane, thereby preventing the presentation of peptides to T lymphocytes by MHC (Rawle et al. 1989; Schowalter et al. 1997). Other E3 proteins inhibit apoptosis elicited by various cellular proteins such as the tumor necrosis factor α (TNFα) (Wold 1993). As an exception, *E3* derived adenoviral death protein (ADP) functions late in the viral cycle to promote cell death, presumably to aid in the release of of the virus after all the replicative functions have been completed (Tollefson et al. 1996).

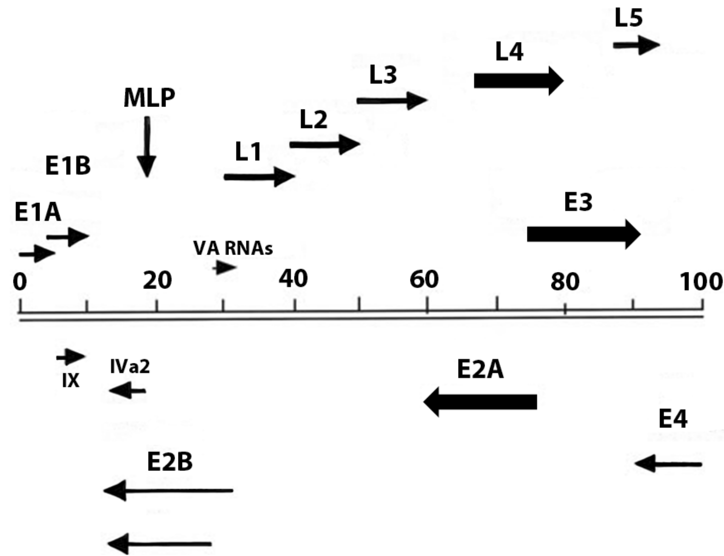

Figure 4. Organization of the adenovirus genome. Early transcripts E1A to E4, intermediate transcripts IX and Iva, and late transcripts L1 to L5. MLP stands for major late promoter. Modified from Russel et al. 2000.

E4 gene products have been implicated in many events that occur as the late program begins. E4 proteins augment viral DNA synthesis and messenger RNA (mRNA) transport, late viral gene expression, shutoff of host protein synthesis, and production of progeny virions (Halbert et al. 1985; Bridge and Ketner 1989; Bridge and Ketner 1990; Goodrum and Ornelles 1999). The late gene transcription leads to the production of viral structural components and the encapsidation and maturation of the viral particles in the nucleus.

3.1.3 Adenoviral transduction

The primary receptor for subgroup C adenoviruses is the coxsackie adenovirus receptor (CAR) (Bergelson et al. 1997). The expression levels of CAR correlate with the susceptibility of a particular cell type to adenoviral infection (Asaoka et al. 2000). Furthermore, the induction of CAR expression leads to improved transduction on cells that are naturally refractory to adenoviral infection (Nalbantoglu et al. 2001). Also, CAR localization on the cell surface affects the infection. CAR has been shown to localize to the basolateral surface of polarized epithelial cells, thereby hindering adenoviral infection through the apical surface, and to the tight junctions, thereby acting as a natural barrier against infection through the basolateral surface as well (Walters et al. 1999; Cohen et al. 2001). CAR expression is variable and often low on cancer cells and CAR levels on cancer tissues inversely correlate with tumor aggressiveness (Kim et al. 2002).

Cellular integrins are cell surface adhesion molecules that many adenoviruses utilize as secondary receptors. The Arg-Gly-Asp (RGD) or Leu-Asp-Val (LDV) motif in the penton base interacts with $\alpha_v\beta3$ and $\alpha v\beta5$ integrins as well as $\alpha v\beta1$, $\alpha5\beta1$, and $\alpha3\beta1$ to activate viral internalization (Wickham et al. 1993) (Davison et al. 2001; Li et al. 2001; Salone et

al. 2003). The importance of the viral interaction with integrins is highlighted by the fact that multiple serotypes from different subgroups contain a conserved RGD motif (Mathias et al. 1994). Nevertheless, adenoviral infection can occur even in the absence of an integrin-penton base interaction, suggesting that the fiber-CAR interaction is sufficient to allow virus entry, albeit at a significantly reduced internalization rate (Bai et al. 1993). The integrins interact with a variety of signaling molecules and the Ad-integrin interaction thereby promotes activation of phosphatidylinositol-3 kinase (PI3K) and Rho family GTPases, which are involved in actin cytoskeleton reorganization (Nemerow and Stewart 1999). Polymerized actin filaments assist dynamin-mediated endosome formation (Doherty and McMahon 2009).

Besides CAR and integrins, various other receptors for adenoviruses have been proposed. CD46, a transmembrane glycoprotein normally acting to prevent complement activation of autologous tissue, has been identified as a cellular receptor for the majority of subgroup B adenoviruses (Segerman et al. 2003; Fleischli et al. 2007), although CD46 usage by serotype 3 remains controversial (Marttila et al. 2005; Tuve et al. 2006; Fleischli et al. 2007). CD80 and CD86 expressed on the surface of antigen presenting cells (APCs), such as dendritic cells and B lymphocytes, have been shown to mediate subgroup B adenoviral infection (Short et al. 2004). Furthermore, the existence of an additional receptor for subgroup B adenoviruses distinct from CD46 and CD80/86 has been suggested and is being referred to as species B adenoviral receptor (sBAR) or receptor X (Sharma et al. 2009).

Proteoglycans, the major histocompatibility complex class 1 (MHC-1), and sialic acid have also been implicated as adenovirus receptors. Proteoglycans form the major component of the extracellular matrix (ECM) and are involved in various cellular functions such as cellular attachment and proliferation (Bishop et al. 2007). Pathogens have evolved to exploit prevalent proteoglycans as receptors and heparin sulphate proteoglycans (HSPG) are involved in the infection of Ad5 and Ad2 (Dechecchi et al. 2001). A consensus sequence Lys-Lys-Thr-Lys (KKTK) in the fiber shaft was suggested as the HSPG binding motif (Dechecchi et al. 2001). Hepatocytes express high amounts of HSPG and the KKTK motif contributes to the hepatotropism of adenoviruses (Smith et al. 2003b). Vitamin K dependent circulating coagulation factors VII, IX and X (FVII, FIX and FX) as well as complement protein C4BP seem to bind adenovirus and direct these complexes to HSPGs, as well as LDL receptor related protein (LRP) expressed on liver cells (Shayakhmetov et al. 2005; Parker et al. 2006). The α2 domain of the MHC-1 heavy chain binds to Ad5 and Ad2 fiber (Hong et al. 1997) and it may play a role in Ad internalization in the absence of CAR or improve the accessibility of CAR to the fiber (Davison et al. 1999). Finally, an epidemic keratoconjunctivitis causing subgroup D adenoviruses use negatively charged sialic acid as a cellular receptor (Arnberg et al. 2000; Sharma et al. 2009).

3.1.4 Adenovirus induced immunity

Adenoviruses have not evolved efficient immune tolerance mechanisms, so the immune system strongly recognizes them as immunogens (Tuve et al. 2009). Although adenoviruses have been proven relatively safe in clinical trials, both innate and adaptive immune responses need to be considered when treating humans. Research and clinical experience with first generation E1/E3-deleted vectors revealed that significant host immune responses limit the utility of adenoviruses in gene therapy (Muruve 2004). Adenoviruses may elicit acute inflammation resulting in significant reduction in gene transfer efficacy and vector persistance (Worgall et al. 1997), and in the worst case scenario, damage to healthy tissue and even death (Raper et al. 2003). Adenovirus-induced acute toxicity is dose-dependent and independent of viral gene expression, which indicates that viral capsid proteins elicit the immune reaction (Muruve et al. 1999).

Adaptive immune responses to adenoviruses may be a major hurdle to the efficacy of adenoviral gene therapy especially if long-term transgene expression is desired, since the cytotoxic T-cell response against infected cells results in transient transgene expression (Leen et al. 2008). On the other hand, adenovirus-specific T-cell responses can be exploited in cancer immunotherapy approaches (Tuve et al. 2009). The adenovirus-specific immunity may be exploited to gain additive or synergistic anti-tumor effects (Prestwich et al. 2008).

3.1.4.1 Innate immunity

The innate immune system represents the first line of defence against invading pathogens and is comprised of cells, immune mediator proteins including chemokines, cytokines and complement proteins, and pattern recognition receptors (PRR), that recognize pathogen associated molecular patterns (PAMPS) (Zaiss et al. 2009). The most studied receptors are the Toll-like receptors (TLRs) that are also linked to the type I interferon (IFN) responses (Vaidya and Cheng 2003). IFNs are cytokines that induce complex antiviral resistance mechanisms in cells. Other adenovirus induced cytokines include the TNF-α and interleukins-1,-6, and -12 (Muruve 2004). Also various chemokines are secreted upon adenoviral infection, including macrophage inflammatory proteins (MIP)-2, -1α, and -1β, as well as IFN-γ-inducible protein 10 (IP-10). The induction of multiple cytokines and chemokines is relatively rapid after adenoviral infection, occurring within a few hours or less (Otake et al. 1998; Muruve et al. 1999). The induction of these immune mediators correlates with the inflammatory response within adenovirus transduced organs (Otake et al. 1998) and has a major role in recruiting and activating innate effector cells to sites of infection (Guidotti and Chisari 2001) and, for example, in subsequent liver damage (Muruve et al. 1999). The acute cytokine response is mediated by dendritic cells (DCs) and macrophages (Zhang et al. 2001).

Kupffer cells (KCs) are resident liver macrophages that act as effector cells and effectively take up systemically administered adenoviruses (Zhang et al. 2001). The blockade of KCs reduces adenovirus-induced cytokine-mediated inflammation and

improves gene transduction (discussed in more detail later) (Kuzmin et al. 1997; Schiedner et al. 2003; Koski et al. 2009). Adenovirus clearance from blood by KCs is saturatable, probably due to exceeding the phagocytic capacity of these cells (Tao et al. 2001).

Along with KCs, adenovirus transduced DCs play a critical role in the early innate immune responses by interacting with other innate immune cells including natural killer cells (NK), mast cells, and neutrophils (Prestwich et al. 2008). Furthermore, DCs are the principal cross-presenting (*i.e.* presenting exogenous antigen in MHC) antigen presenting cells (APC) *in vivo* and act as an important link between the innate and adaptive immunity (Schulz et al. 2005). Upon natural stimuli, such as pathogen encounter, DCs mature and can migrate to T-cell rich areas of secondary lymphoid organs where they cross-present antigens in an appropriate costimulatory environment to the adaptive arm of the immune system (Mercier et al. 2004).

3.1.4.2 Adaptive immunity

T-cell response is provoked upon migration of mature DCs to secondary lymphoid organs. DCs stimulate T-cells via costimulatory molecules CD40, CD86, and CD28/80 and present viral antigens, either processed capsid proteins from random uptake or virus expressed proteins from viral DNA that has reached the nucleus, via MHC I molecules to generate $CD8^+$ cytotoxic T-cells (CTL) or via MHC II molecules to generate $CD4^+$ T-helper (T_H) cells (Perreau and Kremer 2006). Depending on the cytokines produced by DCs, T_H are further differentiated into T_H1 (cellular response) or T_H2 (humoral response). Furthermore, the cytotoxic T-cell generation requires DC secreted costimulatory cytokines and T_H functions in addition to antigen presentation by MHC I. Finally, the $CD4^+$ T_H-cells and $CD8^+$ CTL kill virus infected cell.

TH_2 cells play a central role in the generation of memory B cells, i.e. plasma cells (McHeyzer-Williams and McHeyzer-Williams 2005). Naïve B cells internalize viral particles through B-cell receptors and process them. Thereafter, viral epitopes are presented to T_{H2} cells via MHC II, which finally leads to extensive clonal expansion of antigen-specific B-cells into short-lived antibody-secreting plasma cells, rapidly expanding centroblast or quiescent noncycling centrocytes. Through diverse steps, some of the expanding B-cells develop into memory B-cells. Upon reencountering the specific antigen epitope clonal expansion of the specific memory B-cell population occurs, resulting in a strong and rapid production of specific antibodies.

The majority of the human population has been exposed to Ads and thereby have developed an adenovirus-specific immune response (Bangari and Mittal 2006). The pre-existing vector immunity is mediated by serotype specific B-cell produced neutralizing antibodies (NAbs) that are directed against the capsid components. Of humans, 40-97% have NAbs against Ad5, the most widely used serotype in gene therapy. Nabs bind to capsid proteins and block adenoviral internalization to target cells, and elicit induced uptake to Fc-receptor bearing immune cells such as KCs and DCs, resulting in rapid vector clearance and inflammatory responses (Mercier et al. 2004; Perreau and Kremer

2006). Although pre-existing immunity does not seem to adversely affect the antitumor efficacy of locally delivered adenovirus (Dhar et al. 2009), a high NAb titer may compromise systemic delivery (Chen et al. 2000). Furthermore, NAbs developed in naïve individuals within 1 week after a virus encounter have been suggested to aggravate re-administration attempts. Extensive pre-existing Ad5 immunity is likely to limit clinical utility of adenovirus vectors, unless methods to circumvent neutralizing antibodies can be developed. The successive use of viruses with different capsid modifications has been proposed as a means to circumvent the neutralizing activity of capsid protein specific NAbs to some extent (Sarkioja et al. 2008). Furthermore, serotype switching and the use of non-human adenoviruses may be useful (Perreau and Kremer 2006).

Innate and adaptive immune responses may be exploited in the context of oncolytic virotherapy. Tumors are able to disrupt anti-tumor immune responses by creating an immunosuppressive tumor microenvironment. By secreting soluble immunosuppressive mediators, such as IL-10, and by inducing regulatory T-cells, that are able to abrogate effective antitumor T-cell responses (Mittendorf and Sharma 2010). In addition to their ability to disrupt antitumor immune responses, tumors commonly lack "danger signals" that the immune system responds to. For cross-presentation, virally infected cells are superior at TLR-3 augmented delivery of nonviral antigen (Schulz et al. 2005). Virally induced cell death would be expected to enhance the availability of tumor-associated antigens for uptake by DCs (Moehler et al. 2005). Furthermore, viral infection may alter the balance of cytokine production from the tumor, and subsequently affect the nature of the immune reaction to the tumor, that is, by counteracting the immunosuppressive nature of the tumor microenvironment (Prestwich et al. 2008). Moreover, viruses can be engineered to express highly immunogenic proteins such as granulocyte-macrophage colony-stimulating factor (GM-CSF). When immunogenic proteins are expressed within tumor cells, they are potent stimulators of specific and long-lasting antitumor immunity (Pan et al. 2009).

3.2 Transductional targeting of adenoviral vectors

Loss of CAR expression correlates with tumor progression, which implies low expression levels of CAR in advanced disease (Okegawa et al. 2004). Cells expressing low levels of CAR are refractory to Ad5 infection, at least *in vitro*. CAR dependency results in a scenario in which the target tissue of adenoviral gene therapy is poorly transduced, *i.e.* viruses enter target cells inefficiently, while non-target tissue with high CAR expression is efficiently transduced (Kim et al. 2002).

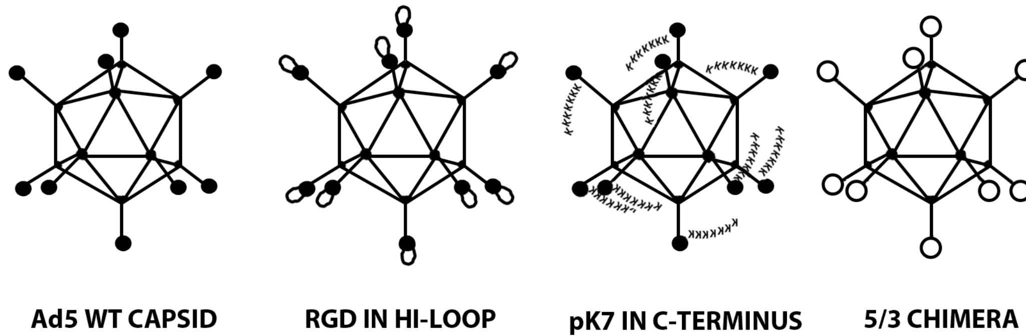

Figure 5. Wt capsid Ad5 and viruses with a single modification in the capsid: RGD-modification in the HI-loop of the knob, pK7 modification in the C-terminus of the knob, and a serotype 5/3 chimeric fiber with a serotype 3 knob.

Adenovirus vector biodistribution *in vivo* is not solely dependent on receptor distribution (Glasgow et al. 2006). High CAR expression does not correlate with tissue transduction after systemic application, since the virus is almost exclusively taken up by the liver and other high CAR and/or integrin expressing tissues are not transduced (Fechner et al. 1999). Various blood coagulation factors play a role in adenovirus tropism by bridging adenovirus to cell surface receptors, especially on hepatocytes and liver KCs (Shayakhmetov et al. 2005; Parker et al. 2006; Schagen et al. 2008). Also, anatomical barriers play a significant role in vector targeting after systemic injection (Fechner et al. 1999). Intratumoral spread of adenoviruses is very limited due to barriers formed by connective tissue or fibroblasts (Li et al. 2004). Large tumors are also often associated with hypoxic and necrotic areas that do not support adenoviral spread.

Taken together, these obstacles make transductional targeting of adenoviral vectors a prerequisite for their systemic use. In the context of systemic cancer treatment it seems necessary to not only redirect adenoviral tropism to tumors by CAR binding ablation and introducing a new attachment molecule, but also to ablate the binding of several blood factors directing adenoviruses to the liver. Otherwise efficient transduction of target tissue might be accomplished, regardless of the tropism expansion, as the vector will be taken up by the liver.

3.2.1 Genetic modification

Genetic modification of the capsid is a conceptually elegant approach to redirect adenoviral tropism. In short, the goal of genetic targeting is to create a single-component vector that can transduce cells via non-native receptors (Noureddini and Curiel 2005). Strict constraints are, however, imposed on such efforts by the structural integrity and the biological functionality of the virus.

Fiber is the primarily exploited capsid locale for genetic engineering (figure 5). Four basic strategies for genetic tropism modification of the fiber exist: fiber pseudotyping,

CAR ablation by short deletions, ligand incorporation into the knob, and "de-knobbing" of the fiber coupled with ligand addition (Glasgow et al. 2006). Other capsid locales hexon, penton base, and pIX have been modified to lesser extent.

3.2.1.1 Fiber pseudotyping

A high degree of structural similarity exists in the fiber protein amino(N)-terminal tail region between different serotypes (Rux and Burnett 2004; Zubieta et al. 2005). This structural similarity enables the creation of chimeric vectors, where the whole fiber or the knob region is switched to that of another serotype. This fiber pseudotyping, or fiber chimerism, results in CAR binding ablation and alternate receptor recognition, but is limited to the tropic behavior of the characterized serotype adenoviruses (Noureddini and Curiel 2005). Previously, altered tropism of the Ad5 vector has been achieved by switching the whole fiber to that of serotype B Ads 7, 35, or 16, resulting in the transduction of various CAR-deficient tissues and cells such as dendritic cells (DCs), hematopoietic stem cells (HSC), and synovial tissues (Gall et al. 1996; Shayakhmetov et al. 2000; Goossens et al. 2001; Rea et al. 2001). The use of the serotype 19p fiber in the Ad5 vector resulted in efficient transduction of renal cell cancer tumors in mice and significantly reduced liver tropism, a useful property for systemic treatment (Diaconu et al. 2009). Increased transduction of ovarian cancer cells was achieved by replacing the Ad5 knob with the knob from Ad3 (Krasnykh et al. 1996; Kanerva et al. 2002).

3.2.1.2 CAR binding ablation by short deletions

Sequence and mutagenesis analysis on the fiber have implicated the AB loop, DE loop, DG loop, Bβ sheet, and β strand F as structures on the knob that have relevance in binding CAR. Yun et al. ablated CAR binding by deleting the Bβ sheet of the knob and reported reduced adenoviral liver tropism and toxicity (Yun et al. 2005). The mutation of the AB and DG loop resulted in the loss of CAR-mediated binding *in vitro* (Leissner et al. 2001).

3.2.1.3 Ligand incorporation to fiber

CAR independent gene delivery can be achieved by incorporating peptide ligands into the knob. This approach does not abrogate the native tropism, as the CAR binding ability is retained, but rather expands the vectors tropism. Incorporation of ligands imposes structural constraints on the folding and trimerization of knob, however, and therefore only a limited number of genetic modifications allow production of functional fiber (Borovjagin et al. 2005). Ligands can be incorporated into two distinct locales of the knob: the HI loop or the carboxy (C)-terminus. An RGD(arg-gly-asp)-ligand, targeting adenoviruses into integrins, or a polylysine (pK) motif, targeting them into HSPGs, have

been incorporated into the C-terminus (Wickham et al. 1993; Borovjagin et al. 2005) and HI-loop (Krasnykh et al. 1998), or both (Borovjagin et al. 2005). Also, an approach termed mosaicism has been utilized, where two distinct ligands were incorporated into the serotype 5 fiber (Wu et al. 2002). Complex mosaicism takes this approach even further with two ligands incorporated into a chimeric fiber (Borovjagin et al. 2005).

3.2.1.4 De-knobbing

Several research groups have developed chimeric attachment molecules, in which the fiber knob or knob and shaft have been replaced with an exogenous trimerization domain such as the α–helix domain from Moloney murine leukemia virus (MoMuLV) envelope glycoprotein (van Beusechem et al. 2000), bacteriophage T4 fibritin protein (Krasnykh et al. 2001), the neck region peptide of human lung surfactant protein D (Magnusson et al. 2001), or the T oligomerization domain of the reovirus attachment protein σ1 (Schagen et al. 2008) coupled with various ligands. With this "de-knobbing" approach, binding to CAR, integrins, HSPGs, as well as to molecules such as various coagulation factors linking adenoviruses to alternative cell surface receptors, is abrogated (Shayakhmetov et al. 2005; Parker et al. 2006; Schagen et al. 2008). In theory, this approach completely abrogates the native tropism and introduces unique binding motifs that have the potential to improve *in vivo* applicability of adenoviruses (Schagen et al. 2008). Recently, however, coagulation factor X (FX) binding to hexon has been claimed to be the major determinant of liver transduction, and thereby de-knobbing approaches would not affect this (Alba et al. 2009).

3.2.1.5 Liver detargeting by genetic modification

The majority of intravenously administered adenoviral vectors are taken up by the liver KCs and hepatocytes, and transgene expression is predominantly found in hepatocytes (Vrancken Peeters et al. 1996; Connelly 1999; Alemany et al. 2000). Hepatocyte transduction ablation has been attempted with various capsid modifications. Smith et al. created a mutation in the Ad5 HSPG binding fiber shaft KKTK motif that resulted in reduced liver tropism in mice, rats, and non-human primates (Smith et al. 2003a; Smith et al. 2003b). Various attempts to modify *in vivo* adenoviral tropism by abolishing CAR and/or integrin interactions have not been successful in detargeting the liver (Alemany and Curiel 2001; Smith et al. 2002; Martin et al. 2003) . This implies that the presence of CAR is not a critical factor in determining the susceptibility of tissues to adenovirus *in vivo*, and that blood factors serving as a bridge between the virus and liver cells may allow efficient liver transduction despite CAR binding ablation (Shayakhmetov et al. 2005; Parker et al. 2006). Hexon is the major determinant of Ad5 liver tropism and recent studies have highlighted the role of the high-affinity interaction of FX with hexon in FX-mediated liver gene transfer (Kalyuzhniy et al. 2008; Waddington et al. 2008). Recently, it has been shown that mutation of critical amino acids from two hypervariable regions in hexon

eliminates FX binding and subsequent FX-mediated liver gene transfer in mice (Alba et al. 2009). If such minimal modifications would indeed eliminate liver transduction in humans as well, this approach would be highly beneficial in oncolytic adenoviruses intended for human use.

3.2.2 Adapter based modification

Adenoviruses can be physically prevented from binding to their native receptor and redirected to other receptors by using bispecific adapter molecules, which bind to the knob with one domain and to a target receptor with another (Li et al. 2007). Usually the detargeting component of such an antibody is either the soluble CAR ectodomain or an antibody to the knob, and the retargeting component is a ligand for cell surface receptor or a cellular antigen, such as epidermal growth factor receptor (EGFR) (Dmitriev et al. 2000), CD40 (Pereboev et al. 2002) or carcinoembryonic antigen (CEA) (Li et al. 2007). Bispecific antibodies have demonstrated infectivity enhancement both *in vitro* and *in vivo*. Their use, however, is limited to non replicating vectors as the non-genetic bispecific antibody modification would not be included in the progeny virions in the context of oncolytic vectors.

3.2.3. KC depletion with pharmaceutical agents

KCs represent the first line of defence against pathogens and can efficiently take up intravenously delivered adenoviruses (Wolff et al. 1997; Alemany et al. 2000; Tao et al. 2001). KC uptake of adenoviruses not only reduces the amount of free virus in the blood that can reach the target tissue, but also provokes toxic responses due to cytokine induction and antigen presentation, which elicit adaptive responses, such as cytotoxic T-cell responses (Zaiss et al. 2009).

Clearance of adenoviruses by KCs is mediated through various routes. Scavenger receptors play a major role in the uptake of adenoviruses (Xu et al. 2008; Haisma et al. 2009), and subsequently preinjections with polyinosinic acid (polyI), a scavenger receptor ligand, can greatly reduce viral uptake by KCs and increase the circulating half-life (Smith et al. 2008). Furthermore, mouse complement and natural antibodies were shown to some extent to contribute to adenovirus uptake by KC (Xu et al. 2008). Other substances used to reduce adenovirus uptake by KCs include gadolinium chloride (GdCl3), empty liposomes, or clodronate liposomes. Gadolinium chloride not only blocks the phagocytic activity of KCs, but also transiently eliminates them (Du et al.). Clodronate is clinically used for osteolytic bone disease and when encapsulated into liposomes, is efficiently delivered into phagocytic cells such as KCs resulting in their damage and apoptosis (Schiedner et al. 2003). The use of empty multilamellar liposomes to block KCs is also based on KC saturation (Snoeys et al. 2006). The use of liposomes represents a non-toxic means for KC blockage, since they do not destroy KCs. In addition to the use of various chemical substances, prior injection of a saturating amount of adenovirus vector has also been

shown to increase the therapeutic window with a subsequent injection of another vector (Shashkova et al. 2008).

3.3 Transcriptional targeting of adenoviral vectors

Upon infection, adenoviruses need to induce a cell cycle S-phase-like state in order to transcribe and replicate the viral genome. E1A is the first viral protein to be expressed in a transduced cell and can activate transcription of other early viral genes by interactions with cellular check point proteins (Nemajerova et al. 2008). Importantly, E1A expression results in the activation of the *E2a* promoter and the *E2* region transcription, leading to the expression of adenoviral replication machinery (Berk 1986a). In oncolytic virotherapy, the replication of Ads has to be controlled in order to avoid side effects in normal tissues. Furthermore, E1A protein is toxic by itself, and therefore it is beneficial to avoid its expression in normal cells.

The transcriptional regulation of E1A can be achieved by introducing specific genetic deletions in the *E1A* gene that makes the virus dependent on the disruption of central cellular regulatory pathways, a typical phenomenon in oncogenesis. Moreover, cellular genetic regulatory elements, such as promoters, enhancers, and silencers, can be inserted into the adenovirus genome to regulate the transcription of viral *E1A* or other key regulatory genes and/or transgenes. This approach aims at obtaining selective and high expression of viral genes in tumor cells as a result of the activity of the cellular regulatory element employed.

3.3.1 Genetic deletions for transcriptional control

Specific deletions on adenoviral key regulatory genes can be utilized to create dysfunctional proteins or the lack of their expression that leads to dependence on a specific genetic feature present in cancer cells. Partial deletions of *E1A* and *E1B* genes result in restricted replication in normal cells but allow replication in cancer cells.

The adenoviral *E1B* region encodes the protein E1B-55K that binds and inactivates p53, thereby inhibiting apoptosis of adenovirus infected cells. The partial deletion of the *E1B* gene targets adenoviral replication to cells with deleted or mutated *p53* TSG, which is a frequent phenomenom in cancer cells (Bischoff et al. 1996). The first genetically modified adenovirus, ONYX-015, featured a deletion in the *E1B* rendering E1B-55K protein truncated and unexpressed. An additional point mutation was introduced to generate a stop codon to prevent expression of the truncated E1B55-K protein. Normally, p53 can counterbalance the E2F dependent activation of the cell cycle progression genes by inducing the expression of p21, an inhibitor of cyclin A and E dependent kinases (Levine 1997). The E1B-55K protein forms a complex with E4orf6 and p53, which leads to proteolysis of p53 (Barker and Berk 1987). Since the first publication describing it, ONYX-015 and its variants have been widely studied and have entered multiple clinical trials. Original studies with ONYX-015 showed that it replicated less efficiently than wt

Ad in cells expressing p53(Bischoff et al. 1996) Various subsequent reports have shown, however, that ONYX-015 does not cause selective death of p53-deficient cells (Goodrum and Ornelles 1998; Rothmann et al. 1998; Harada and Berk 1999), but rather displays different cell killing efficiency in a cell line dependent manner due to differencies in infectivity or permissiveness, rather than p53 status (Steegenga et al. 1999).

The dependence of adenoviral replication on specific structures on the E1A protein can be utilized to restrict adenoviral replication. The adenoviral E1A protein was originally described as a retinoblastoma (pRb) binding protein capable of inducing DNA replication in quiescent normal cells (Ruley 1983). pRb regulates the cell cycle progression by interacting with numerous cellular proteins. The pRb-E2F complex contains an E2F/DP heterodimer stably bound to hypophosphorylated pRb, and acts as a transcriptional repressor of E2F responsive genes, such as adenoviral *E2a*. Under normal proliferative conditions, pRb is phosphorylated by cyclin/cdk complexes, disrupting its ability to bind to its regulatory targets (Dyson 1998). Remarkably, the advancement of the cell cycle to S-phase can arise from the expression of E1A in a similar fashion. Indeed, one of its key functions is to disrupt the pRb-E2F interactions, thereby releasing E2F transcription factors to activate the E2F responsive promoters and transcription of the genes they control. Two conserved regions (CR1 and CR2) in the E1A have been shown to act in the disruption of the pRb-E2F complex (Raychaudhuri et al. 1991). CR2 forms a strong interaction with the pocket binding domain of pRb and CR1 mediates the actual disruption of the E2F binding of pRb (Fattaey et al. 1993).

Conditionally replicating viruses featuring a 24 basepair deletion in the CR2 were created and shown to be potent and selective in the treatment of glioma and breast cancer xenografts (Fueyo et al. 2000; Heise et al. 2000). Their cancer specificity results from the inability of dysfunctional E1A to release E2F1 transcription factor, which leads to the requirement of free E2F1. E2F1 is abundant in cancer cells, where the pRb pathway is most often disrupted (Hanahan and Weinberg 2000). This replication control approach has gained wide interest in the research field during the last decade, and many vectors studied in pre-clinical context feature this deletion.

3.3.2 Tissue and tumor specific promoters

Heterologous cellular promoters with different targeting principles have been employed to target adenoviral gene transcription and subsequent DNA replication to tumors (Nettelbeck 2008). In oncolytic adenoviruses promoters are often utilized to control the expression of *E1A*, the first gene to be transcribed in an infected cell. Furthermore, when cellular promoters are used to drive the expression of essential adenoviral genes, the corresponding viral regulatory sequences are replaced to get rid of inherent activation of viral gene expression. Tissue specific promoters limit the transcription to tumors, but retain the ability to function in the healthy tissue of tumor origin as well, thus perhaps demanding further means for transcriptional regulation.

Human α-Lactalbumin (ala) (Li et al. 2005), cyclo-oxygenase 2 (Cox-2) (Bauerschmitz et al. 2006) and prostate specific antigen (PSA) promoter (Rodriguez et al. 1997) are tissue

specific promoters that have been used to transcriptionally target oncolytic adenoviruses to tumors. α-Lactalbumin is produced in differentiated breast epithelial cells and is expressed in over 60% of breast cancer tissues (Lee et al. 1984). Its expression is low in virgin mammary glands, but rises sharply during pregnancy and in tumors (Li et al. 2005). *Cox-2* is closely linked to carcinogenesis and progression of epithelial tumors (Cao and Prescott 2002), and is consequently overexpressed in ovarian carcinoma, colorectal cancer, as well as prostate cancer with metastatic potential, to name a few (Ali-Fehmi et al. 2003; Richardsen et al. ; Wang and Dubois). PSA is produced in prostate cancer cells and, to a lesser extent, in prostate ductal epithelia as well as in very small amounts in periurethal glands (Aumuller et al. 1990). A rising serum level of PSA is indicative of prostate disease and is used as a diagnostic marker. The first tissue specific promoter driven oncolytic adenovirus, CN706, featured a *PSA* promoter controlling *E1A* (Rodriguez et al. 1997). In addition to the *PSA* core promoter, it now seems that for true tissue-specific expression an upstream enhancer is required (Latham et al. 2000).

Some promoters show high tumor specificity, thereby limiting harmful effects towards the healthy tissue of tumor origin. Tumor-type specific promoters show high selectivity for a certain tumor type. The downside to their use is their limited applicability for other cancer types. Such a promoter is the α-fetoprotein (*AFP*) promoter: the expression of AFP is mainly controlled on the transcriptional level and its promoter is active in fetal liver, but is silenced after birth and remains silent throughout life, except in hepatocellular carcinoma (HCC) tumors, where it is re-activated (Hallenbeck et al. 1999).

3.3.3 Pan-cancer promoters

Pan-cancer promoters target hallmark cancer pathways making them broadly applicable for targeting approaches in various cancer types (Nettelbeck 2008). Examples of such promoters are *E2F1*, the human telomerase reverse transcriptase (*hTERT*) promoter, and the multidrug resistance promoter (*Mdr1*).

The ends of human chromosomes are organized into telomeres, G-rich tandem repeats and associated proteins, which protect chromosomes from end-to-end fusions and degradation (Blackburn 1991). In normal cells, telomeres progressively shorten during cell divisions. Shortened telomeres act as a senescence signal, leading to growth arrest and apoptosis. In contrast, telomeres of cancer cells are maintained due to the activation of telomerase enzyme. hTERT is the catalytic rate-limiting subunit of telomerase (Horikawa et al. 1999) and its expression is highly specific to cancer cells, while the other telomerase subunits are also constitutively expressed in normal cells (Kyo et al. 2008). High telomerase activity is observed in over 90% of human cancers and the *hTERT* promoter is upregulated in cancer cells, but repressed in normal cells (Horikawa et al. 1999; Takakura et al. 1999). The *hTERT* promoter is also tightly regulated on the transcriptional level (Kyo et al. 2008). Modest telomerase activity is observed in proliferative normal tissues with high renewal potential, such as bone marrow (Yui et al. 1998), skin, the gastrointestinal tract, testis, and activated lymphocytes (Kyo et al. 2008), a fact that may

call for further regulation of *hTERT* controlled transcription in the context of oncolytic adenoviruses.

E2F transcription factors regulate the expression of a diverse set of genes involved in key cellular events by binding to their promoters (Johnson and Schneider-Broussard 1998; Muller and Helin 2000). As previously described, the pRb/E2F-1 complex inactivates the *E2F1* promoter and *E2F1* promoter activation requires free E2F1 transcription factor. The pRb pathway is disrupted in nearly all human cancers, creating a broad target spectrum for *E2F1* promoter usage (Hanahan and Weinberg 2000; Johnson et al. 2002).

The multidrug resistance (*Mdr*) phenotype is a major cause of cancer chemotherapy failure and is mainly due to the overexpression or amplification of the *Mdr1* gene encoding p-glycoprotein (Pgp) (Labialle et al. 2002). The *mdr* phenotype is characterized by a decrease in the intracellular accumulation of drugs due to energy-dependent drug efflux by Pgp. *Mdr1* promoter activation is complex, but involves several activation elements that have been reported as tumor inducers, such as TCF elements, or enhancer elements responsive distress, such as SXR element that is responsive for several xenobiotics (Roose and Clevers 1999; Synold et al. 2001). *The mdr1* promoter is activated in several cancer types.

3.3.4 Inducible promoters

Promoters of radiation-inducible genes may be utilized to drive transcription of key viral genes in response to ionizing radiation (Mezhir et al. 2006). Such a radiation-inducible gene is the early growth response gene 1 (*EGR1*), which is induced through distal CArG-elements (Datta et al. 1992). Furthermore, promoters may respond to genotoxic stress such as chemotherapeutics, a characteristic that to some extent can be exploited for replication control, albeit with requirements for other constrictions on replication (Ciribilli et al. 2010).

One useful system for replication control is the tetracycline-derived “Tet-system”, which exists in two opposite configurations, the “Tet-Off” and “Tet-On” (Gossen and Bujard 1992). In the Tet-Off system, addition of tetracycline represses gene expression, while and in the Tet-On system it triggers gene expression. Tet-system has been used to control adenoviral replication (Fender et al. 2002).

Other inducible promoter systems include the heat shock protein promoter (Schweinfest et al. 1998), metallothionine regulatory promoter (Hu and Davidson 1990), and steroid regulatory promoters (Ko et al. 1989). These systems, however, have been shown to suffer from various limitations, such as leakiness, low expression levels, and toxicity of the inducing agents (Yarranton 1992). Therefore, their use with replicating adenoviruses would require further optimization.

3.4 Controlling replication with pharmaceutical agents

Adenoviruses do not inherently contain a mechanism for replication inactivation. Therefore, external compounds that could be introduced to inhibit viral replication may prove useful in case of adverse effects in patients. This negative control over adenoviral replication is referred to as a safety switch system.

Presently, no formally approved compounds are available to treat adenoviral infections. Albeit a number of compounds, mostly nucleotide or nucleoside analogues that target the adenoviral DNA polymerase, with *in vitro* and *in vivo* activity have been described. Cidofovir is an acyclic nucleoside phosphonate that acts as an alternative substrate for viral DNA polymerase, inhibiting adenoviral DNA replication (Naesens et al. 2005). Antiviral selectivity results from the higher affinity for viral DNA polymerase. Ribavirin is another nucleoside analogue with a controversial mechanism of action and perhaps less potency against adenoviruses, as the *in vitro* susceptibility of adenoviruses to Ribavirin has been suggested to be restricted to species C Ads (Morfin et al. 2005). The "off-label" use of Ribavirin and Cidofovir as anti-adenoviral therapeutic modalities in immunocompromized patients is common, though a number of studies show ambiguous results especially with Ribavirin (Lenaerts et al. 2008). Furthermore, resistance to Cidofovir has been reported (Kodama et al. 1996).

Dietary flavonoids are a subject of great interest for their potential use in cancer treatment. Interestingly, they are also proposed to possess antiviral activity (Andres et al. 2009). Guercetin, a flavonoid found in most edible fruits and vegetables, was shown to inhibit adenoviral replication at the early stage of infection rather than to inhibit viral internalization (Chiang et al. 2003). Soy plants are a rich source of flavonoids. Apigenin, another bioflavonoid found in parsley, has been shown to have antiviral activity (Chiang et al. 2005). Apigenin induces G2/M phase cell cycle arrest and reduces the levels of cyclins (Ujiki et al. 2006; Meeran and Katiyar 2008). Cells arrested in G2/M will not go into mitosis and subsequently will not undergo S-phase induction either, which is deleterious for adenoviral replication.

Chlorpromazine is an antipsychotic drug believed to inhibit clathrin-coated pit formation by reversibly translocating clathrin and its adapters from the plasma membrane to intracellular vesicles (Vercauteren et al. 2010). Adenoviruses enter cells through clathrin coated pits and therefore inhibition of their formation inhibits adenoviral trafficking and replication. Recently, it was shown, that chlorpromazine exerted its effect downstream from nuclear delivery (Diaconu et al. 2010), suggesting that some of its various other functions are actually responsible for the inhibition of viral replication instead of its action on chlatrin coated pits (Day and Dimattina 1977). The steroidal compounds dehydroepiandrosterone (DHEA) and epiandrosterone (EA) exhibit a wide range of biologic activities including antiviral actions (Romanutti et al. 2010). They have been shown to adversely affect adenoviral protein synthesis and the formation of mature viral particles.

4 Oncolytic adenoviruses in clinical trials

Type I oncolytic adenoviruses feature short genetic deletions for transcriptional and subsequent replication control purposes. This approach takes advantage of the similarity of reguirements of carcinogenesis and DNA virus replication. Usually these deletions are in the *E1A* or *E1B* adenoviral genes, leading to mutated proteins unable to fulfill functions required for viral replication in normal cells but not in cancer cells that represent genetic alterations in cellular regulatory pathways. Type II oncolytic adenoviruses feature control over replication with various tumor specific promoters (TSP) introduced to regulate transcription of adenoviral key regulatory genes, most often *E1A*, with or without short genetic deletions. This mode of control requires replacement of viral promoters, as well as activity of the TSP in target tissue.

ONYX-015 is the first and most studied type I oncolytic adenovirus that has been the subject of numerous clinical trials starting from 1996 (for more detailed description of the virus see chapter 3.2.1 and for a list of selected clinical trials with oncolytic adenoviruses see table 1) (Bischoff et al. 1996). The general strategy utilized for ONYX-015 has been to progressively demonstrate safety with various routes of administration: intratumoral injections were followed by intraperitoneal administration, intra-arterial infusion, and finally intravenous administration (Aghi and Martuza 2005). Initially, ONYX-015 was studied in head and neck carcinomas that often result in death due to local disease and have accessible tumors for intratumoral injections (Ganly et al. 2000). Intra-arterial delivery of ONYX-015 for metastatic colorectal cancer demonstrated oncolytic viral replication in patients for the first time (Reid et al. 2001). Finally, doses up to 2×10^{12} viral particles were shown to be safe with no dose-limiting toxicity (Nemunaitis et al. 2001). When the safety of ONYX-015 as a single treatment modality was demonstrated in various clinical trials, ONYX-015 underwent clinical trials of combination treatment with chemotherapeutics (Khuri et al. 2000). In head and neck cancer patients the response rate to ONYX-015 treatment in combination with cisplatin and 5-FU was encouraging with several patients having 50 % or more reduction in tumor size and some featuring even a complete response. Disappointingly, long-term survival did not improve (Alemany 2007). Overall, the safety of ONYX-015 was demonstrated in various cancers and with various routes of administration. The clinical data, however, seems to point to the need for more potent viruses.

H101 is a very similar virus to ONYX-015, except that it lacks all E3 proteins, including ADP, rendering it less potent and more immunogenic. The first stage III trial with oncolytic viruses was concluded with H101, where a combination of intratumoral H101 with cisplatin and 5-FU resulted in doubled response rates compared to chemotherapeutics alone in patients with head and neck tumors (Xia et al. 2004). Survival data was inconclusive (Alemany 2007).

Table 1. Selected clinical trials with oncolytic adenoviruses

virus, combination treatment	genetic modification	Phase /number of patients	Cancer	inject. route	Results	Ref.
ONYX-015	E1B-55K deletion[1]	I / 22	SCCHN	i.t.	$1x10^{11}$ pfu 3 PR, 2 MR	(Ganly et al. 2000)
ONYX-015	E1B-55K deletion[1]	I /23	pancreatic cancer	i.t.	$1x10^{11}$ pfu no responses	Mulvihill et al. 2001
ONYX-015	E1B-55K deletion[1]	I / 10	metastatic solid tumor	i.v.	$2x10^{13}$ VP no responses	(Nemunaitis et al. 2001)
ONYX-015	E1B-55K deletion[1]	I / 16	ovarian carcinoma	i.p.	$1x10^{11}$ pfu/day for 5 d	(Vasey et al. 2002)
ONYX-015, 5-FU, cisplatin	E1B-55K deletion[1]	II / 37	SCCHN	i.t.	$1x10^{10}$ pfu/day for 5 d, 8 CR, 11 PR	(Khuri et al. 2000)
ONYX-015, 5-FU, leukovorin	E1B-55K deletion[1]	I-II / 11	metastatic colorectal cancer	i.ha	$2x10^{12}$ VP 2 PR	(Reid et al. 2002)
ONYX-015	E1B-55K deletion[1]	II / 14	SCCHN	i.t.	$1x10^{10}$ pfu/day for 5 d, 3 CR, 3 PR	(Lamont et al. 2000)
ONYX-015	E1B-55K deletion[1]	II / 37	SCCHN	i.t.	$2x10^{11}$ VP twice/day for 10 days, 3 CR, 2 PR	(Nemunaitis et al. 2000)
H101, 5-FU, cisplatin or adriamycin	E1B-55K deletion[1], deletion of E3	III / 123	SCCHN	i.t.	1.5x1012 VP daily for 5 d, 79% response in combination treatment	(Xia et al. 2004)
CV706	PSA promoter contolling E1A	I / 20	prostate cancer	i.t.	1 x 10^{13} pfu, transient decrease in PSA levels	(DeWeese et al. 2001)
CG7870	rat probasin promoter contolling E1A, PSA promoter contolling E1B	I / 23	prostate cancer	i.v.	6 x 10^{12} VP, 25%-49% decrease in serum levels of 5 patients	(Small et al. 2006)

[1]Two deletions in the E1B-55K adenovirus gene

Abbreviations: SCCHN, squamous cell carcinoma of the head and neck; HCC, hepatocellular carcinoma ; i.t. intratumoral; i.v. intravenous; i.p. intraperitoneal; i.ha., intrahepatic artery; 5-FU, 5-fluorouracil; pfu, plaque forming units; VP, viral particles; CR, complete response; PR, partial response; MR, minor response; d, days

CV706 was the first Type II oncolytic adenovirus created and studied in clinical trials (Rodriguez et al. 1997). It contains the *PSA* promoter and enhancer to drive *E1A* expression. In a phase I trial for locally recurrent prostate cancer, CV706 was well tolerated with mild side effects in response to intratumoral injection of 1 x 10^{13} pfu (DeWeese et al. 2001). In subsequent studies, improvements to CV706 were introduced with a final construct, CG7870, containing the rat probasin promoter in addition to the *PSA* promoter and demonstrating good safety in intravenous delivery (Small et al. 2006). Unfortunately, the PSA levels only transiently decreased.

5 Other oncolytic viruses

Other naturally oncolytic viruses or viruses genetically rendered oncolytic include the herpesviruses (HSV), influenza virus (IV), Newcastle disease virus (NDV), parvoviruses, poxviruses, reovirus, vesicular stomatitis virus (VSV), and Vaccinia virus (Sinkovics and Horvath 2008). Some of these viruses and their use in cancer therapy is discussed briefly.

Herpes simplex virus (HSV)-1 has a natural tropism for neuronal tissue, and is thus suitable for treating brain tumors such as glioblastoma (Grandi et al. 2009). HSV genes are classified as essential or nonessential based on their requirement for virus replication. Nonessential viral functions are manipulated to create oncolytic HSV-1 -vectors (Varghese and Rabkin 2002). HSV-1 can establish a latent infection in sensory neurons, or a lytic cycle in cells permissive for replication. Oncolytic HSV-1 vectors are genetically modified so that they can establish a lytic cycle only in cancer cells that either lack cellular functions that normally inhibit mutant virus replication or express high amounts of specific proteins that support replication. HSV-1 is a highly infectious virus and its replication leads to rapid cell death and release of new virus particles upon cell lysis. Four distinct HSV-1 vectors G207, 1716, NV1020 and OncoVEX$^{GM\text{-}CSF}$ have been studied in clinical trials for glioma, melanoma, and metastatic colorectal cancer (Markert et al. 2000; Rampling et al. 2000; MacKie et al. 2001; Senzer et al. 2009). Studies confirmed the safety of intratumoral and intralesional injections and a maximum dose of 1.3 x 10^{7} pfu was tolerated in i.ha injections. Two patients with recurrent glioma treated with G207 intratumorally lived for over 4 years, suggesting therapeutic benefit (Markert et al. 2000). OncoVEX$^{GM\text{-}CSF}$ is a selectively replicating HSV-1 encoding GM-CSF for immune response enhancement (Liu et al. 2003). In a phase II trial of nonresectable metastatic melanoma, 50 patients received median of 6 intratumoral injections of up to 4 x 10^{8} pfu OncoVEX$^{GM\text{-}CSF}$ (Senzer et al. 2009). Eight patients showed complete response, five patients displayed stable disease, and regression of both injected and distant lesions occurred. Overall survival was impressive, 52 % at 24 months.

Newcastle disease virus (NDV) causes Newcastle disease in birds, but has been demonstrated to selectively replicate in and kill human tumor cells sparing normal cells (Reichard et al. 1992). NDVs have been classified as lytic or non-lytic strains. Lytic strains cause lysis of target mammalian cells by inducing changes in the plasma membrane (Pecora et al. 2002), and non-lytic strains cause slow tumor regression by disrupting normal host cell metabolism (Schirrmacher et al. 1999). Furthermore, non-lytic strains can

enhance immune responses against infected tumors by inserting viral proteins on to tumor cell membranes or by inducing the host to produce effector cytokines in response to the virus itself, thereby eliciting NDV-specific immune response that results in NK, macrophage, and T-cell responses against infected cells (Schirrmacher et al. 2001). The most widely studied strain, *Ulster,* is a non-lytic strain, but lytic strains have also been studied as anti-cancer agents (Ravindra et al. 2009). The first clinical trial (stage II) for NDV was conducted in 1975 in patients with stage III melanoma metastasized to regional lymph nodes (Cassel and Murray 1992). Patients received a post-surgery adjuvant vaccine composed of allogeneic and autologous human melanoma cells infected with live NDV. This trial was not prospectively randomized, and therefore the results should be viewed with caution; nevertheless, when compared to contemporary worldwide control patients with stage III melanoma that experience a high relapse rate of 70-80%, a relapse rate of less than 20% of NDV-lysate treated patients in 10 years was encouraging (Batliwalla et al. 1998; Sinkovics and Horvath 2008).

Vaccinia virus from the family *Poxviridae* had a crucial role in the eradication of smallpox (Fenner 1980). Its use as a smallpox vaccination has led to a better understanding of its biology and pathogenesis as well as knowledge of which populations may be at risk for rare adverse events (Enserink 2002). Vaccinia virus replicates and lyses cells rapidly, which is of great importance in terms of efficacy (Wein et al. 2003). Second, they do not require specific cellular receptors for entry – instead, they infect target cells through several membrane fusion pathways and thereby feature broad tumor tissue tropism (Moss 2006). Third, vaccinia are produced in several antigenic forms in which the enveloped virus (EEV) has a host cell-derived envelope with only a few exposed viral proteins, resulting in efficient spread within the host blood stream without immune defence mechanisms recognizing it (Vanderplasschen et al. 1998). Vaccinia viruses can selectively replicate in and lyse cancer cells due to their natural biology and through genetic engineering of viral transformation genes that in a normal situation make normal cells susceptible to viral replication. One targeted and armed oncolytic vaccinia virus, JX-594, has entered clinical trials (Mastrangelo et al. 1999). JX-594 is a Wyeth strain vaccinia featuring an inactivated *thymidine kinase* gene and a *GM-CSF* transgene under a synthetic promoter. In a pilot phase I study of melanoma, intratumoral injections of JX-594 resulted in 71% tumor response. Furthermore, a response in distant skin metastases was also witnessed. In a phase I-II dose escalation study patients with advanced melanoma or hepatocellular, colorectal, or lung cancer received four intratumoral injections of JX-594 every 3 weeks. The virus was well tolerated up to 1 x 109 pfu, and efficacy was shown in diverse tumor types, at both injected and non-injected sites (Park et al. 2008). Seven patients out of ten patients available for evaluation had stable disease. Furthermore, the presence and replication of JX-594 in non-injected tumor sites was witnessed.

6 Xenograft murine tumor models for cancer

The preclinical models predominantly used for cancer research are various murine xenograft transplant models, where *in vitro* cultured human cancer cells are transplanted into immunocompromised mice. Various injection sites are utilized and termed xenograft transplantation, notably intravenous, intraperitoneal, subcutaneous, and orthotopic injections (Kim and Baek 2010). Xenograft tumors are easily replicated, which facilitates the reliability of research results, as the baseline material is comparative. Subcutaneously growing tumors are easy to monitor physically, and various marker genes such as GFP and luciferin may be utilized to allow non-invasive monitoring of tumor growth, when the cancer cells express these markers. Human origin of the tumors is also important for cancer research, and the possibility of tumor metastasis in xenograft models creates an opportunity to study the metastatic potential and behaviour of cancer. Unfortunately, various disadvantages exist. Firstly, an immunocompromised host is necessary in xenograft models (Frijhoff et al. 2004). This is a serious drawback, especially when investigating the systemic behavior of highly immunogenic agents, such as adenoviruses, in a cancer model. Secondly, tumors are growing in host stroma instead of endogenous stroma, probably effecting tumor development in xenograft models. Crosstalk between different cell types within a tumor and the stroma surrounding it is recognized as an important factor in tumor development (Mueller and Fusenig 2004). Furthermore, xenograft models usually require transplantation of large numbers of cells for tumor to develop, and the tumor development is often rapid, with a latency period of only days to weeks. A very different situation exists in a cancer patient, where a tumor may develop for even years. Also, *in vitro* culturing of cells prior to transplantation results in selection of cells displaying such properties that may differ from the properties of the initial tumor cells.

One of the major drawbacks of using any kind of murine models in adenoviral gene therapy research relates to the inability of adenoviruses to replicate in murine tissue. Both normal and cancerous mouse tissue are poorly permissive for adenoviral replication (Duncan et al. 1978; Ginsberg et al. 1991).

AIMS OF THE STUDY

1. To evaluate the effect of capsid modifications and Kupffer cell depleting agents on gene transfer efficacy and oncolytic potency of genetically engineered adenoviruses in breast cancer cell lines, tissues and murine models (**I, II**).

2. To construct Ad5.pK7-Δ24, a replicating oncolytic adenovirus with expanded tropism and selectivity for the pRb pathway defective cells (**I**).

3. To evaluate oncolytic potency of transcriptionally regulated oncolytic adenoviruses on $CD44^{+}$ $CD24^{-/LOW}$ putative cancer stem cell populations derived from pleural effusions of breast cancer patients (**III**).

4. To develop murine models for breast cancer that can be imaged noninvasively for tumor progression (**I, II**).

5. To evaluate the use of pharmaceuticals as a means of adenoviral replication reduction in case of replication associated side effects in patients (**IV**).

MATERIALS AND METHODS

1 Cell lines and fresh human tissues

Cell lines (listed in Table 2) used in this study were cultured in the growth medium recommended by the supplier, supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin-streptomycin. Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2.

Table 2. Human cell lines used in this study.

cell line	Description	Source	Study
293	transformed embryonic kidney cells	ATCC[1]	I-IV
911	transformed human embryonic retinoblasts	Dr Van Der Eb[2]	III
A549	lung adenocarcinoma	ATCC[1]	I-IV
MDA-MB-435	breast ductal carcinoma, derived from pleural effusion	ATCC[1]	I, II
MDA-MB-436	breast adenocarcinoma, derived from pleural effusion	ATCC[1]	I, II
M4A4-LM3	a metastatic derivative of MDA-MB-435, stably transfected with GFP	Dr S. Goodison[2]	I, II
ZR-75-1	breast ductal carcinoma, derived from ascites	ATCC[1]	I, II
MCF7	breast adenocarcinoma, derived from pleural effusion	ATCC[1]	I, II
CAMA-1	brest adenocarcinoma	ATCC[1]	I, II
JIMT-1	breast ductal carcinoma, derived from pleural effusion	Dr J. Isola [3]	III
Hey	Ovarian adenocarcinoma	Dr J. Wolf [4]	IV
Ov-4	Ovarian adenocarcinoma	Dr T.J. Eberlein [5]	IV

[1]Cell line purchased from American Type Culture Collection (ATCC)

[2]Departments of Molecular Cell Biology and Surgery, Leiden University Medical Center, Leiden, The Netherlands

[3] University of Florida, Jacksonville, FL, USA

[4]cell line is a gift from Dr. Minna Tanner (University of Tampere and Tampere University Hospital, Tampere, Finland)

[5]M. D. Anderson Cancer Center, Houston, TX, USA

[6]Harvard Medical School, Boston, MA, USA

2 Adenoviruses

Table 3. Recombinant adenoviruses used in this study.

virus	E1 region status	targeting motif	study	reference
Ad5 (GL)	*GFP, luciferase*	-	I, II	(Wu et al. 2002)
Ad5.LacZ	*β-galactosidase*	-	II	(Yotnda et al. 2004)
Ad5lucI	*luciferase*	-	II	(Kanerva et al. 2002)
Ad5/3lucI	*luciferase*	5/3 serotype chimera	II, III	(Kanerva et al. 2002)
Ad5lucRGD	*luciferase*	RGD	II	(Dmitriev et al. 1998)
Ad5.pK7(GL)	*GFP, luciferase*	polylysine	I, II	(Wu et al. 2002)
Ad5.RGD.pK7(GL)	*GFP, luciferase*	polylysine, RGD	II	(Wu et al. 2002)
Ad5/3mdr-E1[2]	multidrugresistance promoter	5/3 serotype chimera	III	(Bauerschmitz et al. 2008)
Ad5Δ24E3	Δ24[1]	-	I, II	(Suzuki et al. 2001)
Ad5/3-Δ24	Δ24[1]	5/3 serotype chimera	II-IV	(Kanerva et al. 2003)
Ad5-Δ24RGD	Δ24[1]	RGD	II	(Fueyo et al. 2000)
Ad5.pK7-Δ24[2]	Δ24[1]	polylysine	I, II	(Ranki et al. 2007)
Ad5/3ala-Δ24[2]	α-lactalbumin promoter, Δ24[1]	5/3 serotype chimera	III	(Bauerschmitz et al. 2008)
Ad5/3mdr-Δ24[2]	Δ24[1], multidrug resistane promoter	5/3 serotype chimera	III	(Bauerschmitz et al. 2008)
Ad5/3-hTERT-Δgp[2]	Δ24[1], hTERT promoter	5/3 serotype chimera	III	(Bauerschmitz et al. 2008)
Ad5/3-Cox2L-Δ24[2]	Δ24[1], Cox-2 promoter	5/3 serotype chimera	III	(Bauerschmitz et al. 2008)

[1] 24 base pair deletion in the E1A CR2 that targets viral replication to cells with defective pRb pathway
[2] Viruses constructed in the context of this thesis.

An informed consent from patients and permission for studies on human tissue from the ethics committee was obtained prior to any experiments. Breast cancer samples were obtained from patients undergoing surgery and fresh liver samples were obtained from healthy donor livers that were to be implanted into recipients, both by the Department of

Surgery, Helsinki University Hospital. Livers were kept on ice in ViaSpan solution (Bristol-Myers Squibb Ab, Bromma, Sweden) until slicing. Precision-cut slices (250µm) were cut with a Vibratome 1000 plus sectioning system (Vibratome, St Louis, MO, USA) and cultured on a rocker in the same conditions as cell lines. Oxygenated William´s medium E with 25 mM D-glucose and 50 µg/ml was used as a growth medium.

Ad5.pK7-Δ24 (**I**) was constructed by co-transfecting (Effectene Transfection Reagent, Qiagen Corporation, Hilden, Germany) ethanol precipitated Ad5.pK7 (GL) DNA (Wu et al. 2002) with *Pac*I and *Pme*I digested pShuttleΔ24 (Fueyo et al. 2000) plasmid DNA in a 1:1 weight to weight ratio into 293 cells. Individual plaques were collected and purified for 2 rounds on A549 cells.

Ad5/3ala-d24, Ad5/3mdr-E1 and Ad5/3mdr-d24 (**III**) were constructed by cutting promoter sequences from pGL3-ALA (Li et al. 2005) and pM3mdr-p-hTNF (Walther et al. 1997) into pSE1 and pSE1d24 (Nettelbeck et al. 2002), from which the *E1* promoter was removed. The viral backbone was rescued in 911 cells with PacI digested pTU-5/3 (Uil et al. 2003). For construction of Ad5/3-hTERT-Δgp, the hTERT was cloned into pSE1 (Nettelbeck et al. 2002), resulting in pShTERTE1. pAd5/3-hTERT-E1-Δgp was generated by homologous recombination in BJ5183 cells using PmeI digested pShTERTE1 and SrfI linearized pAdEasy-1.5/3-Δgp (serotype 3 knob, a 24 bp deletion in *E1A* and a 965 bp *6.7K/gp19K* deletion in *E3A*) for transfection into 911 cells. The same promoter sequences were cloned to replace CMV in pShuttle-CMV (AdEasy, QBiogene, Carlsbad, CA) and *luciferase* was cut from pGL3-Basic (Promega, Madison, WI) for homologous recombination with pAdEasy-1 to create non replicating control viruses.

All replicating viruses were produced in A549 cells and nonreplicating viruses on 293 cells. Viruses were purified on double CsCl gradients using standard methods.The viruses were titered for viral particles (VP) at 260 nm. The functional titer was determined with the plaque assay or TCID50 assay of 293 cells for nonreplicating viruses and A549 cells for replicating viruses. The modified region on the genome were confirmed by sequencing and polymerase chain reaction (PCR) with appropriate primers.

All recombinant viruses used in this study are listed in table 3 with the appropriate references mentioned for those viruses that we did not create.

3 *In vitro* experiments

3.1 Gene transfer assays

Gene transfer assays were done for various fiber modified (**I, II**) and transcriptionally controlled (**III**) non-replicating Ads in order to evaluate the effect of a particular fiber modification on transductional efficacy of the virus or the effect of a particular promoter on gene expression. Assays were performed in various breast cancer cell lines (**I,II**), JIMT-1 cell line sorted for $CD44^{+}/CD24^{-/LOW}$ expression (**III**), and fresh breast cancer tissue (**II**). Luciferase activity was analyzed and the results were depicted as relative to a

control virus. Protein content was measured when analyzing patient samples and used as an internal control. For more detailed protocols, see “Materials and Methods” from the respective articles.

3.2 Cytotoxicity assay (I-IV)

To study the cell killing efficacy of oncolytic viruses, cells were infected with three virus doses and followed for the cytopathic effect. Cell viability was measured with an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay when one of the viruses had reached complete cell killing with the highest amount and significant cell killing with the second highest virus amount. MTS assay is a non-radioactive cell proliferation assay where tetrazolium compound (MTS) is reduced by cells into formazan. The amount of formazan is measured by absorbance in 492 nm.

The length of the experiment for each cell line was internally timed for maximum dynamic range with the positive and negative controls. When assessing the effect of apigenin and chlorpromazine on replication, these compounds were applied to the cells prior to infection. For more detailed protocols, see “Materials and Methods” from the respective articles (**I-IV**).

3.3 Quantitation of viral replication (IV)

The effect of apigenin and chlorpromazine on viral replication was assessed by quantitating viral replication in ovarian carcinoma cell lines or liver tissue after first preincubating the samples with the compounds. Infected cells and liver slices were collected at different time points and freeze thawed to release the viruses. The supernatant was subsequently used to analyze replication with $TCID_{50}$ on 293 cells. Results were compared to samples without chlorpromazine or apigenin preincubation and mock treated cells.

Furthermore, liver aspartate transaminase (AST) levels were analyzed with a photometric method from the supernatant of infected liver slices. For more detailed protocols, see “Materials and Methods” from the respective article (**IV**).

3.4 Isolating CD44+ CD24-/LOW cell population (III)

Cells from pleural effusion samples and JIMT-1 cell line were sorted with FITC-labeled anti-CD44 and phycoerythrin-labeled anti-CD24 antibodies and collected with magnetic beads (**III**).

3.5 Comparison of receptor levels *in vitro* (I)

To study the CAR and HSPG levels in the M4A4-LM3 cell line, trypsin-EDTA preincubated cells were labeled with the anti-CAR primary antibody RmcB or anti-HSPG primary antibody 10E4. A phycoerythrin-labeled goat anti-mouse antibody was used as a secondary antibody, and labeled cells were analyzed with flow cytometry. For more detailed protocols, see "Materials and Methods" from the respective article (**I**).

4 *In vivo* experiments

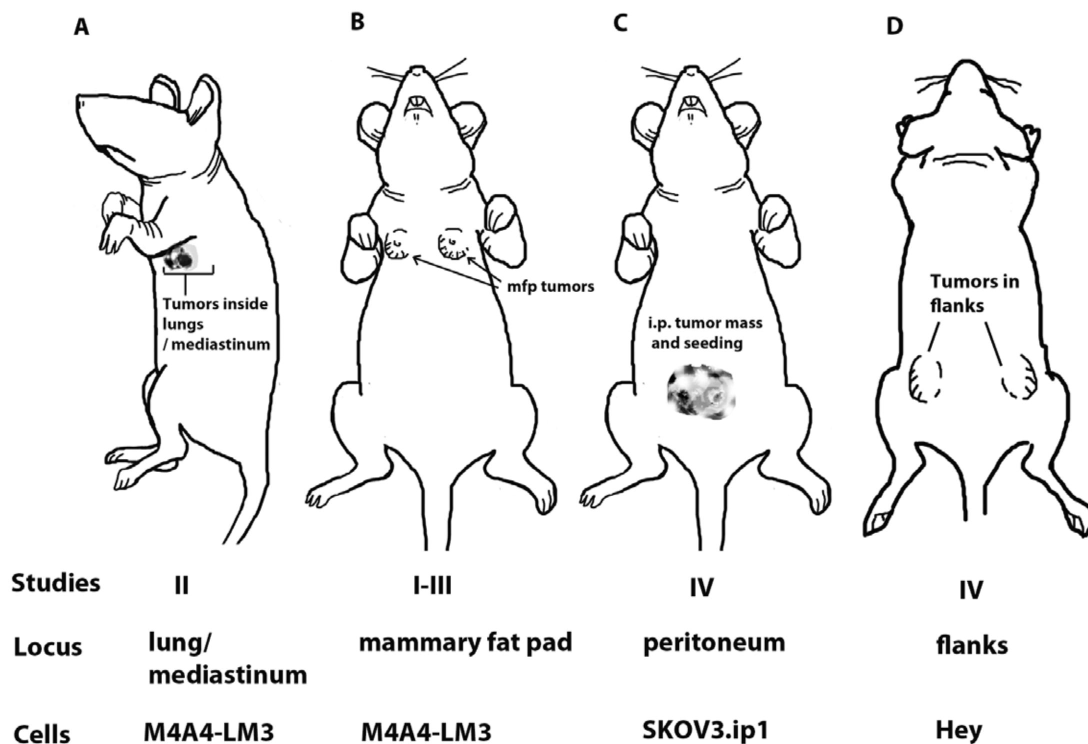

Figure 6. Xenograft murine models utilized in this thesis. A) Systemic treatment model with M4A4-LM3 breast cancer cells inoculated with a midaxillary percutaneous injection under the sixth rib into the left lung or mediastinum. Tumors were imagable with Xenogen IVIS 100 imaging system for GFP expression in the cancer cells. Mice were treated with intravenous injections. Model was used in study II. B) Orthotopic breast cancer model with M4A4-LM3 cells injected into the left and right uppermost mammary fat pad. Tumors were imagable with Xenogen IVIS system for GFP or physically measurable with a caliber. Studies I-III. C) Orthotopic ovarian cancer model with SKOV3.ip1 cells injected into the peritoneum of SCID mice. Tumors were not imagable. Study IV. D) Subcutaneous tumor model with ovarian cancer Hey cells injected into the flanks of SCID mice. Tumors were measured physically with a caliber. Study IV.

Pathogen free 3 to 4-week-old female NMRI (Naval Medical Research Institute) nude or C.B-17 SCID (severe combined immune deficiency) mice were obtained from Taconic (Ejby, Denmark) and quarantined for 2 weeks prior to any experiments. All animal experiments were performed according to the rules of the Provincial Government of Southern Finland.

4.1 Mouse models of breast and ovarian cancer (I – IV)

To create an orthotopic breast cancer model, mice were injected into the left and right uppermost mammary fat pads with 2 x 10^6 sorted JIMT-1 cells or GFP expressing M4A4-LM3 cells and a tumor was allowed to develop (**I-III**). The maximum diameter of a tumor was 1 cm. To study the metastasis of M4A4-LM3 into lymph nodes, mice were euthanized, and axillary lymph nodes were harvested and imaged for GFP expression with the Xenogen IVIS 100 imaging system.

A systemic treatment model mimicking breast cancer lung metastasis was established (**II**). 2 x 10^6 M4A4-LM3 cells were inoculated with a midaxillary percutaneous injection under the sixth rib into the left lung or mediastinum. Mice were imaged as above and survival was followed and mice were euthanized when disease related adverse effects occurred.

For a subcutaneous ovarian cancer model 5 x 10^6 Hey cells were injected into the flanks of nude mice and followed for growth with caliber measurements. The maximum diameter of a tumor was 1 cm. For an orthotopic ovarian cancer model 1 x 10^7 SKOV3.ip1 cells were injected i.p. into SCID mice and a tumor allowed to develop (**IV**).

4.2 Noninvasive imaging (I, II)

M4A4-LM3 cells used in the breast cancer animal models express GFP. Nonreplicating viruses used in biodistribution experiments in mice express luciferase. Mice were imaged with the Xenogen IVIS 100 imaging system to monitor tumor growth in breast cancer animal models or to evaluate viral distribution after intravenous injection. Images were obtained with a charge-coupled device camera cooled to -120 C with the field of view set to 15 cm. Fluorescence images were obtained with an open GFP excitation filter and low level intensity. For bioluminescence images, mice were injected intravenously with D-luciferin and after 10 minutes imaged. For more detailed protocol, see "Material and Methods" of the respective articles (**I, II**)

4.3 Efficacy of replicating viruses *in vivo* (I-IV)

To test the effectiveness of replicating viruses in the mammary fat pad model 3 x 10^8 VP in 50 µl were injected on 3 subsequent days into approximately 0.5 cm wide tumors (**I**). Tumors were measured daily with a caliber and imaged thrice weekly with the IVIS

system. In the systemic treatment model 3 x 10^{10} VP in 100 µl was injected intravenously 10 days after injection of cells and again on day 33. Tumors were imaged and measured as above starting on day 10.

To assess antitumor efficacy of various tumor specific promoter driven viruses in tumors formed by $CD44^{+}CD24^{-/LOW}$ cell populations, sorted JIMT-1 cells were injected as above and tumors were treated with intratumoral virus injections thrice weekly for 5 weeks (**III**). Tumor growth was followed throughout the experiment. To further evaluate the ability of selected viruses to kill putative $CD44^{+}CD24^{-/LOW}$ cancer stem cells in the mammary fat pad model, tumors were analyzed by flow cytometry for CD44 and CD24 expression after 17 days of treatment.

To assess efficacy of replicating viruses in the metastatic breast cancer model, mice were injected intravenously with 2 x 10^{10} VP of virus 5 days after cell injections (**II**). Treatments were repeated on days 10, 15, and 20. Mice were noninvasively imaged and followed for survival. The effect of kupffer cell depleting polyI on survival was evaluated in the same setting. PolyI was injected intranvenously prior to virus injection. The treatment scheme was as above and the survival of the mice was monitored accordingly.

4.4 Biodistribution of adenoviruses (II)

The orthotopic mammary fat pad model was utilized to study the effect of various capsid modifications on viral gene transfer *in vivo*. 5 x 10^{10} VP capsid modified nonreplicating luciferase expressing viruses were injected intravenously to tumor bearing mice. After 48 hours the mice were euthanized and their tumors and organs were collected, homogenized and analyzed for luciferase expression. The protein content was also measured for each sample and used as an internal control.

4.5 KC depletion (II)

The effect of KC depleting agents gadolinium chloride (GdCl3), polyinosinic acid (polyI), and liposomes on gene transfer efficacy was studied in the orthotopic mammary fat pad model. Tumor bearing mice were treated with intravenous injections of the compounds prior to injection of 2 x 10^{10} VP of luciferase expressing virus. Thereafter, analysis of the tumors was performed as in the biodistribution study.

4.6 Inhibition of viral replication (IV)

To assess the effect of chlorpromazine and apigenin on viral replication *in vivo*, the ovarian cancer model was utilized. Tumors were treated with intratumoral injections of virus on days 0, 2, and 4. Mice received daily injections of 200µg chlorpromazine or 250µg apigenin i.p and tumor volume was followed daily.

To assess the effect of apigenin and chlorpromazine on replication, the treatment scheme was as above except for a single virus injection on day 0. Tumors were harvested at different time points, homogenized and freeze thawed repeatedly to release the viruses from cells. The amount of infectious particles was analyzed with $TCID_{50}$.

4.7 Murine toxicity model (IV)

To analyze the effect of chlorpromazine and apigenin on virus related toxicity, the i.p. ovarian cancer model was utilized. Ten days after cell injection mice received a single virus injection i.p. and the previously described dose of either compound. Thereafter, apigenin or chlorpromazine was given daily. Further, mice received 80 mg/kg i.p. on four days. Mice were killed when any pain or distress occurred and tumors and livers analyzed histologically.

5 Histopathology and immunohistochemistry (I, IV)

Livers and tumors were collected in buffered formalin (10 %), embedded in paraffin and cut into 5 µm sections.

Deparaffinized specimens were stained with hematoxylin and eosin to study the histopathology of the specimens. Histopathology was scored blinded by an independent pathologist.

Immunohistochemical stainings with anti-CAR primary antibody RmcB or anti-HSPG primary antibody 10E4 and phycoerythrin-labeled goat anti-mouse secondary antibody were used to study whole-tumor sections for CAR and HSPG expression (**I**).

6 Statistical analysis

The two-tailed student´s t-test was used to analyze the differences in gene transfer efficacy between viruses and their control groups as well as to analyze the difference between a single time point in the tumor size data (**I**). Oncolytic cell killing efficacy (MTS test) data was analyzed by one-way ANOVA (**II**). The survival curves were calculated according to Kaplan-Meier and groups were compared pairwise with the logrank test (SPSS 11.5) (**I, II**).

The effects of chlorpromazine and apigenin on viral replication and cell viability were analyzed using bootstrap multiple comparisons of means in analysis of variance (ANOVA) (PROC MULTTEST SAS v 9.1) to account for multiple testing (**IV**). For statistical analyses the levels of viral replication were log-transformed for normality.

The analysis of tumor size data was performed using a repeated measures growth model with PROC MIXED (SAS v 9.1) (**IV**). The tumor size data was log-transformed for normality. The effects of time, treatment group and interaction between time and treatment

group were shown as F-test results. The a priori planned comparisons of differences in predicted treatment means were computed by t-statistics on the last time-point and averaged over all time points.

A value of $P<0.05$ was deemed statistically significant for all studies (**I-IV**).

RESULTS AND DISCUSSION

1 The effect of capsid modifications and Kupffer cell depletion on gene transfer efficacy and oncolytic activity

Perhaps the most limiting factor in the use of oncolytic adenoviruses for cancer treatment is the limited transduction of target cells. The low expression of Ad5 primary receptor CAR on tumor cells results in poor transduction efficacy, at least *in vitro* (Kim et al. 2002). Most adenoviral gene therapy approaches are based on utilizing serotype 5 viruses, and therefore extensive research efforts have focused on identifying feasible means to circumvent CAR dependence. Receptor expression on cell lines obviously plays a major role in Ad transduction. Nevertheless, because gene transfer is the most relevant endpoint when evaluating the feasibility of different capsid modifications in adenoviral gene therapy, we elected to study functional transgene delivery directly instead of receptor expression. Furthermore, there are published studies concerning CAR and integrin levels on breast cancer cell lines, and our results may be discussed in respect to existing knowledge.

For enhanced gene transfer to cancer cells we chose genetic engineering as the adenovirus capsid modification. With oncolytic cell killing being ultimately the means of action, it is necessary to retain all modifications in the progeny virions and therefore the genetic approach is the only option as the non-genetic bispecific antibody modification would be lost upon viral infection.

1.1 Gene transfer efficacy of capsid modified non-replicating adenoviruses to breast cancer cell lines and patient tissue

Our goal was to evaluate various previously reported capsid modifications for their efficacy in gene transfer to gain insight into their performance in breast cancer treatment. In other words, we sought to identify the most effective capsid modification in terms of gene delivery to breast cancer cells, in order to translate the concept into a replicating, oncolytic agent that would be efficient in breast cancer cell oncolysis.

The fiber knob region is responsible for the binding of the primary receptor CAR, which has led to various attempts to retarget adenoviruses through changes in the knob. We used viruses with targeting ligands incorporated into the C-terminus or the HI-loop, which have previously been reported as feasible locales (Wickham et al. 1993; Krasnykh et al. 1998; Borovjagin et al. 2005). Arg-Gly-Asp (RGD) modification in the HI-loop of the knob allows the virus to utilize cellular integrins for binding and internalization, retaining the CAR binding ability (Dmitriev et al. 1998). Similarly, a polylysine (pK) modification in the C-terminus of the knob allows internalization through HSPGs in addition to CAR (Wu et al. 2002). These features were also combined in hope of gaining additive enhancement of transduction. Importantly, ligand incorporation results in tropism expansion, but not true retargeting with CAR binding ablation, whereas serotype chimeric

viruses display true de- and retargeting. In addition to viruses featuring targeting ligands, we utilized a serotype 3 chimeric Ad5 virus that features a serotype 3 knob domain and targets a yet unknown serotype 3 receptor but not CAR (Kanerva et al. 2002).

Capsid modifications resulted in significant and, in some cases, even dramatic increases in gene transfer efficacy in breast cancer cell lines (**figure 1a in study I**). Gene transfer efficacy was evaluated by analyzing the intensity of bioluminescence resulting from the luciferin converting activity of luciferase gene that nonreplicating adenoviruses express.

The polylysine modification pK7 displayed superior gene transfer efficacy in all but one cell line tested, reaching as high as a 700-fold increase in marker gene expression in MDA-MB-435, a ductal carcinoma cell line and in MCF-7, an adenocarcinoma cell line. The Ad5/3 chimera displayed the highest gene transfer efficacy in CAMA-1, an adenocarcinoma cell line, and a 100-fold increase in ZR-75-1, a ductal carcinoma cell line. MDA-MB-435, MCF-7, and ZR-75-1 reportedly display very low levels of the CAR receptor, which explains the dramatic increase in gene transfer with capsid modified viruses (Lucas et al. 2003). The M4A4-LM3 cell line is a metastatic derivative of MDA-MB-435 and was shown to express *in vitro* very low levels of CAR and sufficient levels of HSPG, explaining the superiority of pK7 modification (**figure 5c in study I**).

The overall gene transfer enhancement was more modest in MDA-MB-436 compared to other cell lines, with a 30-fold increase in marker gene expression with the pK7 and RGD modified viruses. This cell line has been previously described to feature high CAR expression levels, which may partially explain the less pronounced benefit from capsid modifications (Lucas et al. 2003). The double-modified Ad5.RGD.pK7 virus featuring RGD and pK7 modifications in the HI-loop and C-terminus was generally less effective in comparison to single-modified viruses. Double modifications may impose steric hindrance that can interfere with receptor recognition and binding, perhaps explaining the rather unimpressive performance of double-modified viruses. Furthermore, incorporation of ligands imposes structural constraints on the folding and trimerization of the knob (Borovjagin et al. 2005), perhaps resulting in a purified prep with less viable double-modified virions than with single-modified counterparts.

We analyzed gene transfer efficacy to clinical breast cancer samples from patients with advanced disease (all grade 3) (**figure 1b in study II**). Gene transfer enhancement was more modest overall with an approximately 2-7 fold increase with pK7 modification in all the samples and a 2-11 fold increase with the chimeric 5/3 capsid in two samples. Neither RGD nor pK7.RGD double-modification resulted in increased gene transfer to clinical samples. The overall less pronounced increase in gene transfer efficacy compared to that seen in cell lines was logical, as the tumor tissue was not homogenized into a single cell solution and therefore featured characteristics that would impair viral infectivity. Such characteristics would include heterogenous size of the tissue pieces, heterogenous cell population within the tissue, and the possible presence of connective tissue, which would confer an anatomical barrier to viral infection. The use of nonreplicative viruses only allowed infection of the outer layer cells when encountering tissue with a larger volume.

Kupffer cell depletion with poly(I) prior to viral injection resulted in a 10 fold enhancement in gene transfer efficacy to tumors (**figure 4A in study II**). When we

attempted to gain the same effect in combination with viral treatments, however, no signal was obtained from the tumors or livers of the mice imaged with xenogen IVIS 100 (**figure 4B in study II**).

In conclusion, capsid-modified viruses displayed significantly enhanced gene transfer efficacy to breast cancer cell lines and breast cancer tissues.

1.2 Biodistribution of capsid modified viruses

We evaluated the effect of capsid modifications on the biodistribution of viruses after intravenous injections to mice bearing mammary fat pad tumors. After intravenous virus injection tumor transduction was demonstrated (**figure 3c in study II**). Most of the marker gene expression was seen in the liver, however, which was to be expected as intravenously delivered virus usually homes to Kupffer cells and hepatocytes and most of the transgene expression is detected from hepatocytes (Vrancken Peeters et al. 1996; Connelly 1999; Alemany et al. 2000), and because the capsid modifications were not designed to confer any liver detargeting properties. No significant differences were seen in the biodistribution profiles between capsid modified and unmodified viruses. Furthermore, no differences were seen in the ratio between tumor and liver homing, as all the viruses displayed effective liver transduction and less impressive tumor transduction (**figure 3f in study II**). Nevertheless, capsid modified viruses displayed less liver transduction compared to Ad5, though no significance due to high variability emerged. For example, the mean marker gene expression level from the liver was over 30 times less with RGD-modified virus than with Ad5, suggesting a trend towards favorable tumor to liver transduction. Interestingly, M4A4-LM3 cell line derived tumors were found to upregulate CAR, a phenomenon that would diminish any favorable effects capsid modifications would display in a CAR deficient setting often associated with tumors (**figure 5 d-f in study I**) (Kim et al. 2002). A similar phenomenon previously demonstrated in a study where the CAR level and location were different when the same breast cancer cells were grown on plates versus in a three-dimensional model system (Anders et al. 2003). Furthermore, with brain tumors it was demonstrated that xenografts had a higher CAR mRNA expression than the parental tumor cells (Fuxe et al. 2003).

In conclusion, capsid modifications did not result in significant differences in biodistribution in mice bearing M4A4-LM3 derived xenograft tumors.

1.3 Oncolytic potency of capsid modified replicating viruses *in vitro*

Oncolytic adenoviruses convey their anticancer effect through replication and subsequent oncolysis of target cells. Obviously, the presence of a replicating virus within the cell is a prerequisite for oncolysis, and therefore transductional efficacy of the virus is of great importance to the cell killing ability.

To evaluate the cell killing potency of oncolytic viruses featuring capsid modifications we utilized replicative viruses that have a 24 bp deletion in the viral *E1A* CR2, which

ultimately targets these viruses to cells with a defective pRb/p16 pathway (Fueyo et al. 2000; Heise et al. 2000). The pRb/p16 pathway is mutated in a majority of human tumors, including advanced breast cancers (Anderson et al. 1996; Sherr 1996). We studied the cell killing potency in breast cancer cell lines and *in vivo* in murine models of breast cancer.

In all but one cell line (MDA-MB-436) significantly enhanced cell killing was observed with one or more of the capsid-modified replicating viruses compared to the wt-capsid virus featuring the 24 bp deletion (**figure 2 in studies I and II**). The Ad5/3-Δ24 displayed the most pronounced enhancement in cell killing activity, being the most effective in 3 (MCF-7, CAMA-1 and ZR-75-1) of the 6 cell lines tested. The Ad5.pK7-Δ24 was the most effective virus in the MDA-MB-435 and almost as effective as the Ad5/3-Δ24 in other cell lines. Ad5-Δ24RGD showed the most effective cell killing potency in M4A4-LM3 cell line. In the MDA-MB-436 no significant difference between wt capsid control virus and any of the capsid modified viruses emerged, which corresponds to the situation in the gene transfer efficacy study. Overall, these results coincide quite well with the gene transfer efficacy results, with perhaps the chimeric Ad5/3-Δ24 displaying a better cell killing activity than would have been expected based on gene transfer efficacy.

Similar results were seen previously in renal cancer cell lines, where dramatically enhanced gene transfer efficacy with pK7-modified nonreplicating adenovirus did not convert into a dramatic enhancement in cell killing, though efficient cell killing was observed (Guse et al. 2007). Moreover, in the same setting the chimeric 5/3 modified virus displayed the most potent cell killing activity. Furthermore, the same effect was seen in non-small cell lung cancer (NSCLC) cell lines displaying high infectivity enhancement with pK7 modification, but less pronounced enhancement in cell killing, and *vice versa* with the chimeric 5/3 virus (Sarkioja et al. 2006). This suggests that *in vitro*, the superior cell killing activity of Ad5/3-Δ24 seems to be mostly related to the speed of production of new viral particles instead of transductional enhancement. Perhaps the concept of fiber chimerism is more feasible than the insertion of ligands which may interfere with the trimerization of the fiber in the pool of progeny virions, thus slowing down the process of oncolysis and subsequent infection of neighboring cells.

1.4 Oncolytic potency of capsid modified replicating viruses *in vivo*

The antitumor activity of Ad5.pK7-Δ24 was evaluated in a mammary fat pad breast cancer model in comparison to Ad5-Δ24E3. No significant difference existed between the viruses when they were injected intratumorally (**figure 3a in studyI**) or intravenously (**figure 6a in study I**). These results suggest that the previously mentioned upregulation of CAR on M4A4-LM3 derived tumors hampers any advantage the pK7 modification might have in a different setting, and that the infectivity of cells *in vitro* is not the whole truth with regard to gene transfer and oncolysis *in vivo*. Furthermore, in immunohistochemical staining the bulk of the tumor stained less intensively for HSPG than tumor vasculature, which also may play a role in the less than impressive tumor efficacy of Ad5.pK7-Δ24 if the virus gets stuck to the vasculature and does not have as ample choice for receptors in the tumor

(**figue 5g and 5h in study I**). Interestingly, there seemed to be some difference between the viruses at later time points with Ad5.pK7-Δ24 showing somewhat enhanced efficacy in comparison to Ad5-Δ24E3. Perhaps the polylysine modification binding to HSPG resulted in extended localization in the vicinity of the tumor and a subsequent re-infection of tumor cells. Any further speculations are not warranted, however, as the actual situation was not studied.

Poly(I) injection prior to virus injection was studied as a means to enhance gene transfer efficacy and subsequent viral replication and survival (**figures 4B and 4C in study II**). No signal emitted from the mice given a prior Poly(I) injection compared to mice that did not receive poly(I) (**figure 4B**). Furthermore, some mice treated with poly(I) seemed to die due to toxic effects of the substance. Perhaps this explains why no signal was obtained from the livers of the mice when imaged for luciferin. In case of extensive liver damage there might not be viable cells to express the transgene. We concluded, that poly(I) may not be the optimum compound for KC depletion. Furthermore, based on our data, it is not clear, whether KC depletion *per se* leads to enhanced gene expression in tumors after systemic delivery of adenoviruses, and whether that enhancement of gene expression would result in enhanced tumor cell killing with replicating viruses.

In the systemic treatment model of mice bearing the M4A4-LM3 cell line derived tumors in the lungs all replicating viruses showed significant survival benefit compared to no treatment (**figure 5A in study II**). Neither Ad5/3-Δ24 nor Ad5.pK7-Δ24 displayed significant survival benefit compared to Ad5-Δ24E3, the control virus with non-modified capsid. Ad5-Δ24RGD showed significant survival benefit compared to the control virus, however, which may suggest superiority of the RGD-modification as a retargeting moiety in this treatment model and CAR expressing tumors. Interestingly, Ad5-Δ24RGD previously resulted in significant survival benefit in a systemic treatment model of NSCLC tumors after showing less impressive cell killing enhancement in cell lines (Sarkioja et al. 2006).

Upregulation and activation of αvβ integrin reportedly to contribute to metastasis in human breast cancer (Felding-Habermann et al. 2001). It was also suggested that integrins are present in a nonactivated state in MDA-MB-435, the parental cell line of M4A4-LM3, but in an activated state in various *in vivo* selected cell variants, such as M4A4-LM3. These differences in integrin expression and activation may in part explain why RGD-modified virus was superior to the highly effective control virus Ad5-Δ24E3.

The relative differences between biodistribution data and the efficacy data can in part be explained by different murine models. Tumors develop in different sites, with the mammary fat pad model resembling a subcutaneous tumor model, where the tumor is somewhat “external”, and the metastatic model may better represent the disease as it is growing in the lungs or mediastinum and thereby is “within” the animal. With regard to RGD-modification this difference may play a significant role, since differences may exist in the tumor vasculature of the two models. RGD targets αvβ integrins that are induced in angiogenic vessels (Brooks et al. 1994). Their inhibition has been suggested to also inhibit pathologic angiogenesis. The possible difference in neovascularization of the models may play a role if integrin expression is different and if the lung metastasis model thereby

presents more available receptors for RGD-modified viruses. The vasculature of the tumors, however, was not studied and therefore further speculation is unwarranted.
We utilized only xenograft murine models in this study. Xenograft models allow the use of human cancer cells, which is important for transductional and transcriptional studies of adenoviruses. However, there are various limiting aspects in using xenograft murine models (discussed in chapter 6), that encourage the use of other models, such as syngeneic tumor models.

Syngeneic tumor models allow the use of immunocompetent animals. Tumors grow in endogenous stroma and display the same advantages of easy monitoring and replication of tumors as xenograft models do. Tumors are not of human origin, which is a downside to using a syngeneic model.

Syrian hamsters are an interesting alternative for murine models which are not permissive for adenovirus replication. Syrian hamster model is permissive for human adenovirus both *in vitro* and *in vivo*. Importantly, adenoviral replication occurs not only in hamster cell lines and in tumors, but also in normal tissues (Thomas et al. 2006).The permissivity for adenoviral replication allows better assessment of the safety of replicating vectors (Ying et al. 2009), as well as the effect of the immune system on the vector and on the tumor (Dhar et al. 2009).

2 Transcriptionally and transductionally targeted oncolytic adenoviruses in CD44$^+$ CD24$^{-/LOW}$ cell population

Putative breast cancer stem cells have been proposed to reside in the CD44$^+$CD24$^-$/^{LOW} cell population (Al-Hajj et al. 2003). Cancer stem cells are thought to be highly drug-resistant and subsequently to have a central role in post-treatment relapse. The drug-resistant phenotype is postulated to result from the presence of transporters that have a well-defined role in drug efflux and the maintenance of quiescence in a subset of CSCs resulting in evasion of most current treatment methods targeted to proliferative cells (Gottesman et al. 2002; Sneddon and Werb 2007; Saini and Shoemaker). Moreover, enhanced DNA repair machinery and a higher tolerance for mutations due to disrupted apoptosis machinery presumably play a role in CSC drug-resistance (Johannessen et al. 2008).

Adenoviruses infect both quiescent and proliferative cells. Genetically engineered adenoviruses also take advantage of various cancer related cellular defects to feature cancer specific and effective replication. These characteristics are likely to exist also in CSCs, making them potential targets for adenoviruses. Furthermore, adenoviruses themselves inhibit apoptosis through expression of *E3* gene products (Wold 1993), and therefore the disrupted apoptosis machinery of CSCs is perhaps irrelevant to the cytolysis elicited by adenovirus. The mechanism by which adenoviruses actually cause cytolysis is still poorly understood, but autophagy, a form of programmed cell death distinct from apoptosis, has been proposed to play a role (Ito et al. 2006; Rajecki et al. 2009). Furthermore, adenoviruses express *E3* derived adenoviral death protein (ADP) that functions late in the viral cycle to promote cell death (Tollefson et al. 1996). These

characteristics help to explain how replicating adenoviruses may be effective in killing CSCs.

2.1 Activity of tissue specific promoters in $CD44^{+}CD24^{-}/^{LOW}$ breast cancer cell population

Marker gene expression was evaluated in sorted $CD44^{+}CD24^{-}/^{LOW}$ JIMT-1 cells infected with nonreplicating, luciferase expressing viruses that featured the wt capsid, chimeric 5/3 fiber, or an RGD-modification (**figure 1B in study III**). Virus with the chimeric fiber showed the highest gene transfer efficacy, and based on this result and a previous study, where the 5/3 fiber modified virus was shown to be effective in the context of treating $CD44^{+}CD24^{-}/^{LOW}$ breast cancer cells (Eriksson et al. 2007), the 5/3 fiber was chosen as the capsid modification for the viruses constructed for further studies.

We evaluated various tissue specific promoters in $CD44^{+}CD24^{-}/^{LOW}$ breast cancer cell populations. A multidrug resistance (*mdr1*), human telomerase reverse transcriptase (*hTERT*), human α-lactalbumin (*ala*), or cyclo-oxygenase 2 (*Cox-2*) promoter was introduced into a non-replicating 5/3-chimeric adenovirus to control the expression of a marker gene *luciferace*. Sorted $CD44^{+}CD24^{-}/^{LOW}$ JIMT-1 or pleural effusion cells from breast cancer patients were infected and analyzed in order to evaluate the marker gene expression controlled by each promoter in putative breast cancer CSCs (**figure 1A in study III**). The *Mdr1* promoter showed the highest activity reaching marker gene expression levels of nearly 18% and 6% of the highly active but nonselective cytomegalovirus (*CMV*) promoter in JIMT-1 cells and pleural effusion cells. Corresponding gene expression levels were 3% and 12.8% for the *hTERT* promoter, 5.2% and 7.9% for the *Cox-2* promoter and only 0.4% and 1.8% for the *ala* promoter. In fact, the activity of *ala* promoter may be too low for successful control of adenoviruses.

To further evaluate promoter activities, we performed RT-PCR analysis to assess the expression of the genes related to each promoter (**figure 1A insert in study III**). Correlation was not perfect for the assays, suggesting that the gene expression is not solely regulated in transcriptional level. The mRNA expression level was highest for *hTERT*, which seems logical as it is known that *hTERT* gene expression is tightly regulated on the transcriptional level (Kyo et al. 2008).

These results suggested that *mdr1*, *hTERT* and *Cox-2* promoters are active in $CD44^{+}CD24^{-}/^{LOW}$ breast cencer cells, and further evaluation of the promoters in replicating viruses is warranted.

2.2 Oncolytic potency of TSP controlled adenoviruses *in vitro*

Oncolytic, capsid modified viruses have previously been shown to be effective in killing $CD44^{+}CD24^{-}/^{LOW}$ breast cancer cells (Eriksson et al. 2007). In order to avoid side effects in normal tissues, replication of oncolytic viruses needs to be tightly regulated. Also, E1A protein is expressed every time an adenovirus enters a cell, even if the E1A gene is partly

deleted. Regardless of partial deletions in the *E1A* gene, the E1A protein is toxic by itself, and therefore its expression in normal cells should be avoided.

Our hypothesis was that we would gain dual control over replication by introducing the 24 bp deletion in the *E1A* to target viruses to pRb/p16 defective cells and by inserting a tissue specific promoter (TSP) to regulate *E1A* transcription. Furthermore, by introducing a TSP in front of *E1A*, we would be able to diminish the E1A protein expression in normal cells to very low levels. In CSCs it is notable, that normal stem cells and some normal highly proliferative tissues also express hTERT (Yui et al. 1998), and therefore promoter control would not be sufficient to gain specificity. The pRb/p16 pathway is expected to be intact in normal cells, however, and thereby additional control in terms of partial E1A deletion conveys additional safety to the concept of transcriptional regulation of oncolytic viruses in normal stem cells. The normal stem cells occupy two distinct "niches", *i.e.* the quiescent and the activated niche (Sneddon and Werb 2007). These niches may be highly dynamic in nature, suggesting that stem cells from the quiescent niche act as a protective cell pool sustaining the balance between these niches, protecting the overall stem cell pool from adenoviruses.

All TSP controlled chimeric viruses were more effective in cell killing than the Ad5 wt virus in $CD44^{+}CD24^{-}/^{LOW}$ cell populations of pleural effusion samples (**figure 2a-c in study III**). In 1 out of 3 samples tested, the control virus Ad5/3-Δ24 featuring the wt *E1A* promoter, was the most effective. Impressively, in 2 out of 3 samples, the *mdr1*, *hTERT* and *Cox-2* promoter-controlled viruses displayed similar or superior oncolytic potency in comparison to Ad5/3-Δ24, with Ad5/3-mdr-Δ24 being most oncolytic. Ad5/3-mdr-Δ24 was also superior to Ad5/3-mdr-E1, which had an intact *E1A* gene. Therefore, we concluded that introducing the 24 bp deletion or TSP to the viral genome did not slow down viral replication.

In order to evaluate kinetics between cell killing by TSP controlled viruses, sorted JIMT-1 cells were infected and we performed viability assays daily (**figure 3a and b in study III**). JIMT-1 is a cell line derived from sorted $CD44^{+}$ $CD24^{-/LOW}$ pleural effusion cells (Tanner et al. 2004). Ad5/3-mdr-Δ24 and Ad5/3-hTERT-Δgp were the most effective and similar in efficacy to Ad5/3-Δ24, the highly active positive control. It was notable that 100 % of the sorted JIMT-1 cells were killed. This result suggests, that even in the presence of progenitor cells in the $CD44^{+}$ $CD24^{-/LOW}$ cell population, the TSP controlled replicating adenoviruses are effective in killing CSCs.

2.3 Oncolytic potency of TSP controlled adenoviruses *in vivo*

To assess antitumor activity of TSP controlled viruses, $CD44^{+}$ $CD24^{-/LOW}$ sorted JIMT-1 cells were injected into mammary fat pads of mice and tumors were subsequently treated intratumorally. Virus injections resulted in significantly smaller tumors when compared to mock treatments (**figure 4a in study III**). Ad5/3-mdr-Δ24 and Ad5/3Cox2LΔ24 were most oncolytic and Ad5/3-mdr-Δ24 was superior even to the control virus Ad5/3-Δ24. Interestingly, the *hTERT* promoter controlled virus was less effective than the control virus, albeit high efficacy *in vitro*.

CSCs are expected to divide in an asymmetric fashion, resulting in either two stem cells or one new stem cell and one progenitor cell that would subsequently proliferate to form heterogeneously differentiated tumor cell populations (Sagar et al. 2007). To test this further, $CD44^{+}CD24^{-/LOW}$ mammary fat pad tumors were established and treated with Ad5/3-mdr-Δ24 (active in $CD44^{+}$ $CD24^{-/LOW}$ cells *in vitro*) or Ad5/3-ala-Δ24 (not active in $CD44^{+}CD24^{-/LOW}$ cells *in vitro*) and subsequently analyzed for CD44 and CD24 expression with fluorescence activated cell sorting (FACS) (**figure 3b and c in study III**). Importantly, Ad5/3-mdr-Δ24 reduced tumor size and the total number of $CD44^{+}CD24^{-/LOW}$ cells. The mean proportion of the $CD44^{+}$ $CD24^{-/LOW}$ cells was 2.6% in the Ad5/3-mdr-Δ24 treated tumors that were smaller in volume than the mock treated tumors showing a proportion of 3.1% of $CD44^{+}CD24^{-/LOW}$ cells. Ad5/3-ala-Δ24 neither reduced tumor size nor the proportion of $CD44^{+}CD24^{-/LOW}$ cells. If the viruses were only able to kill fast dividing differentiated progenitor cells, the proportion of $CD44^{+}CD24^{-/LOW}$ cells in a smaller tumor would be higher instead of similar or smaller than in a bigger tumor. Furthermore, this result is in accordance with the *in vitro* data, where the *ala* promoter controlled virus was not effective in cell killing, whereas the *mdr1* promoter controlled virus was highly effective (**figures 2a-c and 3 a and b in study III**). The JIMT-1 cells utilized in this thesis have the appearance of epitheloid cells with variable nuclear size (Tanner et al. 2004). Stromal to epithelial interactions mediate normal breast development and the initiation and progression of breast cancer. Epithelial cells growing in 2-dimensional cultures are more susceptible to cytotoxic drugs than cells grown in 3-dimensional cultures or *in vivo* (dit Faute et al. 2002; Krause et al. 2010), emphasizing the importance of tumor microenvironment on the effectiveness of cancer therapies. Further, this also highlites the significance of the putative ability of adenoviruses to kill tumors formed by epithelial like breast CSCs.

In conclusion, the *Mdr1*, *Cox-2* and *hTERT* promoters are active in $CD44^{+}CD24^{-/LOW}$ cell populations. More importantly, 5/3-chimeric oncolytic adenoviruses featuring dual control over replication in terms of TSP and 24 bp deletion in *E1A* are effective in killing this cell population. These putative breast cancer stem cells are thought to be responsible for tumor formation, metastasis and post-treatment relapses and therefore represent the cell population that needs to be most desperately eradicated within a tumor. This approach may be interesting in a clinical context, especially for treating breast cancer patients with recurrent disease.

3 Inhibition of viral replication with pharmacological agents

The safety of adenoviral gene therapy has been validated in clinical trials with E1B-55K deleted viruses, of which ONYX-015 is probably the most widely studied (Khuri et al. 2000; Xia et al. 2004). E1B-55K deleted viruses, however, have shown somewhat attenuated replication compared to wt virus even in tumor cells and the studies of their replication specificity have given contradictory results (Goodrum and Ornelles 1998; Rothmann et al. 1998). A similar approach for replication control featuring a 24 bp deletion in the *E1A* that targets viral replication to pRb/p16 defective cells has resulted in

higher replicative potency in tumor cells (Fueyo et al. 2000; Heise et al. 2000; Kanerva et al. 2003). With higher replicative potency, however, increase in side effects is also possible, especially in immunosuppressed patients (Kojaoghlanian et al. 2003).

Our hypothesis was that the pharmacological agents apigenin and chlorpromazine could be used to inhibit viral replication in case of severe side effects. The antipsychotic agent chlorpromazine is suggested to inhibit the formation of chlatrin-coated pits which are needed for virus trafficking (Wang et al. 1993; Dimitrov 2004). Apigenin is a natural bioflavonoid that mediates cell cycle arrest to G2/M, thereby inhibiting S-phase induction required for adenoviral DNA replication (Sato et al. 1994).

3.1 Inhibition of replication *in vitro*

Ad5 wt and Ad5/3-Δ24 were used to study the effect of apigenin and chlorpromazine on viral replication in fresh human liver samples. The wt virus displayed replication over 48 hours (**figure 1A in study IV**). Interestingly, adding chlorpromazine reduced replication 8-fold. Ad5/3-Δ24 did not replicate productively in the liver samples (**figure 1B in study IV**), which is of utmost importance as Ad5/3-Δ24 is in development for human trials. Nevertheless, the marginal replication was even further attenuated by chlorpromazine at later time points. Furthermore, lower liver aspartate transaminase (AST) levels were measured, suggesting reduced hepatocyte damage in chlorpromazine treated liver samples (**figure 1E in study IV**). In non-malignant *E1*-transformed 293 cells both viruses replicated effectively and chlorpromazine reduced replication up to 1960 fold at the latest time point (48 h) (**figures 1C and D in study IV**).

The effect of apigenin and chlorpromazine on viral replication in cancer cells was studied next. Apigenin reduced replication 100 fold in adenocarcinoma Hey cells (**figures 2A and B in study IV**), but in OV-4 cells the effect was less pronounced (**figures 2C and D in study IV**). Furthermore, the close association of cell killing to replication was corroborated in a longitudinal assay (MTS), where apigenin reduced Ad5/3-Δ24 activity, albeit not statistically significantly (**figure 2E in study IV**). Chlorpromazine did not reduce replication significantly in Hey or OV-4 cell lines. This result may reflect the genetic heterogeneity between these cell lines. Differences in the activity of alternative entry mechanisms, such as caveolae, might explain the cell line specific differences in the results. Caveolin-1-deficient MDCK cells that lack caveolae display significantly reduced uptake of adenoviruses (Manninen et al. 2005). Importantly, caveolae have been suggested as an alternative entry route for a chimeric Ad5/F35 vector (Drouin et al. 2010), corroborating the possibility that cell line specific differences in alternative entry mechanisms for adenoviruses may effect our results.

Previously chlorpromazine has been shown to reduce oncolytic adenovirus DNA copy numbers in 3 to 4 orders of magnitude and also result in as high as a 4 log reduction in transgene expression in various cancer cell lines (Carette et al. 2005). The chlorpromazine levels used by Carette *et al.*, however, were 8 fold higher than the concentration we utilized.

Apigenin inhibited viral replication in cancer cells, but had a less pronounced effect in normal cells. The opposite was true for chlorpromazine. Apigenin induces G2/M phase cell cycle arrest and reduces the levels of cyclins (Ujiki et al. 2006; Meeran and Katiyar 2008). A cell arrested in G2/M will not go into mitosis and subsequently S-phase will not be induced either. S-phase induction is critical for adenoviral replication. Hey cells proliferate rapidly *in vitro*, and thus the effect of cell cycle arresting apigenin may be more clearly seen.

3.2 Inhibition of replication and toxicity *in vivo*

The use of chlorpromazine and apigenin were further studied *in vivo* in a subcutaneous ovarian cancer model. Human xenografts were used because adenoviruses do not replicate productively in mouse tissue. Both substances were found to reduce the antitumor efficacy of Ad5/3-Δ24 by inhibiting replication, with chlorpromazine displaying a 36 fold reduction and apigenin an 11 fold reduction in the production of new viruses (**figures 3A and B in study IV**). Recently, chlorpromazine has been shown to significantly inhibit viral replication in an immunocompetent syrian hamster model, which allows productive replication of adenoviruses (Diaconu et al. 2010). Replication was inhibited in tumors derived from hamster cell lines as well as in hamster livers, but the effect was significant only in the 24 hours post viral injections, which may suggest that chlorpromazine is merely slowing down the replication instead of truly inhibiting it.

To assess the general toxicity of the treatments and to follow the overall health of the mice, their body weight was followed (**figure 4A in study IV**). No weight loss occurred in treated mice and the lowest body weights were seen in mock treated mice. Furthermore, the effects of chlorpromazine and apigenin were evaluated with regard to liver toxicity in a mouse model, where a combination therapy of Ad5/3-Δ24 and gemcitabine was previously shown to cause treatment-related toxicity (Raki et al. 2005). In that study mice died due to fulminant liver necrosis caused by persistant viral replication and subsequent sustained liver damage that was aggravated by gemcitabine, a deoxycytidine analog used to treat solid tumors (Fowler and Van Le 2003). When this combination treatment was repeated here, mice succumbed to liver necrosis, foamy degeneration, and steatosis, and the surviving hepatocytes displayed large nuclei (**figure 4B in study IV**). Analysis of evaluable livers in a blinded manner revealed that apigenin and chlorpromazine reduced liver toxicity, as toxicity was more frequently seen in PBS treated mice (**figure 4C and D in study IV**).

A discrepancy between the effect of chlorpromazine in Hey cells *in vitro* and in Hey cell derived tumors exists. Ad5/3-Δ24 is a very potent virus in cell killing *in vitro*, and thereby the speed of its replication may become counteractive to packaging new virions. The cells might be lysed before the maximum number of progeny virions is produced and slowing the speed of replication may not be seen as reduction of *in vitro* virion production. In tumors, the rapid replication and viral release might actually improve tumor penetration and viral dissemination, and in this setting the effect of chlorpromazine may be more easily seen. As previously mentioned, the results *in vitro* do not always predict results *in*

vivo. Although the subcutaneous tumor model is constrained as a representation of the disease, it is still more truthful than any setting *in vitro*, and therefore we feel that the results *in vivo* give a better interpretation of the effect of the drugs.

In this study, chlorpromazine and apigenin were delivered to cells and animals before any toxic events were evident. If chlorpromazine and apigenin were used in patients, the drugs would be delivered only after replication-associated toxicities were encountered. This might effect the outcome of the use of these compounds, *i.e.* they might not have strong enough effect in case viral replication had already proceeded to such levels that toxic effects were indeed encountered. Thus the timing of drug delivery should be looked at more carefully in case the use of these compounds as a safety switch was contemplated further.

4 Noninvasively imagable murine models of breast cancer

It is generally accepted that the tissue environment in which malignant cells reside affects tumor growth and response to therapy (Mueller and Fusenig 2004). Many proteins that cells express, both *in vitro* and *in vivo*, are pro survival only in the tissue context. For example, transforming growth factor β (TGFβ) expression has been shown to protect malignant cells from cytotoxic therapies in mice xenografts, but not *in vitro* (Teicher et al. 1997). Various physical barriers to adenoviral cancer therapy exist in live animals, which hinder the target tissue transduction, tumor penetration, and viral replication. Interactions between tumor and matrix cells, signaling between cells residing in different niches within the tumor, and the formation of central hypoxia within the tumor are poorly reflected by *in vitro* 2-dimensional studies (Fischbach et al. 2007), and undoubtedly affect the gene transfer efficacy and spread of adenoviruses *in vivo*. Therefore, any results obtained *in vitro* have to be considered preliminary and further studies need to be conducted *in vivo* in order to have a better view on efficacy as well as treatment safety (comparison between most *in vitro* and *in vivo* models is represented in table 4).

We developed two murine models of breast cancer that featured green fluorescent protein (GFP) expressing M4A4-LM3 metastatic breast cancer cells inoculated either in the left lung or in mediastinum to represent disseminated disease or orthotopically in the mammary fat pad (see figure 6 for a schematic presentation of the models). The tumor growth was followed by measuring with a caliber and by imaging the GFP expression with Xenogen IVIS 100 imaging system that can detect the light emitted by GFP excitation.

Tumor growth in the orthotopic model was easily followed by fluorescence imaging, and the results were well in accordance with the physical measurements (**figures 3A and B in study I**). A significant correlation emerged between the photon emission and tumor size in the orthotopic model (**figure 3C in study I**). In the systemic treatment model with the tumor growing in lungs, however, no significant differences existed between treatment groups in mean fluorescence values over time, although a significant difference in survival between different treatment regimes occurred (**figure2 5A and B in study II**). GFP is a surface weighted molecule, *i.e.* the deeper the signal is emitted from, the weaker it is and

the more background signals from autofluorescing particles there will be (Hoffman 2002). Therefore, GFP may not be the optimum choice for deep-tissue imaging, although it is sufficient for imaging objects near the surface (**figures 3A, B and D in study II**).

Table 4. Comparison between in vitro and in vivo models used in this study.

Model	Study objectives	Advantages	Disadvantages
In vitro cancer cell lines	* Viral gene transfer efficacy * Viral cell killing potential * Promoter activity * Receptor expression * The effect of pharmaceutical agents on viral replication	* Easy production and maintenance of study material * Various different cell lines and cancer types available * Various available study methods	* 2-dimensional system * selection of characters that promote survival *in vitro* * Lack of host surroundings and interactions such as immune system and blood circulation
In vitro patient tissue samples	* Viral gene transfer efficacy * Viral cell killing potential	* Fresh samples without prior *in vitro* cultivation * 3-dimensional structure	* Lack of host surroundings and interactions * Difficulty of maintenance
In vivo orthotopic mfp model	* Viral gene transfer efficacy * Viral cell killing potential after local and systemic injections, effect on survival * non-invasive imaging of tumor progression	* Tumors with vasculature, blood flow and naturally occurring barriers such as stromal cells * Tumors easy to replicate * Tumors easy to measure and image * Human tumors * Systemic and local delivery possible	* Immunocompromised mice * Mouse tissue non-permissive for viral replication * Tumor initiation usually requires a considerable number of cells (CSCs can be an exception) * Only some cancer cell lines form tumors
In vivo lung model	* Viral cell killing potential after systemic injections, effect on survival * non-invasive imaging of tumor progression	* Tumors with vasculature, blood flow and naturally occurring barriers such as stromal cells * Human tumors * Systemic and local delivery possible * Better resemblance of disseminated disease	* Disadvantages as above * Difficulty of tumor inoculation * Physical tumor measurements impossible * Imaging with GFP difficult
In vivo orthotopic ovarian cancer model	* The effect of pharmaceutical agents on viral replication	* Advantages as in lung model	* Disadvantages as in mfp model * Physical tumor measurements impossible

Importantly, murine models suffer from various drawbacks. Mouse tissues are not permissive for adenoviral replication (Ginsberg et al. 1991), preventing reliable

assessment of replication related toxicity *in vivo*. Mice used in xenograft models are immunocompromised, and therefore studies assessing the effect of immune defence mechanisms on safety and efficacy of adenoviral vectors are impossible. In addition, biodistribution studies that would span a longer period than just a few days and would give a better view on the behavior of replicating agent after systemic delivery cannot be performed. Therefore, permissive immunocompetent animal models, such as the Syrian hamster model (Thomas et al. 2006), might be a superior model for preclinical *in vivo* studies with replicating adenoviruses, though immunocompetent models do not permit the use of human tissue.

SUMMARY AND CONCLUSIONS

We evaluated various genetic modifications for controlling transduction and transcription of replicating adenoviruses with the overall goal being improvement of efficacy and safety of oncolytic virotherapy. With this goal in mind, we studied various capsid modified adenoviruses for their gene transfer potential *in vitro* and saw dramatic improvements compared to viruses utilizing the primary Ad5 receptor, CAR. Improved gene transfer efficacy translated into enhanced cell killing potential *in vitro*. As predicted based on literature, the situation *in vivo* proved to be much more complicated. Capsid modifications did not significantly change the viral biodistribution profile after intravenous delivery, though a trend towards less liver transduction was seen. Clearly, our results with xenograft murine models show that the use of permissive and immunocompetent animal models is recommendable for preclinical studies with replicating adenoviruses. Nevertheless, capsid modified oncolytic viruses showed efficient antitumor activity, and the treatment with Ad5-Δ24RGD resulted in significant survival benefit compared to the positive control virus in a highly aggressive model of disseminated disease.

The 5/3 serotype chimeric capsid modification proved to be efficient in gene transfer in putative CSCs. Furthermore, various TSPs were successfully utilized in this context with *Mdr1*, *hTERT* and *Cox-2* promoter controlled viruses showing even more efficient cell killing of $CD44^{+}$ $CD24^{-/LOW}$ breast cancer cell populations *in vitro* than the highly active positive control virus Ad5/3-Δ24. TSP controlled viruses were able to kill all sorted pleural effusion cells from breast cancer patients as well as sorted pleural effusion derived JIMT-1 cells. More importantly, TSP controlled viruses were able to reduce the size of tumors derived from $CD44^{+}CD24^{-/LOW}$ cells, as well as the total number of $CD44^{+}CD24^{-/LOW}$ cells. This result is encouraging, as the putative CSCs are thought to be responsible for post-treatment relapses due to various characteristics that make them insensitive to conventional therapies.

When highly potent oncolytic adenoviruses are used in patients, it is beneficial to have an additional safety switch in case of replication related adverse effects. An antipsychotic chlorpromazine and a natural bioflavonoid apigenin were evaluated for their ability to inhibit viral replication *in vitro* and *in vivo*. Both substances inhibited replication of Ad5/3-Δ24 *in vitro*, but had different activities in cancer and normal cells, probably reflecting their different mechanisms of action. Furthermore, both substances inhibited viral replication *in vivo* and decreased treatment related toxicity in a murine ovarian cancer model.

In conclusion, various capsid modifications can be used to gain expanded tropism of oncolytic adenoviruses and to circumvent their dependence on CAR. Furthermore, TSPs and short genomic deletion can be utilized to gain efficient viral replication in target cells, and oncolytic adenoviruses featuring all these genetic modifications are able to kill putative CSCs. An additional safety switch is achieved with pharmacologic inhibition of replication. Overall, oncolytic adenoviruses represent a powerful addition to the treatment regime for cancer and may prove to be beneficial in the treatment of relapsed disease.

ACKNOWLEDGEMENTS

This work begun in 2003 and was carried out in Biomedicum Helsinki, in Cancer Gene Therapy Group (CGTG). During these years many people have participated in this project or have otherwise helped me finish my work and for that are owed my sincerest gratitude.

I am grateful to the chairman of the Transplantation Laboratory and the Dean of the Medical Faculty, Professor Risto Renkonen, the former chairman of the Transplantation Laboratory, Professor Pekka Häyry, the directors of the Molecular Cancer Biology Program, Academy Professor Kari Alitalo and Professor Jorma Keski-Oja, the head of the Haartman Institute, Seppo Meri, the head of FIMM, Professor Olli Kallioniemi, and the head of Biomedicum Helsinki and Faculty Research Programs, Olli Jänne, for providing excellent research facilities. I also wish to thank Päivi Ojala and Tomi Mäkelä, the current and former Deans of the Helsinki Biomedical Graduate School, for maintaining an excellent school for graduate students.

I am most grateful to my supervisor, Research Professor Akseli Hemminki, MD PhD, for leading me to the fascinating world of adenoviruses, for providing me endless supply of ideas and showing such enthusiasm and passion for this work, that one cannot help but be nearly equally enthusiastic. Most importantly, I wish to thank Akseli for giving me a chance.

I also wish to thank my other supervisor, Docent Anna Kanerva, MD PhD, for all the help she has provided me during this research and for being such a down-to-earth supervisor.

Docent Aki Manninen, PhD, and Docent Johan Lundin, MD PhD, are most warmly thanked for their thorough revision of my thesis and their valuable comments on it. I also thank Shannon Kuismanen, PhD, for the linguistic revision of this thesis.

Ari Ristimäki, Pirjo Laakkonen, Steve Goodison, Renee A Desmond, Karl Von Smitten, Aija Helin, Ulf-Håkan Stenman, Helena Isoniemi, Krister Höckerstedt, Marius Porten, Isabell Herrmann, Pekka Virkkunen, Maija Tarkkanen and Daniel Rein are thanked for collaboration and their contributions to this work.

My sincere gratitude is owed to the whole CGTG-team, present and former members – you all know who you are – for creating such a fun and warm working environment. Without the refreshing coffee breaks and discussions with topics ranging from the state of the world to fake lashes one would not have the energy to do science. Special thanks go to the technicians, Eerika, Aila, Saila, and Kikka, for taking good care of us all, and to Mari, Merja and Lotta, who have travelled through this all with me since day one.

I wish to thank my friends outside the lab for reminding me to take a break from time to time. I especially want to thank Jenni for always being so interested – in everything, really.

I thank Pirjo and Pertti for helping in day-to-day life – your help has made many practical dilemmas seem much easier to conquer.

My deepest gratitude is owed to my family: to my parents for always believing in me, for supporting and loving me and being there for me, and to my sister's family for love and support and a place to crash. And most importantly, to Ari, my beloved husband and companion, for his endless support, love, and trust that keep me going, and to my little girls, Venla and Siiri, who make my life complete.

For the financial support I wish to acknowledge Helsinki Biomedical Graduate School, Sigrid Juselius Foundation, Emil Aaltonen Foundation, HUCH Research Funds (EVO), the K. Albin Johansson Foundation, the Finnish Cancer Organization, the Breast Cancer Group and Farmos Research and Science Foundation.

Helsinki, November 2010

Tuuli Ranki

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