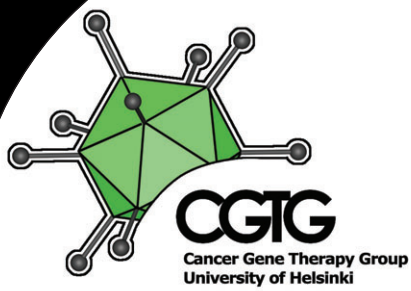

GENETICALLY ENGINEERED ONCOLYTIC ADENOVIRUSES FOR THE TREATMENT OF KIDNEY AND BREAST CANCER

Kilian Guse

GENETICALLY ENGINEERED ONCOLYTIC ADENOVIRUSES FOR THE TREATMENT OF KIDNEY AND BREAST CANCER

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ACADEMIC DISSERTATION

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“People love chopping wood. In this activity one immediately sees results.”

- Albert Einstein

To my parents

Table of Contents

PART A

i. List of original publications

The thesis is based on the following original publications, which are referred to in the text by their roman numerals.

I. Guse K, Dias JD, Bauerschmitz GJ, Hakkarainen T, Aavik E, Ranki T, Pisto T, Särkioja M, Desmond RA, Kanerva A, Hemminki A.
Luciferase imaging for evaluation of oncolytic adenovirus replication *in vivo*.
Gene Ther. 2007 Jun;14(11):902-11.

II. Guse K, Ranki T, Ala-Opas M, Bono P, Särkioja M, Rajecki M, Kanerva A, Hakkarainen T, Hemminki A.
Treatment of metastatic renal cell cancer with capsid modified oncolytic adenoviruses.
Mol Cancer Ther. 2007 Oct;6(10):2728-36.

III. Guse K, Diaconu I, Rajecki M, Sloniecka M, Hakkarainen T, Kanerva A, Pesonen S, Hemminki A.
Ad5/3-9HIF-Δ24-VEGFR-1-Ig, an infectivity enhanced, dual-targeted and antiangiogenic oncolytic adenovirus for treatment of renal cell cancer.
Accepted for publication in *Gene Therapy*.

IV. Eriksson M, Guse K, Bauerschmitz G, Virkkunen P, Tarkkanen M, Tanner M, Hakkarainen T, Kanerva A, Desmond RA, Pesonen S, Hemminki A.
Oncolytic Adenoviruses Kill Breast Cancer Initiating $CD44^{+}CD24^{-/low}$ cells.
Mol Ther. 2007 Dec;15(12):2088-93.

ii. Abbreviations

5-FU	5-fluorouracil
Ad	adenovirus
bp	base pair
CAR	coxsackie-adenovirus receptor
CD	cytosine deaminase
Cox-2	cyclooxygenase-2
CBGr	click beetle green
CBr	click beetle red
CMV	cytomegalovirus
CR	constant region
CRAd	conditionally replicating adenovirus
CTL	cytotoxic T-lymphocytes
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
GCV	ganciclovir
HCC	hepatocellular carcinoma
HSV-TK	herpes simplex thymidine kinase
i.ha.	intrahepatic artery
i.p.	intraperitoneal
i.t.	intratumoral
ITR	inverted terminal repeat
i.v.	intravenous
LacZ	β-galactosidase
luc	luciferase
MAP	mitomycin C + doxorubicin + cisplatin
MOI	multiplicity of infection
NF-κB	nuclear factor κB
oct4	octamer-4
pfu	plaque forming unit
pK	polylysine
Rb	Retinoblastoma
RGD	arginine-glycine-aspartic acid
SCCHN	squamous cell carcinoma of the head and neck

sox2	sex determining region Y box 2
TCID50	tissue culture infective dose 50
VEGF	vascular endothelial growth factor
vp	virus particle

iii. Abstract

Metastatic kidney and breast cancer are devastating diseases currently lacking efficient treatment options. One promising developmental approach in cancer treatment are oncolytic adenoviruses, which have demonstrated excellent safety in many clinical trials. However, antitumor efficacy needs to be improved in order to make oncolytic viruses a viable treatment alternative. To be able to follow oncolytic virus replication *in vivo*, we set up a non-invasive imaging system based on coinjection of a replication deficient luciferase expressing virus and a replication competent virus. The system was validated *in vitro* and *in vivo* and used in other projects of the thesis. In another study we showed that capsid modifications on adenoviruses result in enhanced gene transfer and increased oncolytic effect on renal cancer cells *in vitro*. Moreover, capsid modified oncolytic adenoviruses demonstrated significantly improved antitumor efficacy in murine kidney cancer models. To transcriptionally target kidney cancer tissue we evaluated two hypoxia response elements for their usability as tissue specific promoters using a novel dual luciferase imaging system. Based on the results of the promoter evaluation and the studies on capsid modifications, we constructed a transcriptionally and transductionally targeted oncolytic adenovirus armed with an antiangiogenic transgene for enhanced renal cell cancer specificity and improved antitumor efficacy. This virus exhibited kidney cancer specific replication and significantly improved antitumor effect in a murine model of intraperitoneal disseminated renal cell cancer. Cancer stem cells are thought to be resistant to conventional cancer drugs and might play an important role in breast cancer relapse and the formation of metastasis. Therefore, we examined if capsid modified oncolytic adenoviruses are able to kill these cells proposed to be breast cancer initiating. Efficient oncolytic effect and significant antitumor efficacy on tumors established with breast cancer initiating cells was observed, suggesting that oncolytic adenoviruses might be able to prevent breast cancer relapse and could be used in the treatment of metastatic disease.

In conclusion, the results presented in this thesis suggest that genetically engineered oncolytic adenoviruses have great potential in the treatment of metastatic kidney and breast cancer.

PART B

1 REVIEW OF THE LITERATURE

1.1 Introduction

Cancer is a devastating disease, which is still mostly incurable especially in advanced stages when metastatic. Researchers all over the world are investigating new approaches in an effort to treat cancer patients more effectively, and this has led to many promising preliminary results. However, the great breakthrough is still to come.

Surgery had been the predominant form of cancer treatment until radiotherapy was introduced in the early 1900s and chemotherapy in the 1950s. Only in the past 20 years more efficient and less toxic treatments such as targeted therapies (small molecule inhibitors and monoclonal antibodies including angiogenesis inhibitors) and immunotherapy have been developed.

The role of viruses in cancer treatment has a long history. Already from the mid 1800s on several case reports appeared describing tumor regressions in coincidence with natural virus infections (Bierman et al., 1953; Dock, 1904; Pelner et al., 1958). Based on these observations oncolytic Hepatitis B virus (Hoster et al., 1949), West Nile virus (Egypt 101)(Southam and Moore, 1952), adenovirus (Huebner et al., 1956) and many other viruses were enthusiastically administered to cancer patients in early clinical trials performed in the 1940s and 1950s and anticancer efficacy could be observed. However, severe and sometimes fatal side effects were also seen. In the 1970s and 1980s the number of reported clinical trials employing oncolytic viruses fell dramatically. Among other issues and considerations it became apparent that a high dose application of non-attenuated replicating viruses can cause uncontrollable side effects posing a danger to patients and

therefore it would be difficult to gain regulatory approval for these agents (Kelly and Russell, 2007).

With the rapid development of modern biotechnology in the 1980s it became possible to genetically engineer viruses. In the last 15 years researchers attenuated wild type viruses by deleting or mutating those virus genes necessary for replication (Kelly and Russell, 2007). Besides replication deficient viruses as gene transfer vectors, oncolytic viruses featuring cancer selective replication were generated to improve the safety of virotherapeutics (Bischoff et al., 1996). Other approaches to render oncolytic viruses more cancer selective include the employment of cancer tissue specific promoters to drive viral genes necessary for replication and genetic modifications of the viral capsid to make virus transduction more preferential for tumor tissue (Liu and Kirn, 2008). In recent years more than 50 phase I or II clinical trials have been conducted with different engineered oncolytic viruses (Kelly and Russell, 2007) and one phase III trial has been published with the oncolytic adenovirus H101 (Xia et al., 2004). The Chinese regulatory agencies subsequently granted market approval for H101 to be used in combination with chemotherapy for the treatment of head and neck cancers, making H101 the first approved oncolytic virus product ever worldwide.

Oncolytic viruses have shown encouraging safety results in clinical trials over the past decade. However, antitumor efficacy as a single agent was limited (Liu et al., 2007) for a number of reasons. Thus, to overcome obstacles towards efficacious virotherapeutics novel virus constructs with improved antitumor efficacy and further enhanced cancer selectivity have to be generated.

1.2 Cancer

Cancer is a global life threatening disease with an estimated 10.9 million new cases and 6.7 million deaths worldwide in 2002 (Parkin et al., 2005) being the second most common cause of death in developed countries and the fourth most common worldwide. Moreover, it is

estimated that global cancer rates will double by 2020 unless preventive measures are adopted (Eaton, 2003).

1.2.1 Renal Cell Cancer

In 2002 renal cell cancer (RCC) accounted for more than 100,000 deaths worldwide and more than 200,000 new cases were diagnosed during the same year (Parkin et al., 2005). Histological classification divides RCC into four different subtypes: clear cell, papillary, chromophobe and collecting duct. Clear cell RCC which make up 70-80% of the cases, is the most common form of kidney cancer and it is also the most aggressive subtype (Amin et al., 2002).

RCC presents with about 30% metastatic cases at initial diagnosis (Levy et al., 1998) and another 30% of initially organ confined cases develop metastasis during follow-up (Uchida et al., 2002). The median survival for patients with metastatic RCC is 10-12 months (Motzer et al., 1999).

1.2.1.1 Molecular mechanisms of renal cell cancer

Carcinogenesis usually involves a series of mutations in the genome of normal cells, ultimately transforming them into cancer cells. In most cancers, mutations in tumor suppressor genes (*p53, retinoblastoma (Rb)* and others) and proto-oncogenes (e.g. *ras* family genes) are found. Mutated proto-oncogenes can become oncogenes, which are factors that lead to increased cell survival by promoting cell propagation, inducing loss of the ability to undergo apoptosis and other mechanisms. Altered tumor suppressor genes lead to loss of cell cycle control, a requisite for carcinogenesis. Whereas most cancers feature mutations in the *p53* and *Rb* pathways, the predominantly mutated tumor suppressor gene in kidney cancer is *Von-Hippel-Lindau* (*VHL*) (Shuin et al., 1994). In fact, it has been shown that a total of 40-80% of sporadic clear cell carcinomas are linked to biallelic VHL inactivation (Kaelin, 2004). The VHL gene product, pVHL, has multiple functions, but the one that has been most

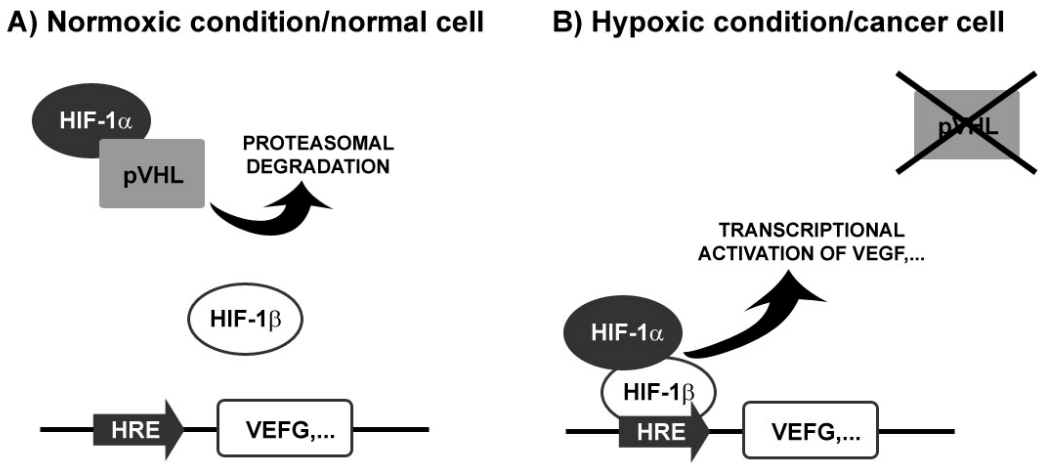

Figure 1:

Under normal oxygen supply (normoxic condition) in normal cells pVHL binds to HIF-1α and the complex is degraded at the proteasome (**A**). When oxygen supply is insufficient (hypoxic condition) or in renal cancer cells with defective pVHL degradation of HIF-1α does not take place (**B**). HIF-1α heterodimerizes with HIF-1β and the complex act as a transcription factor on hypoxia response elements (HRE) which activates expression of VEGF and other factors.

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extensively studied relates to the regulation of the transcription factor hypoxia inducible factor (HIF) (Kaelin, 2004). HIF is a heterodimer that consists of an unstable α-subunit (HIF-1α) and a stable β-subunit (HIF-1β). Under normal conditions (in the presence of oxygen) pVHL binds to HIF-1α which leads to proteasomal degradation of the complex (**Figure 1**). In cells that lack functional pVHL, or that are exposed to low oxygen (hypoxia), HIF-1α accumulates and binds to the HIF-1β partner protein (Maxwell et al., 1999). This HIF heterodimer binds to specific DNA sequences, called hypoxia response elements (HRE), and transcriptionally activates genes involved in acute or chronic adaptation to hypoxia, such as vascular endothelial growth factor (VEGF) and other mitogenic factors (TGFα, TGFβ, cyclin D, etc.) (Kaelin, 2004). In renal cell cancer with non-functional pVHL, HIF permanently activates HREs independently from the oxygen supply. The expressed HRE regulated proteins significantly contribute to carcinogenesis and tumor growth.

1.2.1.2 Angiogenesis in kidney tumors

Tumors need to build up a vast network of blood vessels to be sufficiently supplied with blood (Ferrara and Kerbel, 2005). This is especially true in the case of renal cell cancer which is characterized by high vascularization, due to strong angiogenic activity (Fukata et al., 2005), mediated in part by VHL/HIF pathway defects (Kim and Kaelin, 2004). A major player in tumor angiogenesis is VEGF which was found to be highly expressed in renal cell cancers (Nicol et al., 1997). VEGF binds to fms-like-tyrosine-kinase receptor (flt-1 or VEGFR-1) and kinase domain region receptor (KDR or VEGFR-2) with high affinity (Ferrara, 1999). Moreover, the naturally occurring soluble VEGFR-1 (sFlt) also binds VEGF but does not induce vascular endothelial cells mitogenesis (Kendall et al., 1996).

Antiangiogenic therapy approaches have been shown to be effective in many cancer types but especially in kidney cancer (Escudier et al., 2007b).

1.2.1.3 Treatment options for renal cell cancer

Surgery is effective for localized RCC. However, metastatic disease poses a therapeutic problem because it is chemotherapy resistant and radiotherapy is only palliative (Godley and Kim, 2002; Longo et al., 2007). Aggressive treatment of metastatic RCC with radical nephrectomy and interleukin-2 or interferon alpha immunotherapy seems to provide survival benefit in a subset of patients, although this has not yet been shown prospectively (Pantuck et al., 2001). Recent advances in elucidating deregulated cancer pathways have led to the development of targeted therapeutics such as sorafenib, sunitinib and temsirolimus.

Sorafenib and sunitinib are broad spectrum tyrosine kinase inhibitors targeting VEGF receptors, PDGF receptors and others (Stadler, 2005). These agents are active against various types of cancer and have demonstrated significant improvements in progression free survival in phase III clinical trials with advanced kidney cancer patients (Escudier et al., 2007a; Motzer et al., 2007).

Temsirolimus is an inhibitor of mammalian target of rapamycin (mTOR) and has been shown to lead to G1 cell cycle arrest in cancer cells (Stadler, 2005). Temsirolimus has demonstrated prolonged overall survival in phase III clinical trials with advanced kidney cancer patients (Hudes et al., 2007).

Another promising agent for the treatment of kidney cancer is bevacizumab, a humanized monoclonal antibody directed against VEGF. Bevacizumab has shown significantly improved median progression free survival in two phase III clinical trials (Escudier et al., 2007b; Rini et al., 2008). Other VEGF blocking molecules, like VEGF-trap (aflibercept), are being evaluated in preclinical studies for the treatment of kidney cancer (Verheul et al., 2007).

1.2.2 Breast Cancer

Breast cancer is by far the most frequent form of cancer afflicting women (23% of all cancers) with 1.15 million new cases worldwide in 2002 (Parkin et al., 2005). Moreover, it is the leading cause of cancer mortality in women, totalling 411,000 annual deaths (Parkin et al., 2005). The 5-year survival rate for localized breast cancer is 89%, which is very favorable. However, metastasis will develop in 20-85% of diagnosed patients depending on the initial disease stage, tumor biology, and treatment strategy used (Greenberg et al., 1996). Despite extensive research, metastatic breast cancer remains essentially incurable with a median survival of 2 years (Bernard-Marty et al., 2004).

1.2.2.1 Molecular mechanisms of breast cancer

Control of cell proliferation in the normal mammary gland is steroid hormone dependent and involves complex interactions with other hormones, growth factors and cytokines. Normal cell cycle progression is also dependent on the activation of three proto-oncogenes (c-Myc, cyclin D1 and cyclin E1) that are rate limiting for the G1 to S phase transition (Butt et al., 2008). Mammary epithelial cell-specific overexpression of these oncogenes is involved in the induction of breast cancer. Besides these oncogenes, inactivation of the tumor

suppressor p53 and Rb is connected to carcinogenesis. p53 has been shown to be non-functional in approximately 30-40% of breast cancers (Oesterreich and Fuqua, 1999) and Rb was reported to be inactivated in 20-30% of the cases (T'Ang et al., 1988).

Mutations of the tumor suppressors BRCA1 and BRCA2 are involved in familial hereditary breast cancer. In fact, carriers of mutations in *BRCA1* and *BRCA2* are considered to be at high risk (30-40%) for breast and ovarian cancer (Venkitaraman, 2002).

Human epidermal growth factor receptor 2 (HER-2, also known as EGFR2 or erbB2/neu) is a member of the epidermal growth factor receptor (EGFR) tyrosine kinase family, which is involved in regulation of cell growth and proliferation. HER-2 is highly expressed in 20-30% of breast cancers, typically because of amplification of the gene, and is associated with an aggressive phenotype and a poor prognosis for breast cancer patients (Meric-Bernstam and Hung, 2006).

Estrogen is a hormone that signals cells to grow and divide which is important in the normal development of the breast but also in breast tumor development and growth (Yager and Davidson, 2006). In fact, about 80% of breast cancers are dependent on estrogen supply for growth.

1.2.2.2 Breast cancer initiating cells

The rather new theory of cancer stem cells is based on the idea that there are cancerous cells present within solid tumors that have the same or similar properties as normal stem cells (Jordan et al., 2006). The main features of these cells are capacity for self-renewal, asymmetric replication, the potential to develop into any cell in the overall tumor population, and the proliferative ability to drive continued expansion of the malignant cell population (Jordan et al., 2006). Given these features, it is thought that cancer stem cells arise by mutation or epigenetic changes from normal stem cells or progenitors (**Figure 2A**) (Cozzio et al., 2003; Krivtsov et al., 2006). These relatively rare populations of "tumor-initiating" cancer stem cells have been identified in cancers of the hematopoietic system, brain, breast and many others (Al-Hajj et al., 2003; Lapidot et al., 1994; Singh et al., 2004).

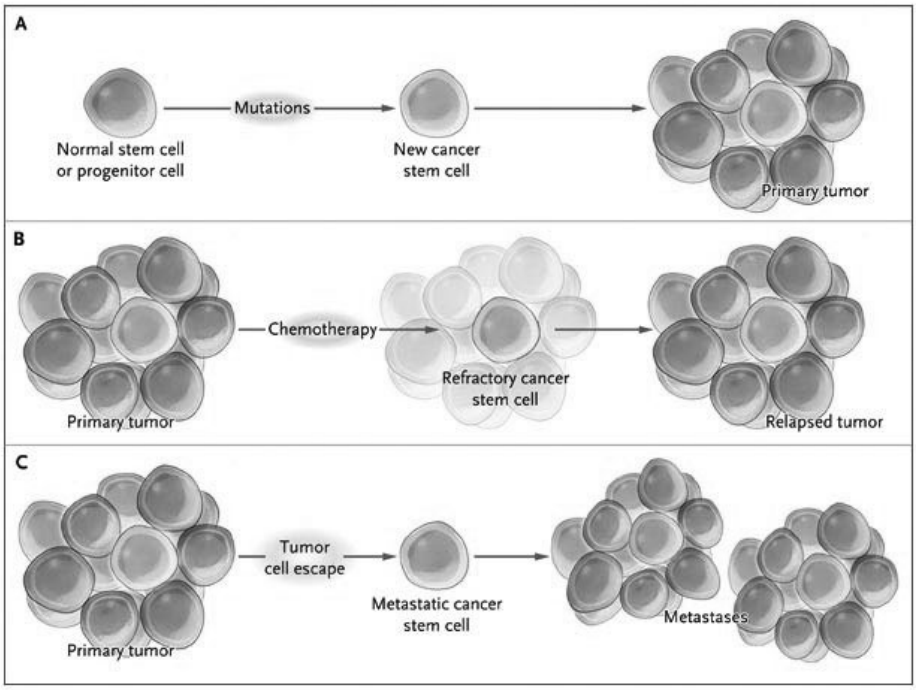

Figure 2: Scenarios Involving Cancer Stem Cells.

For tumors in which cancer stem cells play a role, at least three scenarios are possible. First, mutation of a normal stem cell or progenitor cell may create a cancer stem cell, which will then generate a primary tumor (A). Second, during treatment with chemotherapy, the majority of cells in a primary tumor may be destroyed, but if the cancer stem cells are not eradicated, the tumor may regrow and cause a relapse (B). Third, cancer stem cells arising from a primary tumor may emigrate to distal sites and create metastatic lesions (C). (Adapted from Jordan *et al*, 2006)

Since these cells might possess the ability to travel from the primary tumor to distant sites and because very low cell numbers are thought to be sufficient to form tumors, cancer stem cells could play an important role in the formation of metastases (**Figure 2C**) (Jordan et al., 2006). Furthermore, the fact that cancer stem cells self-renew themselves slowly and that they have the ability to expel chemical compounds, might make them resistant to conventional antitumor agents (**Figure 2B**) (Ischenko et al., 2008). Therefore, these cells may play an important role in cancer relapse following treatment and could be the reason for the incurable nature of metastatic breast cancer.

For many tumor types it has proven difficult to clearly identify cancer stem cell populations because of the lack of unique cellular markers. However, Al-Hajj *et al* showed that the $CD44^{+}CD24^{-/low}$ cell population found in many breast cancers exhibits stem cell characteristics, such as self-renewal and differentiation along various mammary epithelial lineages as well as resistance to conventional anti-tumor drug treatments (Al-Hajj et al., 2003; Ponti et al., 2005). In comparison to unsorted cells, a low number of $CD44^{+}CD24^{-/low}$ cells are sufficient for initiation of tumors in mice (Ponti et al., 2005). Based on these properties, the $CD44^{+}CD24^{-/low}$ cell population might harbor actual cancer stem cells (Al-Hajj et al., 2003).

1.2.2.3 Treatment options for breast cancer

Breast tumors are usually surgically removed along with sentinel lymph nodes. After surgery most breast cancer patients receive adjuvant therapy being chemotherapy, radiotherapy, endocrine therapy or biological therapy or combinations of these (Colozza et al., 2006).

Chemotherapy was proven to significantly reduce the risk for relapse and death in operated breast cancer patients (1998). Combination regimens including adriamycin, cyclophosphamide, epirubicin and taxanes are usually used.

Also radiotherapy significantly lowers the risk for relapse in operated breast cancer patients (Colozza et al., 2006).

Hormone receptor positive breast cancer patients usually undergo endocrine therapy with tamoxifen or aromatase inhibitors. Tamoxifen is a selective estrogen receptor modulator (SERM), which competes with estrogen for binding sites on the estrogen receptor. Thereby, tamoxifen blocks the cell proliferative effect that estrogen has on cancer and precancerous cells which was shown to significantly decrease relapses in breast cancers (Howell et al., 2005). Aromatase inhibitors block the enzyme aromatase, which is responsible for the conversion of androgens into estrogens. Lowering estrogen levels in this way has been proven to be effective in the treatment of breast cancer (Howell et al., 2005).

Overexpression of HER-2 on breast cancer cells, found in roughly a quarter of the patients, is associated with an aggressive phenotype and poor prognosis (Meric-Bernstam and Hung, 2006). The approved monoclonal antibody trastuzumab, directed against HER2/neu receptor, has demonstrated a 46% reduced risk for relapse in HER2 positive patients (Piccart-Gebhart et al., 2005).

1.3 Cancer Gene Therapy

Cancer is a life-threatening disease that in most cases lacks curative therapy options especially when metastatic. Therefore, researchers are investigating a variety of new treatment approaches to improve anticancer efficacy and reduce side effects. One approach, that appears promising, is 'cancer gene therapy' which can be divided into different categories:

- In one approach, missing or altered genes are replaced with their healthy 'copies'. Because some missing or altered genes (e.g. p53) are involved in carcinogenesis and tumor growth, replacing them with their intact copies may be used to treat cancer.
- Researchers are also trying to insert genes into cancer cells that make them more susceptible to chemotherapy, radiotherapy or other treatments.
- In another approach 'suicide genes' are introduced into cancer cells. A pro-drug is then given to the patient, which is converted into a toxic drug by the pro-drug converting enzyme that is produced by the suicide gene. The toxic drug then kills the cancer cells containing the 'suicide gene' and tumor cells surrounding them by the so-called bystander effect.
- Gene therapy can also be used to improve the patient's immune response to cancer.

- Furthermore, tumor angiogenesis can be inhibited with gene therapy approaches. This should deprive the tumor of sufficient blood supply and therefore inhibit cancer development.
- Finally, oncolytic viruses are another promising approach for treating cancers.

1.4 Oncolytic Viruses

Oncolytic viruses are replicating viruses that infect and lyse cancer cells. Most oncolytic viruses preferentially replicate in cancer cells, which are destroyed at the end of the replication cycle thereby releasing viral progeny that is able to infect and lyse other tumor cells. Depending on the virus species the number of released viruses can be several thousand times the number of viruses that originally infected the cell. Many different virus species have been shown to possess oncolytic properties. Among the most important oncolytic viruses, which are studied for their application in cancer therapy are: Adenovirus, herpes simplex virus, vaccinia, newcastle disease virus, reovirus, measles, mumps, west nile and vesicular stomatitis virus.

1.4.1 Adenoviruses

1.4.1.1 General virology

Adenoviruses have been extensively characterized since their initial description in the early 1950s (Rowe et al., 1953). They are generally not considered to be highly pathogenic because adenoviruses are mostly associated with self-contained respiratory infections, epidemic conjunctivitis and infantile gastroenteritis (Berk, 2006). However, in children and immune suppressed individuals, adenoviruses can cause severe infections.

The family of adenoviruses (adenoviridae) is divided into 4 genera (aviadenovirus, atadenovirus, mastadenovirus, siadenovirus) with further subdivision into subgroups A-F.

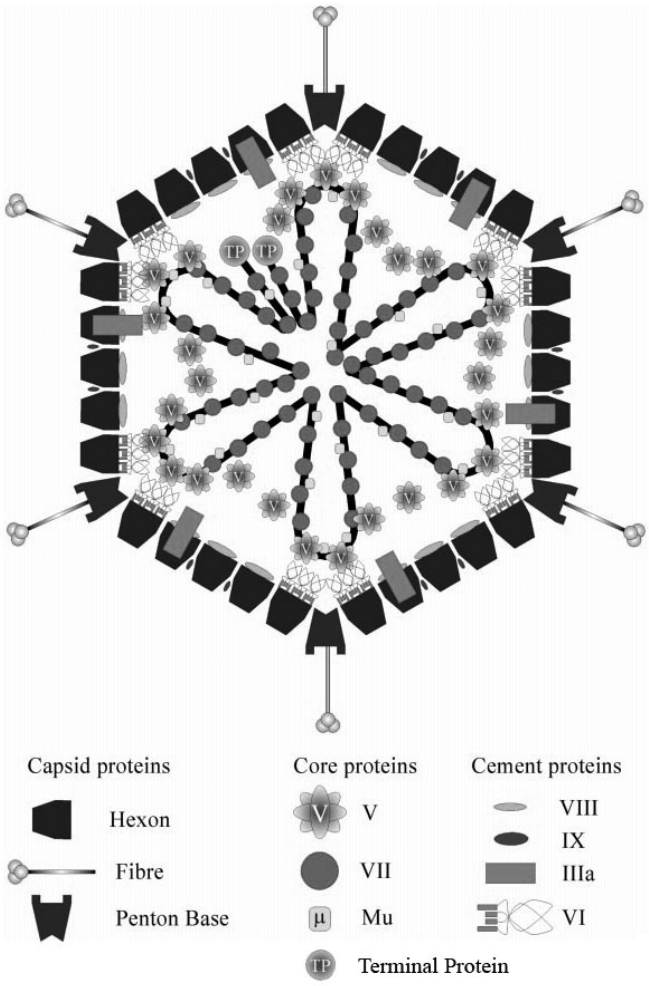

Figure 3:
Adenovirus structure; adapted from (Russell, 2000).

Division into serotypes, of which so far 51 have been identified (De Jong et al., 1999), has historically been the basis of classification (Russell, 2000).

Adenoviruses are nonenveloped icosahedral particles, approximately 90 nm in diameter, with fibers projecting from the vertices of the icosahedron. The virions contain protein (87% of mass), DNA (13% of mass) and trace amounts of carbohydrate but no lipids (Rux and Burnett, 2004). The protein fraction consists of three major proteins (hexon (II), penton base (III) and knobbed fiber (IV)) and five minor proteins (VI, VIII, XI, IIIa and IVa2) (**Figure 3**). The virus genome is a linear, double-stranded DNA with a terminal protein (TP) covalently

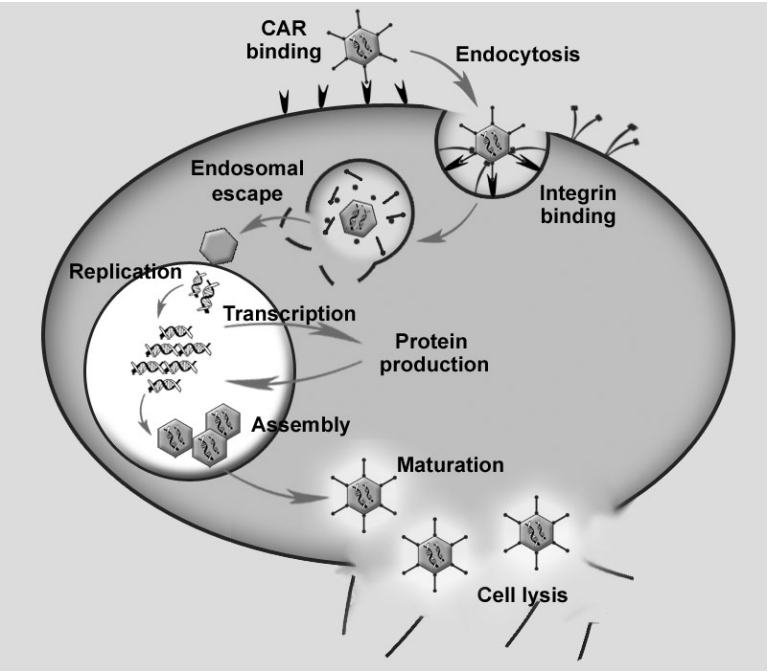

Figure 4:
Adenovirus life cycle (see text for detailed explanation)

attached to the 5' termini (Rekosh et al., 1977) which have inverted terminal repeats (ITRs). Also protein VII and the small peptide mu are directly associated with the virus DNA (Anderson et al., 1989). Protein V is packaged with this DNA-protein complex and seems to provide a structural link to the capsid via protein VI (Matthews and Russell, 1995). Furthermore, the virus contains a protease, which is necessary for processing some of the structural proteins to produce mature infectious virus.

The adenovirus infectious cycle can be divided into early and late phases. The early phase covers the entry of the virus into the host cell and the passage of the virus genome to the nucleus, followed by the selective transcription and translation of the early genes (**Figure 4**)(Russell, 2000). The entry of the virus is initiated through knob binding to specific receptors on the target cell surface. The binding receptor for adenoviruses of the subgroups A and C-F was shown to be the coxsackie-adenovirus-receptor (CAR) in *in vitro* settings (Roelvink et al., 1998). However, cell entry *in vivo* seems to be more complex but the exact mechanisms have not been completely elucidated yet. After the interaction with the primary binding receptor cellular $\alpha_v\beta$ integrins interact with the viral penton base arginine-glycine-

aspartic acid (RGD) motif, thereby initiating endocytosis in clathrin coated pits (Berk, 2006). While the virus containing endosome moves towards the nucleus, a pH change occurs which leads to partial disassembly of the virion. Eventually the endosomal membrane is disrupted and the virus particle attaches to the nuclear pore complex of the nucleus (Berk, 2006). Here, the virus DNA is injected into the nucleus and transcription is initiated.

Adenovirus transcription can be defined as a two-phase event, early and late, respectively occurring before and after viral DNA replication. Early transcription cassettes are termed E1-E4 and late transcription cassettes are divided into L1-L5 (Berk, 2006). E1 gene products can be subdivided into E1A and E1B. E1A proteins are primarily concerned with modulating cellular metabolism to make the cell more susceptible to virus replication. Cells respond to virus infections with mechanisms that invoke innate and adaptive immune response largely mediated by the transcription factor NF-κB (Berk, 2006). Moreover, infected cells may induce apoptosis via a number of routes, most importantly through p53 or retinoblastoma (Rb) pathways. E1A and E1B proteins are able to block these cellular defense mechanisms (e.g. binding of E1A to Rb) to ensure virus replication (Berk, 2006). One important factor that is released from Rb upon E1A binding is E2F inducing S-phase which is necessary for production of a range of viral proteins (Brehm et al., 1998). E2 gene products provide the machinery for virus DNA replication (Hay et al., 1995). E3 genes, which are dispensable for virus replication in tissue culture, provide a compendium of proteins that subverts the host defense mechanisms (Russell, 2000). One of these E3 gene products has been termed the adenovirus death protein (ADP) since it facilitates late cytolysis of the infected cell and thereby releases viral progeny more efficiently (Tollefson et al., 1996). The E3 gp19K is localized in the ER membrane and binds the MHC class I heavy chain thereby preventing transport to the cell surface, where it would be recognized by cytotoxic T-lymphocytes (CTLs). In addition, the gp19K protein delays expression of MHC I (Bennett et al., 1999). E4 gene products mainly facilitate virus messenger RNA metabolism (Goodrum and Ornelles, 1999) and provide functions to promote virus DNA replication and shut-off of host protein synthesis (Halbert et al., 1985). Further, they are associated with resistance to lysis by CTLs (Kaplan et al., 1999). Transcription of the late genes (L1-L5) leads to production of the virus

structural components and the encapsidation and maturation of the particles in the nucleus (Russell, 2000).

1.4.1.2 Adenoviruses as gene transfer vehicles

Genetically engineered adenoviruses based on serotype 5 are a widely used tool for gene transfer in many fields of basic research. Moreover, they hold great promise as gene therapy vectors for the treatment of various diseases (Russell, 2000).

In first generation adenoviral vectors, the E1 gene cassette is replaced with the gene of interest, which results in replication deficient viruses expressing the desired protein (Shen, 2006). These vectors are among the most commonly used gene transfer vehicles for transient gene expression in basic research (Russell, 2000) as well as for gene therapy trials (Bainbridge et al., 2008; Immonen et al., 2004; Li et al., 2007; Shirakawa et al., 2007; Stewart et al., 2006).

Second generation adenovirus vectors typically have deletions in E2 or E4 in addition to deleted E1 and E3 regions (Shen, 2006). These vectors are supposed to cause a milder host immune response and therefore transgene expression is supposed to be prolonged compared to the more immunogenic first generation vectors (Shen, 2006). Moreover, due to additional deletions in the virus genome, larger and/or more genes of interest can be inserted into second generation vectors.

To further decrease immunogenicity and increase genetic payload, helper-dependant also known as gutless adenoviral vectors have been constructed representing the third generation (Shen, 2006). In these vectors, basically all viral genes except the ITRs and the packaging signal were deleted.

1.4.1.3 Transductional targeting to cancer cells

Adenoviruses efficiently transduce a wide range of epithelial tissues. Virus tropism is mainly determined by recognition of the primary receptor, which is the coxsackie-adenovirus-

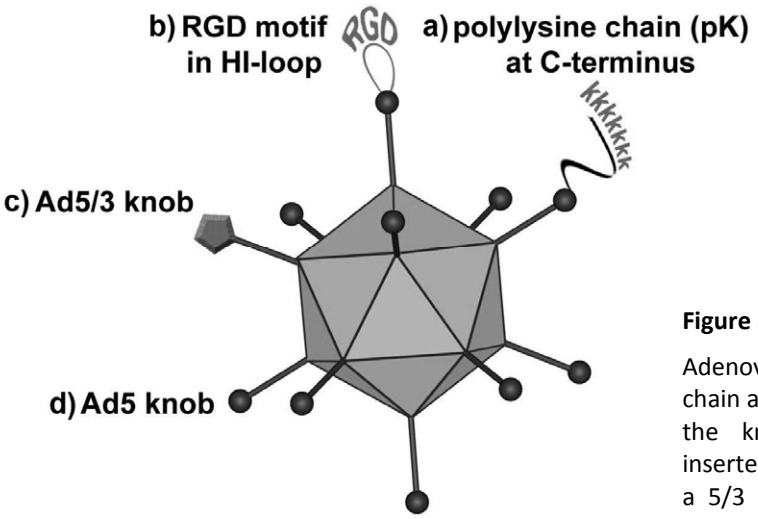

Figure 5:

Adenovirus displaying a polylysine chain attached to the C terminus of the knob (**a**), the RGD motif inserted in HI loop of the knob (**b**), a 5/3 serotype chimeric knob (**c**) and a serotype 5 wild type knob for comparison (**d**).

receptor (CAR) for the widely used serotype 5 adenoviruses (Roelvink et al., 1998). Increased CAR expression seems to inhibit growth of some cancer cell lines, while decreased CAR expression correlates with tumor progression and advanced cancer stage (Okegawa et al., 2001). Furthermore, CAR appears to play a role in cell adhesion and its expression may be cell-cycle dependent (Cohen et al., 2001). Since efficient gene transfer is the basis for successful cancer gene therapy, low CAR expression on tumor cells is a major challenge (Okegawa et al., 2001). To overcome CAR deficiency, adenoviruses can be transductionally retargeted by adapter-molecule based approaches or genetic manipulation of the virus capsid.

Adapter-molecule based targeting is based on a molecule that crosslinks the adenovirus particle with an alternative cell surface receptor. This targeting approach therefore represents a two-component system, which is a potential drawback. The stability of such two-component systems in humans is not well known and the effects the adapter molecule itself has in organisms should be studied first. A one-component system, such as genetic capsid modifications, which is more stable, might therefore be safer.

So far, three different genetic capsid manipulation strategies for retargeting adenoviruses have been developed: the so-called 'fiber-pseudotyping', ligand incorporation into the fiber knob and 'de-knobbing' of the fiber coupled with ligand addition (Glasgow et al., 2006).

Fiber-pseudotyping was first accomplished by Krasnykh *et al* who replaced the knob of a serotype 5 adenovirus with a serotype 3 knob (**Figure 5c**)(Krasnykh et al., 1996). The adenovirus 3 receptor is still disputed but CD46 (Sirena et al., 2004), CD80 and CD86 (Short et al., 2004) as well as an additional unknown receptor (Tuve et al., 2006) and heparan sulfate proteoglycans (Tuve et al., 2008) were shown to be involved in cell entry. 5/3 chimera viruses have displayed significantly enhanced transduction to tumor cells *in vitro* and *in vivo* in many types of cancer (Kanerva et al., 2002a; Kangasniemi et al., 2006; Volk et al., 2003; Zheng et al., 2007).

Other studies have revealed the feasibility of manipulating the C-terminus and the HI-loop within the fiber (**Figure 5**). Wickham *et al* (Wickham et al., 1997) added a polylysine tail to the C-terminus (**Figure 5a**) to mediate adenovirus binding to heparan sulfate proteoglycans (HSPGs), which are highly expressed on cancer cells (Matsuda et al., 2001). Adenoviruses with 7 lysine residues at the C-terminus (pK7) have demonstrated improved transduction to cancer cells (Kangasniemi et al., 2006; Ranki et al., 2007a; Stoff-Khalili et al., 2005; Zheng et al., 2007). Another promising location for incorporation of targeting moieties is the HI-loop of the knob which is exposed towards the outside and can tolerate up to 100 amino acids (Krasnykh et al., 1998). Dmitriev *et al* (Dmitriev et al., 1998) inserted an arginine-lysine-aspartic acid (RGD) motif into the HI-loop (**Figure 5b**), which resulted in enhanced infectivity of various cancer cell types (Kanerva et al., 2002b; Kangasniemi et al., 2006; Volk et al., 2003; Zheng et al., 2007). An asparagine-glycine-arginine (NGR) motif incorporated in the HI-loop also demonstrated improved adenovirus transduction to cancer cells (Mizuguchi et al., 2001). Wu *et al* combined the polylysine tail in C-terminus modification with RGD motif in the HI-loop incorporation and achieved increased transduction to CAR deficient cells (Wu et al., 2002).

Also other locations for incorporation of targeting moieties have been explored. Vigne *et al* incorporated an RGD motif into the hexon monomer protein achieving enhanced gene delivery to CAR deficient cells (Vigne et al., 1999). Furthermore, replacing the RGD motif of the penton base with receptor specific peptide motifs can target the adenovirus to different kinds of cancer tissue (Wickham et al., 1995). Also the pIX location was found to be useful for incorporating peptide motifs for retargeting or imaging purposes (Dmitriev et al., 2002; Le et al., 2004).

1.4.1.4 Transcriptional targeting to cancer cells

To achieve cancer cells selective adenoviral gene expression, transcriptional targeting can be employed. For cancer gene therapy purposes, tumor specific promoters can be used to control the expression of genes coding for peptides with antitumor activity. A vast number of tumor specific promoters have been used for cancer gene therapy (Glasgow et al., 2004). Notable examples are carcinoembryonic antigen (CEA) promoter for gastric and lung cancer (Brand et al., 1998; Osaki et al., 1994), cyclooxygenase 2 (COX-2) promoter for gastric, pancreatic and ovarian cancer (Casado et al., 2001; Wesseling et al., 2001; Yamamoto et al., 2001), and hypoxia response elements (HREs) for kidney cancer (Binley et al., 2003).

As described in chapter 1.2.1.1 most kidney cancers feature *Von-Hippel-Lindau* (*VHL*) mutations, which lead to a permanently high expression of the hypoxia inducible factor (HIF). HIF in turn acts as a transcription factor on hypoxia response elements (HRE) (Kaelin, 2004). Under non-pathogenic conditions, HIF expression can only be detected in the eye (Ashton et al., 1954). Therefore, HREs appear to be useful tissue specific promoters for targeting kidney cancer. In fact, Binley *et al* showed that an HRE was more than 1000 times more active in tumor environment when compared to normal tissue (Binley et al., 2003). Also other groups have shown renal cell cancer selective expression of genes under the control of HREs (Cuevas et al., 2003; Ogura et al., 2005).

Most researchers have used tissue specific promoters in first generation adenoviral vectors to express certain transgenes. However, this kind of transcriptional targeting can also be

applied to replicating adenoviruses, where the genes necessary for replication are placed under the control of the tissue specific promoters. Replication and oncolytic effect should then be restricted to tumor tissue thereby reducing possible toxicity. In these transcriptional targeting approaches the E1A gene cassette of oncolytic adenoviruses was placed under the control of various tissue specific promoters, such as E2F (Tsukuda et al., 2002), CXCR4 (Haviv et al., 2004) and hTERT (Hashimoto et al., 2008). Cuevas *et al* and Cho *et al* were able to generate oncolytic adenoviruses controlled by HREs and demonstrated selective replicating in kidney cancer models (Cho et al., 2004; Cuevas et al., 2003).

1.4.1.5 Targeted conditionally replicating adenoviruses for cancer therapy

Solid tumor masses are large and complex and therefore difficult to efficiently transduce with first generation, replication deficient viruses. Thus, oncolytic viruses may be more useful having the advantage of multiplying themselves inside the tumor and are therefore able to spread more efficiently and subsequently transduce more cancer cells. Transduction of cancer cells as the first step in the adenovirus life cycle will finally lead to cell lysis resulting in antitumor efficacy. However, administering wild type adenoviruses to human cancer patients might not be acceptable because of possible uncontrollable replication in healthy tissue which could lead to severe toxicity. Therefore, transductional and/or transcriptional targeting methods have to be employed. Moreover, replication can be restricted to tumor cells by deleting adenoviral genes that are necessary for replication in normal cells but not in cancer cells. Viruses that have been rendered tumor specific in this way are called 'conditionally replicating adenoviruses' or CRAds.

The first published CRAd, which was named dl1520 and is nowadays better known as ONYX-015, has two mutations in the E1B gene, which codes for the E1B-55kD protein (Bischoff et al., 1996). p53 is one of the major tumor suppressor proteins and is activated upon virus infection causing cell cycle arrest or apoptosis. To avoid this cellular shutdown, adenoviruses have evolved countermeasures in form of the expression of E1B-55kD. This protein binds and inactivates p53 leading to induction of S-phase-like state which is required for viral

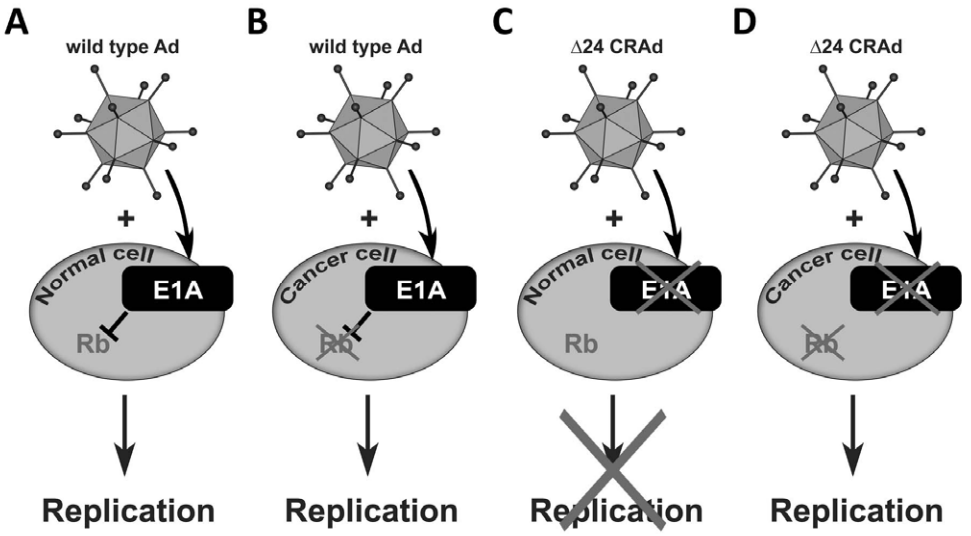

Figure 6: Mechanism for cancer cell selective replication.

Wild type adenovirus is able to replicate in normal cells since Rb is inhibited by the E1A protein (**A**). In a cancer cell with non-functional Rb replication occurs as well (**B**). A CRAd with a 24 bp deletion in E1A is unable to inhibit Rb and therefore no replication in normal cells occurs (**C**). However, a Δ24 CRAd can replicate in cancer cells with mutated Rb (**D**).

replication (Berk, 2006). Deletion of E1B-55kD will render the virus unable to replicate in normal cells since p53 will initiate cell cycle arrest or apoptosis. However, since most tumor cells have a defective p53 pathway no cellular shutdown occurs and the virus will be able to replicate (Bischoff et al., 1996). It turned out that some tumor cells fail to support replication of E1B-55kD deleted viruses. One reason might be that the E1B-55kD protein is also responsible for preventing host mRNA nuclear export and therefore the E1B mutant viruses might fail to initiate host protein shutoff (O'Shea et al., 2005).

Another strategy to create CRAds is to delete 24 bps in the constant region 2 of the *E1A*. The resulting E1A protein is not able to inactivate the function of the tumor suppressor/cell cycle regulator Rb anymore. The result is very similar to that caused by dl1520, which was described in the previous paragraph: replication is attenuated in normal cells, however, in cancer cells with mostly defective Rb pathways (Sherr, 1996) replication is unhampered

(**Figure 6**). Viruses featuring this 24 bp deletion were shown to be selective for cancer cells without losing their oncolytic potential (Fueyo et al., 2000; Heise et al., 2000).

To maximize cancer selective replication and minimize side effects, the described targeting methods can be combined. Bauerschmitz *et al* showed in a triple targeting approach that a conditionally replicating adenovirus, which in addition is transductionally and transcriptionally targeted, exhibited increased tumor cell selectivity while retaining oncolytic potency (Bauerschmitz et al., 2006).

In theory, a targeted oncolytic adenovirus would selectively replicate in the tumor cells of a cancer patient until all of them are lysed. It could spread through the system, find metastases, replicate in them and lyse them as well. Subsequently, the virus would not find any cells that allow replication anymore and therefore be cleared out from the system.

1.4.1.6 Arming approaches for enhanced antitumor efficacy

Targeted oncolytic adenoviruses have been shown to be safe in many preclinical models as well as human clinical trials (see chapter 1.5). However, treatment with virus alone has only rarely led to significant responses in patients with advanced cancers. This might be due to the complexity of large human tumors featuring stromal barriers as well as necrotic, hyperbaric, acidic and hypoxic region, which are difficult to penetrate by oncolytic adenoviruses (Cheng et al., 2007; Hay, 2005). Furthermore, ambitious approaches to restrict replication through over-stringent methods might have resulted in 'overly safe' adenoviruses that no longer have sufficient oncolytic potency to act as effective anticancer agents.

The efficacy of oncolytic adenoviruses can be enhanced by arming them with transgenes coding for therapeutic proteins. The advantage of this approach is that the expressed therapeutic protein has a different tumor cell killing mechanism than the oncolytic virus itself. Therefore, a wider range of cancer cell populations can be affected which might improve the overall antitumor efficacy.

Prodrug converting enzyme also known as 'suicide genes' have been used in several studies as therapeutic transgenes (Hermiston and Kuhn, 2002). One of the most famous suicide genes is herpes simplex thymidine kinase (HSV-TK), which converts the non-toxic drug ganciclovir (GCV) into a cytotoxic metabolite. The active metabolite can spread into surrounding cells causing the so-called cytotoxic bystander effect. Some studies have shown that GCV enhances the antitumor efficacy of HSV-TK armed oncolytic adenoviruses (Nanda et al., 2001; Raki et al., 2007). However, others have reported that activated GCV might inhibit virus replication and therefore does not augment antitumor efficacy (Hakkarainen et al., 2006; Lambright et al., 2001). Cytosine deaminase (CD) is another suicide gene that converts 5-fluorocytosine into a toxic metabolite. Oncolytic adenoviruses armed with CD have shown improved antitumor efficacy in several cancer models (Liu and Deisseroth, 2006; Zhan et al., 2005). Oncolytic adenoviruses with both HSV-TK and CD combined with radiotherapy have also shown promising results in preclinical models (Freytag et al., 1998; Rogulski et al., 2000) and in a clinical trial (Freytag et al., 2003).

Another promising approach is to arm oncolytic adenoviruses with antiangiogenic molecules since tumors are often highly vascularized and antiangiogenic therapies have demonstrated efficacy in cancer therapy (Ferrara and Kerbel, 2005). Several groups have demonstrated improved antitumor efficacy with oncolytic adenoviruses featuring different antiangiogenic transgenes that target VEGF (Yoo et al., 2007; Zhang et al., 2005).

Furthermore, oncolytic adenoviruses expressing interleukins (Lee et al., 2006; Post et al., 2007) and p53 (Idema et al., 2007; Wang et al., 2008) have shown enhanced anticancer activity.

1.5 Clinical Trials with Oncolytic Viruses

Oncolytic viruses have a long history in the treatment of cancer (Kelly and Russell, 2007) as described in chapter 1.1. Modern-era phase I-III clinical trials with non-engineered and

engineered oncolytic viruses were initiated in the 1990s using different virus species such as adenovirus, vaccinia, measles, new castle disease virus, reovirus and herpes simplex virus (Liu and Kirn, 2007). ONYX-015 (see chapter 1.4.1.5 for description of the virus) was the first targeted oncolytic adenovirus used in a phase I study resulting in 14% regression rate in head and neck cancer patients (Ganly et al., 2000). In combination with cisplatin and 5-FU, ONYX-015 resulted in tumor regression in 65% of the patients in a phase II trial (Khuri et al., 2000). A randomized phase III trial with H101 (an oncolytic adenovirus closely related to ONYX-015) in combination with chemotherapy was performed in 2004 in China reporting 79% response rate in the combination group versus 40% in the chemotherapy only group (Xia et al., 2004). The Chinese regulatory agencies subsequently granted market approval for H101 to be used in combination with chemotherapy for the treatment of head and neck cancers, making H101 the first approved oncolytic virus product ever worldwide. Other selected clinical trials with oncolytic adenoviruses are listed in **Table 1**.

Also other oncolytic viruses have shown promising results during clinical testing. In a phase I trial for metastatic melanoma JX-594, an oncolytic vaccinia virus armed with GM-CSF, resulted in 71% objective responses at injection sites (Mastrangelo et al., 1999). Moreover, 4 of 7 patients showed regressions of non-injected dermal metastasis and two patients with complete tumor eradication were disease free for at least 1.5 years. JX-594 showed also promising results in a phase I trial for hepatocellular cancer (Park et al., 2008).

In summary, clinical trials with oncolytic viruses have demonstrated excellent safety. However, antitumor efficacy greatly varied depending on treated tumor type and administration route.

Table 1: List of selected clinical trials with oncolytic adenoviruses

Virus/ treatment agents	Genetic modification	Phase	Administration route	Max. dose	Cancer type	Responses/ total number of patients	Reference
ONYX-015	E1B-55kD deletion	I	i.t.	$1x10^{11}$ pfu	SCCHN	2/22	(Ganly et al., 2000)
ONYX-015	E1B-55kD deletion	I	i.t.	$1x10^{11}$ pfu	Pancreatic cancer	0/23	(Mulvihill et al., 2001)
ONYX-015	E1B-55kD deletion	I	i.v.	$2x10^{13}$ vp	Cancer metastatic to the lung	0/10	(Nemunaitis et al., 2001a)
ONYX-015	E1B-55kD deletion	I	i.p.	$1x10^{11}$ pfu/d on 5 days	Ovarian cancer	0/16	(Vasey et al., 2002)
ONYX-015	E1B-55kD deletion	I	i.v., i.t.	$3x10^{11}$ pfu	HCC	1/5	(Habib et al., 2002)
ONYX-015 + 5-FU + leucovorin	E1B-55kD deletion	I	i.ha.	$2x10^{12}$ vp	Colorectal cancer metastatic to the liver	1/11	(Reid et al., 2001)
ONYX-015	E1B-55kD deletion	I	i.t.	$1x10^{10}$ pfu	Glioma	3/24	(Chiocca et al., 2004)
ONYX-015 + etarnercept	E1B-55kD deletion	I	i.v.	$1x10^{12}$ pfu	Advanced cancers	0/9	(Nemunaitis et al., 2007)
CV706	PSA promoter controlling E1A	I	i.t.	$1x10^{13}$ vp	Prostate cancer	5/20	(DeWeese et al., 2001)
Ad5-CD/TKrep + GCV/5-FU + radiation	E1B-55kD deletion + TK/CD transgene	I	i.t.	$1x10^{12}$ vp	Prostate cancer	15/15	(Freytag et al., 2003)
ONYX-015 + 5-FU	E1B-55kD deletion	I-II	i.t., i.ha., i.v.	$3x10^{11}$ pfu	HCC and colorectal cancer metastatic to the liver	3/16	(Habib et al., 2001)
ONYX-015	E1B-55kD deletion	II	i.t.	$2x10^{11}$ vp on 10 days	SCCHN	5/40	(Nemunaitis et al., 2001b)
ONYX-015 + cisplatin + 5-FU	E1B-55kD deletion	II	i.t.	$1x10^{10}$ vp/d on 5 days	SCCHN	19/37	(Khuri et al., 2000)

ONYX-015 + gemcitabine	E1B-55kD deletion	I-II	i.t.	$2x10^{11}$ vp/wk; 8 cycles	Pancreatic cancer	2/21	(Hecht et al., 2003)
ONYX-015	E1B-55kD deletion	II	i.v.	$2x10^{12}$ vp every 2 weeks	Metastatic colorectal cancer	0/18	(Hamid et al., 2003)
H101 + cisplatin/adriamycin + 5-FU	E1B-55kD deletion	III	i.t.	$1.5x10^{12}$ vp/d on 5 days	SCCHN	71/160	(Xia et al., 2004)
ONYX-015 + MAP chemotherapy	E1B-55kD deletion	I-II	i.t.	$5x10^{10}$ pfu	Sarcoma	1/6	(Galanis et al., 2005)

CD: cytosine deaminase; HSV-TK: herpes simplex virus thymidine kinase; i.t.: intratumoral; i.v.: intravenous; i.p.: intraperitoneal; i.ha.: intrahepatic artery; 5-FU: 5-fluorouracil; MAP: mitomycin C + doxorubicin + cisplatin; pfu: plaque forming units; SCCHN: squamous cell carcinoma of the head and neck; vp: virus particles

1.6 *In vivo* Bioluminescence Imaging

In vivo bioluminescence imaging has become a powerful tool in gene therapy and other fields of research.

Bioluminescence is defined as the production and emission of light by a living organism as the result of a chemical reaction during which chemical energy is converted into light energy. This phenomenon naturally occurs in marine vertebrates and invertebrates, terrestrial insects, mushrooms and microorganisms. Bioluminescence has been widely used in biological research. Especially firefly *luciferase*, derived from *photinus pyralis*, is commonly employed as a reporter gene in biomedical research but other *luciferases* such as click beetle red (CBr) and click beetle green (CBGr) *luciferase* can also be used. Firefly, CBr and CBGr luciferase all convert the same substrate (D-luciferin), however the light emission spectra differ from each other (**Figure 7**).

Cells transfected with *luciferase* emit light upon addition of the substrate luciferin and the light intensity correlates with the amount of luciferase mRNA present in the cell. Therefore, luciferase can be used to assess the transcriptional activity in cells that are transfected with

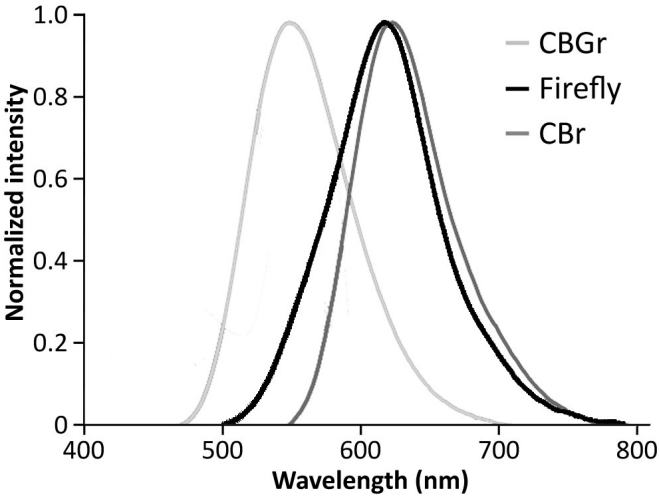

Figure 7: Emission spectra of click beetle green (CBGr), firefly and click beetle red (CBr) luciferases

a genetic construct containing *luciferase* under the control of a promoter of interest. Furthermore transfection and transduction efficiency can be evaluated with *luciferase* as a reporter gene.

Luciferase has also become a powerful technique for *in vivo* imaging purposes. Different types of cells (e.g. cancer cells) or viruses can be engineered to express luciferase allowing their non-invasive visualization inside a live animal using a sensitive charge-coupled device (CCD) camera. The main advantages over *in vivo* fluorescence imaging are the high sensitivity of bioluminescence systems and the low background of non-transfected tissue.

2 AIMS OF THE STUDY

1. To set up an imaging system for following adenovirus replication kinetics *in vivo*. (I)
2. To evaluate whether capsid modification can improve the antitumor efficacy of oncolytic adenoviruses for the treatment of kidney cancer. (II)
3. To evaluate tissue specific promoters for renal cell cancer and to generate a targeted and armed oncolytic adenovirus for enhanced selectivity and improved antitumor efficacy in kidney cancer models. (III)
4. To evaluate whether breast cancer initiating cells can be killed by oncolytic adenoviruses for improving treatment of metastatic breast cancer. (IV)

3 MATERIALS AND METHODS

3.1 Cell lines, tumor samples and isolation of breast cancer stem cells

Characteristics of the cell lines used in the studies are described in **Table 2**.

Table 2: List of human cell lines used in the studies

Cell line name	Description	Used in
293	Transformed embryonic kidney cells	I, II, III
911	Transformed embryonic retinoblasts	III
A549	Lung adenocarcinoma	I, II, III
HEY	Ovarian adenocarcinoma	I
786-O	Renal cell adenocarcinoma	I, II, III
786-O-CBGr	Renal cell adenocarcinoma stably transfected with click beetle green luciferase	III
ACHN	Renal cell adenocarcinoma	II, III
Caki-2	Renal cell carcinoma	II, III
769-P	Renal cell adenocarcinoma	II, III
Sv7tert	Renal cell carcinoma	III
SN12C	Renal cell carcinoma	III
SN12L1	Renal cell carcinoma	III
SN12L1-luc	Renal cell carcinoma stably transfected with firefly luciferase	III
FHS173WE	fibroblasts	III
HUVEC	Human umbilical vein endothelial cells	III
JIMT-1	Human breast carcinoma	IV

Cells were subcultured under recommended conditions up to a passage number of 30.

786-O-CBGr, which stably expresses click beetle green luciferase, was generated by transfection of 786-O cells with a plasmid carrying the puromycin resistance gene and the click beetle green luciferase gene and subsequent antibiotic selection of surviving cell clones.

Kidney tumor samples were obtained with signed informed consent and ethical committee approval from patients undergoing surgery at Helsinki University Central Hospital.

Breast cancer pleural effusion samples were obtained (with ethics committee approval and after obtaining an informed consent) directly from thoracocentesis and washed with Dulbecco's modified Eagle's medium-F12 supplemented with 10 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.4% bovine serum albumin (all from Sigma, St. Louis, MO). Cells from pleural effusion samples and JIMT-1 cells were sorted with fluorescein isothiocyanate–labeled anti-CD44 and phycoerythrin-labeled anti-CD24 antibodies (BD Pharmingen, Franklin Lakes, NJ), which were collected with fluorescein isothiocyanate- and phycoerythrin-conjugated magnetic beads, respectively (Miltenyi Biotech, Bergisch Gladbach, Germany). The collected cell populations were confirmed to be CD24 negative and CD44 positive by flow cytometry. Both unsorted and $CD44^{+}CD24^{-/low}$ living cell populations were stained with Hoechst 33342 (5 μg/ml; Sigma, St. Louis, MO) at 37 °C, mounted on glass slides and viewed under a fluorescence microscope.

3.2 Adenoviruses

3.2.1 Replication deficient viruses (I, II, III, IV)

Main features of the replication deficient adenoviruses used in the studies are described in **Table 3**.

For large scale production, adenoviruses were amplified on 293 cells and purified on double cesium chloride gradients. Virus particle (vp) concentrations were assessed by measuring absorbance at 260 nm and plaque forming unit titers were determined with standard TCID50

assay on 293 cells. The presence of inserted genes and absence of wild type virus was confirmed by PCR and sequencing.

Table 3: List of replication deficient adenoviruses used in the studies

Virus name	E1 *	Fiber	Used in	Reference
Ad5luc1	Luciferase	Wild type serotype 5	II, III, IV	(Kanerva et al., 2002a)
Ad5/3luc1	Luciferase	5/3 serotype chimerism	I, II, III	(Kanerva et al., 2002a)
Ad5lucRGD	Luciferase	RGD motif in HI loop	II	(Dmitriev et al., 1998)
Ad5(GL)	GFP + luciferase	Wild type serotype 5	II	(Wu et al., 2002)
Ad5.pK7(GL)	GFP + luciferase	7 lysine residues at C-terminus	II	(Wu et al., 2002)
Ad5.RGD.pK7 (GL)	GFP + luciferase	RGD motif in HI loop and 7 lysine residues at C-terminus	II	(Wu et al., 2002)
Ad5LacZ	LacZ	Wild type serotype 5	II	(Yotnda et al., 2004)
Ad5pK21-LacZ	LacZ	21 lysine residues at C-terminus	II	(Yotnda et al., 2004)
Ad5-9HIF-luc	Luciferase under control of 9HIF promoter	Wild type serotype 5	III	Study III
Ad5-OB36-luc	Luciferase under control of OB36 promoter	Wild type serotype 5	III	Study III

* The marker genes in E1 are under control of the CMV promoter if not stated otherwise. The luciferase gene in these viruses codes for the firefly luciferase enzyme.

3.2.2 Replication competent adenoviruses (I, II, III, IV)

Main features of the replication competent adenoviruses used in the studies are described in **Table 4**.

For large scale amplification, adenoviruses were amplified on A549 cells and purified on double cesium chloride gradients. VP concentrations were assessed by measuring absorbance at 260nm and plaque forming unit titers were determined with standard TCID50

assay on 293 cells. Presence of inserted genes and absence of wild type virus was confirmed by PCR and sequencing.

Table 4: List of replication competent adenoviruses used in the studies

Virus name	E1	E3	Fiber	Used in	Reference
Ad300wt	Wild type	Wild type	Wild type serotype 5	I, II, III, IV	ATCC[1]
Ad5-Δ24E3	24 bp deletion[2]	Wild type	Wild type serotype 5	I, II, IV	(Kanerva et al., 2003)
Ad5/3-Δ24	24 bp deletion[2]	Wild type	5/3 serotype chimerism	I, II, III, IV	(Kanerva et al., 2003)
Ad5-Δ24RGD	24 bp deletion[2]	Wild type	RGD motif in HI loop	I, II, IV	(Suzuki et al., 2001)
Ad5.pK7-Δ24	24 bp deletion[2]	Wild type	7 lysine residues at C-terminus	I, II, IV	(Ranki et al., 2007a)
Ad5luc3	Wild type	Luciferase under control of CMV promoter	Wild type serotype 5	I	(Krasnykh et al., 1996)
Ad5/3cox2LE1	Cox-2 promoter	Wild type	5/3 serotype chimerism	I	(Bauerschmitz et al., 2006)
Ad5/3cox2Ld24	Cox-2 promoter and 24 bp deletion[2]	Wild type	5/3 serotype chimerism	I	(Bauerschmitz et al., 2006)
Ad5/3cox2Ld2d24	Cox-2 promoter, 2 bp[3] and 24 bp deletion [2]	Wild type	5/3 serotype chimerism	I	(Bauerschmitz et al., 2006)
Ad5/3-9HIF-Δ24-E3	9HIF promoter and 24 bp deletion[2]	Wild type	5/3 serotype chimerism	III	Study III
Ad5/3-9HIF-Δ24-VEGFR-1-Ig	9HIF promoter and 24 bp deletion[2]	VEGFR-1-Ig	5/3 serotype chimerism	III	Study III

[1] virus purchased from American Type Culture Collection (ATCC)
[2] 24 bps deleted in the constant region 2 (CR2) of the E1A gene
[3] 2 bps deleted in the constant region 1 (CR1) of the E1A gene

3.2.3 Construction of Ad5-9HIF-luc, Ad5-OB36-luc, Ad5/3-9HIF-Δ24-VEGFR-1-Ig and Ad5/3-9HIF-Δ24-E3 (III)

For construction of Ad5-9HIF-luc and Ad5-OB36-luc, expression cassettes with either 9HIF (Aragones et al., 2001) or OB36 (Boast et al., 1999) hypoxia response elements controlling firefly *luciferase* were inserted into the multiple cloning site of pShuttle (Stratagene, La Jolla, CA, USA). Shuttle plasmids were recombined with pAdeasy-1 plasmid (Stratagene), which carries the whole adenovirus genome, and resulting rescue plasmids were transfected to 293 cells to generate Ad5-9HIF-luc and Ad5-OB36-luc.

For construction of oncolytic Ad5/3-9HIF-Δ24-VEGFR-1-Ig and Ad5/3-9HIF-Δ24-E3, the gene for VEGFR-1-Ig (first five domains of VEGF receptor 1 fused to Fc tail of human IgG antibody, kindly provided by Dr. Kari Alitalo, University of Helsinki, Finland) was cloned into pTHSN plasmid that contains the E3 region of the adenoviral genome replacing the 6.7K/gp19K genes (Kanerva et al., 2005). The resulting plasmid was recombined with pAdeasy-1.5/3-Δ24, an adenovirus rescue plasmid containing the serotype 3 knob and a 24 bp deletion in E1A (Kanerva et al., 2005), resulting in pAdeasy-1.5/3-Δ24-VEGFR-1-Ig. 9HIF was inserted into pSEΔ24 (Bauerschmitz et al., 2006), a shuttle plasmid containing the E1 region and a 24 bp deletion in E1A, to construct pSEΔ24-9HIF. This shuttle plasmid was then recombined with pAdeasy-1.5/3-Δ24-VEGFR-1-Ig and pAdeasy-1.5/3- Δ24 resulting in pAdeasy-1.5/3-9HIF-Δ24-VEGFR-1-Ig and pAdeasy-1.5/3-9HIF-Δ24-E3, which were transfected to 911 cells for generation of Ad5/3-9HIF-Δ24-VEGFR-1-Ig and Ad5/3-9HIF-Δ24-E3.

3.3 *In vitro* studies

3.3.1 Replication kinetics analysis of virus combinations (I)

250,000 HEY or 786-O cells per well were infected with $1.25x10^6$ vp of the respective replicating adenovirus or control virus in combination with $1.25x10^6$ vp of Ad5/3luc1. For the assay with the replicating, luciferase expressing virus, $1.25x10^6$ vp of either Ad5luc3 or Ad5luc1 alone was used to infect cells. On day 1, 3, 5 and 7 after infection cells were harvested and analyzed by TCID50 assays with 293 cells (Adeasy application manual, QBiogene)., luciferase expression (Luciferase Assay System, Promega, Madison, WI) and qPCR for *luciferase*. For TCID50 assays cell samples were freeze-thawed thrice to release the virus, for luciferase expression assays the provided reporter lysis buffer was used and for qPCR DNA was extracted from samples using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Analyses were done in triplicates for each virus, day and analytical method.

3.3.2 qPCR for luciferase (I)

Luciferase specific primers (forward 5'- GAAATCCCTGGTAATCCGTT -3' and reverse 5'-ATCACAGAATCGTCGTATGC -3') were used to perform real time quantitative PCR with a SYBR green assay using a RotorGene system. The efficiency of the reaction was assumed to be 2, therefore values were calculated using the formula: $2^{(\text{MockCycleNumber - SampleCycleNumber})}$.

3.3.3 Quantification of infectious particles of *in vivo* samples (I)

Tumors were minced, suspended in 1ml DMEM growth medium without FCS and freeze-thawed three times (Kanerva et al., 2005). The supernatant was then used for duplicate TCID50 assays with 293 cells.

3.3.4 Flow cytometric analysis for receptor expression (II)

Renal cancer cells were incubated with anti-CD46 (BD Biosciences, Franklin Lakes, NJ, USA), anti-integrin $\alpha_v\beta_3$ (Chemicon International, Temecula, CA, USA), anti-integrin $\alpha_v\beta_5$ (Chemicon International), anti-HSPG (Seikagaku, Falmouth, MA, USA), anti-CAR RmcB antibodies or FACS buffer. Cells were then washed and incubated with phycoerythrin (PE) labeled goat anti-mouse immunoglobulin polyclonal antibody (BD Biosciences) prior to flow cytometry.

For analysis of clinical samples, tumor pieces were minced, suspended in growth medium with 0.2 Wünsch units/mL Liberase Blendzyme (Roche Diagnostics, Indianapolis, IN, USA) and incubated at 37°C for 2 hours for enzymatic dissociation prior to FACS.

3.3.5 Marker gene transfer assays (II, III)

Cells were infected with replication deficient, marker gene expressing viruses for 30 min. and then washed and incubated with complete growth medium at 37°C. 24 hours later luciferase (Luciferase Assay System, Promega) or β-gal (Galacto Light Plus, Tropix, Bedford, MA, USA) assays were performed according to the manufacturer's manual.

Clinical samples were minced and washed twice. Samples were resuspended in 2% RPMI and then infected with 5000 VP/cell. Luciferase or b-gal assays were performed as described above.

3.3.6 Cytotoxicity assays (II, III, IV)

10^4 cells per well on 96 well plates were infected with indicated viruses. After 1 hour, infection medium was replaced with growth medium containing 5% FCS, which was changed every other day. 5-14 days later cell viability was analyzed with MTS assay (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega).

For study IV, cytotoxicity assays were done with samples containing 100% $CD44^+CD24^{-/low}$ cells because virus infection was performed after sorting, except PL1, which was analyzed

without sorting. The complete PL1 sample was used for infection and therefore the proportion of $CD44^{+}CD24^{-/low}$ cell could not be analyzed. The total number and proportion of viable cells in patient sample PL11 was also studied with trypan blue staining (Invitrogen, Carlsbad, CA) 8 days after infection.

3.3.7 Western blot for VEGFR-1-Ig (III)

Cells were infected with Ad5/3-9HIF-Δ24-VEGFR-1-Ig, Ad5/3-9HIF-Δ24-E3 or mock at an MOI of 10 vp/cell, medium was changed after one hour and cells were incubated for 72 hours. Western blot was done with cell culture supernatant using anti-human-IgG antibody (GE Healthcare, Barrington, IL, USA) for detection of VEGFR-1-Ig protein.

3.3.8 Immunofluorescence staining (III)

Tumor cryosections of 4-5µm thickness were prepared and fixed in acetone for ten minutes at -20^{0}C. Sections were incubated with normal donkey serum for 15 minutes, and then reacted with primary polyclonal rabbit anti Von Willebrand Factor (1:200 dilution, DakoCytomation, Denmark) overnight. After washing with PBS, sections were incubated with Alexa Fluor 594 labeled secondary antibody (1:250 dilution, Molecular Probes, Invitrogen) for 30 minutes. Sections were fixed in 4% paraformaldehyde and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Representative pictures of areas of the tumors with the highest microvessel density were captured at 20x magnification.

3.3.9 Expression of stem cell markers (IV)

Messenger RNA was isolated using RNAeasy (Qiagen). The following primers were used for real-time-PCR; oct4 forward 5′-CGCACCACTGGCATTGTCAT-3′, reverse 5′-TTCTCCTTGATGTCACGCAC-3′, sox2 forward 5′-GGCAGCTACGCATGATGCAGGAGC-3′, reverse 5′-CTGGTCATGGAGTTGTACTGCACG-3′ β-actin forward 5′-CGAGGCCCAGAGCAAGACA-3′,

reverse 5′-CACAGCTTCTCCTTAATGTCACG-3′. Immunofluorescence staining of paraformaldehyde-fixed $CD44^{+}CD24^{-/low}$ cells was performed with Oct3/4 and CD44 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; BD Pharmingen, Franklin Lakes, NJ).

3.4 *In vivo* studies

All experiments were approved by the Experimental Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice at the age of 4-5 weeks were purchased from Taconic (Ejby, Denmark) and quarantined for at least one week. The animals were frequently monitored for their health status and euthanized as soon as any sign of pain or distress was noticed.

For tumor volume determination, the largest diameter of the tumor and the diameter perpendicular to it were measured with calipers. Volumes were calculated using the formula: (larger diameter) x (smaller diameter)^2 x 0.52.

3.4.1 Bioluminescence imaging (I, II, III)

Mice were injected intraperitoneally with 4.5 mg of D-Luciferin (Promega, Madison, WI) diluted in 100 µl RPMI without FCS and after 10 min images were captured with the IVIS imaging system series 100 (Xenogen, Alameda, CA). Photon emission values were calculated with Living Image v2.5 software (Xenogen).

In the experiment with the dual luciferase imaging system GFP, DsRed or no emission filter were applied to separately measure cells (expressing click beetle green luciferase) or virus (expressing firefly luciferase).

3.4.2 Animals models in study I

For the ovarian cancer xenograft model, female NMRI nude mice were subcutaneously injected with $5x10^6$ Hey ovarian adenocarcinoma cells in both flanks. When tumors reached a volume of ca. 65 mm^3, mice were randomized into seven groups of four animals each receiving indicated virus or no virus intratumorally on three consecutive days (days 0, 1, 2) and again on day 10 (n=8 tumors per group). Each injection contained $3x10^8$ vp of the replicating virus and $3x10^8$ vp of Ad5/3luc1. Half of the mice were killed on day 3 and the rest on day 17. All tumors were collected and stored at -80°C.

For the renal cancer model, subcutaneous tumors were induced in female NMRI nude mice by injecting $5x10^6$ 786-O renal cancer cells as above. The mice were randomized into six groups and treated on three consecutive days (days 0, 1, 2) with $3x10^8$ vp of the replicating virus and $3x10^8$ vp of Ad5/3luc1 (n=8 tumors per group). In another experiment $3x10^8$ vp of Ad5luc3 alone was injected intratumorally on three consecutive days. Half of the mice were killed on day 3 and the rest on day 9.

3.4.3 Animals models in study II

3.4.3.1 Biodistribution experiment

ACHN cells were injected intraperitoneally into Fox chase SCID mice and 35 days later 10^8 vp of indicated replication deficient, marker gene expressing viruses were administered intraperitoneally. 48 hours later mice were killed and selected organs collected and analyzed for marker gene expression as described earlier (Kanerva et al., 2002b).

3.4.3.2 Subcutaneous tumor growth inhibition experiment

786-O tumors were grown in nude mice and a mixture of $3x10^8$ vp of indicated oncolytic virus and $3x10^8$ vp of Ad5/3luc1 was injected intratumorally on three consecutive days (day 1, 2 and 3). On day 4 and 10 mice were imaged for luciferase.

3.4.3.3 Survival experiment

ACHN cells were injected intraperitoneally into SCID mice. 7, 14 and 21 days later 10^8 vp of indicated viruses were injected also intraperitoneally. 50 μg bevacizumab (Avastin, Genentech, South San Francisco, CA, USA) was given intraperitoneally once weekly for 5 weeks starting at day 11.

3.4.4 Animals models in study III

3.4.4.1 Luciferase activity experiment

For luciferase expression experiments, nude mice were injected subcutaneously with $5x10^6$ 786-O cells. When diameters of tumors were approximately 5mm, $3x10^8$ vp was injected intratumorally. Two days later, mice were imaged and then killed, tumors were excised, ground and resuspended in lysis buffer and analyzed for luciferase expression as described above.

For the intraperitoneal models, tumors were induced with 10^7 786-O or 786-O-CBGr cells. After 20 days mice were imaged and 10^8 vp was administered intraperitoneally. Two days later mice were imaged again for tumor and virus location. Mice were then killed, livers were excised and prepared and analyzed for luciferase expression as described above.

3.4.4.2 Subcutaneous tumor growth inhibition experiment

In the oncolytic efficacy experiments, nude mice were injected subcutaneously with $5x10^6$ 786-O cells. When diameters of tumors were approximately 5mm, 10^8 vp were injected intratumorally. Blood samples were taken on day 7, 11 and 15 after virus injection and VEGFR-1-Ig concentration in the collected mouse serum was determined with a human IgG elisa kit (Immunology Consultants Laboratory, Newberg, OR, USA). At the end of the experiment on day 17, mice were killed and tumors were excised and frozen for immunofluorescence staining.

3.4.4.3 Survival experiment

For the survival experiment, SCID mice were injected intraperitoneally with 10^7 SN12L1-luc cells. A single intraperitoneal virus injection of $5x10^8$ vp was performed on day 10 after cell injection. Mice were monitored for survival and imaged on day 9, 18, 25 and 32 after cell injection.

3.4.5 Animals models in study IV

2×10^6 sorted or unsorted patient pleural effusion or JIMT-1 cells were injected together with Matrigel (BD Pharmingen, Franklin Lakes, NJ) into the uppermost mammary fat pads of non-obese diabetic/severe combined immunodeficient mice. The quantity of $CD44^+CD24^{-/low}$ cells available limited the number of mice that could be included in the experiments. Mice were also injected with 1 mg/kg Estradurin (Pfizer, New York, NY) every 3 weeks throughout the experiment. Intratumoral injections were performed with 3×10^9 vp of indicated viruses at different time points.

3.5 Statistics

3.5.1 Statistical analysis for study I

The significance of antitumor efficacy for the ovarian cancer model experiment was calculated using a non-parametric change-point test to show a systematic change in the pattern of observations as opposed to fluctuation due to chance. The Proc Mixed procedure in SAS v.6.12 (SAS Institute, Cary, NC) was used to examine the effects of group and time on tumor growth. Pairwise comparisons were performed to compare groups.

RLU, qPCR, TCID50, *in vivo* photon emission and tumor volume values for all CRAds were entered into scatter plots and correlation (two tailed Pearson t-test) was determined using GraphPad Prism 4 software (GraphPad Software Inc, San Diego, CA).

3.5.2 Statistical analysis for study II and III

To compare differences between groups in *in vitro* assays, two tailed student's t-test was used and a p-value of <0.05 was considered significant. P-values of the *in vivo* subcutaneous experiment were calculated by Mann-Whitney test (SPSS 13.0). Data of survival experiments was plotted as Kaplan-Meier graphs and a log rank t-test (SPSS 13.0) was used for pairwise comparison of groups.

3.5.3 Statistical analysis for study IV

The repeated measures growth model in PROC MIXED (SAS 9.1; SAS, Cary, NC) was used for comparison of tumor sizes. Data was log-transformed for normality. The effects of the treatment group, time and the interaction between the treatment group and time were evaluated by F tests. Curvature in the growth curves was tested by a quadratic term for time. *A priori* planned comparisons of differences in the predicted treatment means of all groups to mock were computed by Tukey–Kramer adjusted two-sided t-statistics averaged over all time points.

4 RESULTS AND DISCUSSION

4.1 Coinfection of a replicating with a non-replicating luciferase expressing adenovirus allows monitoring of virus replication *in vivo* (I)

The efficacy of oncolytic adenoviruses is linked to infection of target cells and subsequent productive replication. Other variables include intratumoral barriers, access to target cells, uptake by non-target organs and immune response. Each of these aspects relates to the location and degree of virus replication. Unfortunately, detection of *in vivo* replication has been difficult, labor intensive and costly and therefore not much studied. We hypothesized that by co-infection of a luciferase expressing E1-deleted virus with an oncolytic virus, both viruses would replicate when present in the same cell. Subsequent imaging for luciferase could be used for quantitation of the amplitude, persistence and dynamics of oncolytic virus replication *in vivo*, which would be helpful for the development of more effective and safe agents.

In vitro replication kinetics of a panel of CRAds and control viruses (Table 1 in study I) in combination with the replication deficient, luciferase expressing Ad5/3luc1 were assessed on HEY and 786-O cells. The samples were analyzed for viral titers by TCID50 assay, for luciferase activity by luminescence measurement and for luciferase gene copy number by qPCR. Significant correlation of the measured parameters was found (Fig.1d-f and 2d-f in study I).

The experiments were repeated with Ad5luc3, a replicating adenovirus expressing luciferase representing a one component system as opposed to the two component system evaluated earlier. Ad5luc3 showed similar results compared to the system with two viruses (Figure 3 in

study I) suggesting that luciferase imaging could track replication regardless of E1 provided in *trans* or by the same virus.

The coinfection approach was subsequently analyzed in a subcutaneous *in vivo* model of ovarian cancer induced with HEY cells. Tumors were injected with CRAds featuring Cox-2 promoters and/or E1A region mutations and control viruses (Table 1 in study I) in combination with the replication deficient Ad5/3luc1. Tumor sizes were measured, mice were imaged and tumors were harvested for determination of viral titers (Figures 4 and 5a-c in study I). Ad5/3-Δ24 and Ad5/3Cox2Ld24 exhibited significant antitumor efficacy and significant correlations between production of infectious virions, photon emission values and tumor size were observed (Figures 5a and d-f in study I).

To verify the findings, we repeated the experiment using a subcutaneous renal cell cancer model with 786-O cells. In this model, all CRAds exhibited significant antitumor efficacy compared to the replication deficient control virus and again significant correlations between infectious virions titers, photon emission values and tumor size were found (Figures 7a and d-f in study I). In another *in vivo* experiment, Ad5luc3 was injected subcutaneously and the obtained results were comparable to those of the two component coinjection system (Figure 8 in study I).

These data suggest that *in vivo* luciferase imaging can be used to estimate virus replication in live animals. Interestingly, we also saw positive correlation between tumor volume and infectious virus production, and between tumor volume and photon emission, which initially seemed partly counterintuitive. One would expect more replication to result in more antitumor activity and therefore smaller tumors. However, we assume that the strongest virus replication took place in the first days (days 0-2) in the tumors which in the end showed the best antitumor responses. Therefore, correlation of tumor size with photon and virus counts occurs mostly at later time points and presumably reflecting a situation where the tumor is not completely eradicated (due to intratumoral barriers), but virus replication persists.

In conclusion, the proposed system of coinjecting a luciferase expressing, replication deficient adenovirus with a replication competent adenovirus could be employed for non-invasive evaluation of virus replication. Laborious TCID50 assays with tissue samples from euthanized animals could be replaced with the proposed system, which would reduce the amount of work, costs and the number of test animals needed.

4.2 Capsid modified adenoviruses display enhance transduction to renal cancer cells and kidney cancer specimens with high levels of target receptors (II)

Adenoviruses have great potential in gene therapy approaches for the treatment of cancer. However, preclinical studies and clinical trials have demonstrated that high transduction of all areas of the tumor is difficult to achieve. Besides intratumoral barriers, one major reason for insufficient transduction has probably been downregulation of the primary receptor for adenovirus entry (CAR), which was reported for many types of cancer, including kidney cancer (Bauerschmitz et al., 2002; Haviv et al., 2002). To circumvent CAR dependency, adenoviruses with genetically engineered capsid modifications, which transductionally target them to non-CAR receptors, can be constructed. We hypothesized that capsid modifications (5/3, pK7, pK21 and RGD capsid modifications; see chapter 1.4.1.3 for description of these modifications) on adenoviruses would enhance transduction to renal cancer cells and kidney cancer specimens. Because expression of the receptors relevant for entry of these viruses can vary between individual tumors, we sought to evaluate if fluorescence activated cell sorting (FACS) could be used to predict which virus would be most useful for each tumor.

FACS analysis of renal cancer cell lines, clinical tumor specimens and tumor xenografts showed high expression of CD46 (proposed receptor for Ad5/3 based viruses), HSPGs (target receptor for Ad5.pK based viruses) and integrins ($\alpha v\beta_3$ and $\alpha v\beta_5$ integrins are target receptors for Ad5-RGD viruses)(Supplementary Figures 1, 2 and 3 in study II). Consequently,

transductional targeting with 5/3, pK7, pK21 and RGD capsid modifications resulted in high levels of gene delivery. Ad5.pK7 and Ad5/3luc1 showed more than 300 and 400 fold increased transduction respectively compared to a virus with wild type capsid (Figure 1a and b in study II). For all cell lines, viruses with polylysine modifications or 5/3 chimerism demonstrated significantly enhanced gene delivery compared to the respective wild type capsid control viruses, which correlated with high target receptor expressions seen by FACS.

With one of the analyzed kidney cancer tumor samples, a 17 fold increase in gene delivery was seen with Ad5.pK21-LacZ (Figure 2a in study II), whose target receptor was shown to have high expression levels (Supplementary Figure 2 in study II).

In summary, adenoviruses with capsid modifications transduce renal cancer cells and kidney tumor samples significantly better than wild type capsid viruses and their target receptors were shown to be highly expressed. These findings suggests that in a clinical setting it might be useful to analyze receptor expression before deciding which virus would be most useful for each cancer patient, assuming a panel of treatment agents were available.

4.3 Improving oncolytic effect *in vitro* and antitumor effect *in vivo* with capsid modified oncolytic adenoviruses (II)

Next we hypothesized that the enhanced transduction of capsid modified adenoviruses would translate into increased oncolytic effect of oncolytic adenoviruses featuring the same capsid modifications.

In vitro, all capsid modified viruses exhibited increased oncolytic effect compared to wild type capsid viruses (Figure 3 in study II). In particular, Ad5/3-Δ24 showed the highest cell killing effect on all tested kidney cancer cell lines.

In vivo, in a subcutaneous murine model of kidney cancer, intratumoral injection of capsid modified viruses resulted in significantly better antitumor efficacy compared to wild type

virus (Figure 4a in study II). The *in vivo* imaging system for monitoring replication (described in chapter 4.1) was applied to this model demonstrating highest replication activity for Ad5/3-Δ24 (Figure 4b and c in study II).

Since kidney tumors are highly vascularized (Fukata et al., 2005) and antiangiogenic treatments have proven efficacy in this disease (Escudier et al., 2007b) we sought to evaluate whether combining oncolytic adenoviruses with bevacizumab (a monoclonal antibody against VEGF) would result in additive or synergistic antitumor efficacy. Therefore, we set up an intraperitoneal murine model of disseminated kidney cancer, where we injected capsid modified oncolytic viruses alone or in combination with bevacizumab. All capsid modified viruses resulted in significantly prolonged survival compared to wild type virus (Figure 5b in study II). In particular, Ad5/3-Δ24 demonstrated the best therapeutic effect with 50% of the mice being alive after 320 days, suggesting that these mice were cured. Combination treatment with bevacizumab was generally not as efficient as virus alone. This might be due to bevacizumab mediated collapse of the tumor vasculature that would prevent intratumoral dissemination of the virus. Moreover, oncolytic viruses may exert part of their effect via release into the systemic circulation and subsequent reinfection of tumors, which might be compromised if vessels are no longer available (Jain, 2005).

In conclusion, we could show that capsid modified CRAds have improved antitumor efficacy in murine renal cell cancer models. Therefore, these viruses might be valuable agents for the treatment of kidney cancer in humans.

4.4 Dual luciferase imaging system for simultaneous *in vivo* imaging of viruses and tumor cells (III)

We sought to develop a dual luciferase imaging system based on viruses coding for firefly luciferase and a cell line stably transfected with click beetle green luciferase to localize virus gene expression in an *in vivo* kidney cancer model.

Both click beetle green luciferase and firefly luciferase convert D-luciferin but they emit light with different peak wavelengths, 550nm and 610nm respectively, which can be filtered out with the GFP and DsRed filters (emission passbands: 515-575 nm and 575-650 nm). To create a luciferase expressing cell line, we stably transfected the kidney cancer cell line 786-O with the gene for click beetle green luciferase, thereby creating 786-O-CBGr. 786-O and 786-O-CBGr cells were infected with Ad5luc1, which expresses firefly luciferase under the ubiquitous CMV promoter. With the DsRed filter only in the wells infected with 1000 vp/cell of virus light emission could be detected (Figure 3a in study III). Using the GFP filter, light emission from all 786-O-CBGr wells was seen. No light was detected in the 786-O wells with the GFP filter, as expected. These results suggest that the DsRed and GFP filters are useful to separate light signals from firefly and CBGr luciferases.

For *in vivo* evaluation of the system, SCID mice were injected intraperitoneally with 786-O or 786-O-CBGr cells. When the mice were imaged 20 days later without a filter, light emission was detected in 786-O-CBGr injected mice (Figure 3b in study III). Using the (virus specific) DsRed filter, basically no light was detected, whereas with the (cell specific) GFP filter light emission was comparable to that observed without filters.

Immediately after imaging, the mice were injected intraperitoneally with Ad5luc1 and imaged again two days later. Without a filter and with the virus specific DsRed filter strong light emission from the liver and peritoneum was detected (Figure 3c in study III). When applying the CBGr luciferase specific GFP filter, no light was detected from 786-O injected mice, whereas 786-O-CBGr mice showed similar light emission as before virus injection.

In summary, it was possible to specifically image for virus mediated firefly luciferase or tumor cell mediated CBGr luciferase *in vivo*, and therefore, this system can be used to localize tumors and viruses in live animals.

4.5 Promoter evaluation for transcriptional targeting to renal cell cancer (III)

For tumor tissue specific gene expression and replication of oncolytic viruses, tissue specific promoters can be used. Because of high HIF expression in renal cell cancer tissue (see chapter 1.2.1.1), hypoxia response elements are promising candidates for controlling kidney cancer specific gene expression. Therefore, we sought to evaluate the activity of two different hypoxia response elements in kidney cancer and normal cells and tissues.

Replication deficient adenoviruses with 9HIF and OB36 hypoxia response elements controlling firefly luciferase (Ad5-9HIF-luc and Ad5-OB36-luc) were constructed and their luciferase activity was compared to Ad5luc1 with the highly active CMV promoter driving luciferase. 9HIF demonstrated rather low but highly selective activity in kidney cancer cells *in vitro* (Figure 1 in study III). *In vivo,* in a subcutaneous kidney cancer model both elements exhibited activity, which was higher than with the control virus with CMV driven luciferase, suggesting efficient induction of hypoxia response elements in the *in vivo* setting (Figure 2 in study III). In an intraperitoneal kidney cancer model, luciferase expression specific to the tumors was demonstrated for Ad5-9HIF-luc (Figure 4a in study III) using the dual luciferase imaging system (see chapter 4.4). Moreover, when livers of the injected mice were analyzed *ex vivo*, significantly reduced luciferase activity was seen for Ad5-9HIF-luc (Figure 4b in study III).

In conclusion, we were able to identify 9HIF as a useful promoter for targeting kidney cancer.

4.6 An infectivity enhanced, targeted and armed oncolytic adenovirus exhibits high specificity for renal cell cancer and improved antitumor effect (III)

We hypothesized that targeted oncolytic adenovirus armed with an antiangiogenic transgene would exhibit enhanced specificity and increased antitumor effect with regard to renal cell cancer. Therefore, based on the transductional targeting studies and the promoter evaluation we generated Ad5/3-9HIF-Δ24-VEGFR-1-Ig, a dual-targeted, infectivity enhanced and antiangiogenic oncolytic adenovirus for the treatment of kidney cancer. This virus features the 5/3 capsid modification for infectivity enhancement and transductional targeting, 9HIF controlling E1A for transcriptional targeting and the antiangiogenic molecule VEGFR-1-Ig in the E3 region for improved antitumor efficacy (Figure 5a in study III). Ad5/3-9HIF-Δ24-E3, an isogenic unarmed control virus was also constructed.

Expression of VEGFR-1-Ig from infected cells was confirmed by western blot (Figure 5b in study III). *In vitro* with kidney cancer cells, oncolytic effect of Ad5/3-9HIF-Δ24-VEGFR-1-Ig and Ad5/3-9HIF-Δ24-E3 was weaker than that of the non-targeted, non-armed control Ad5/3-Δ24 but usually stronger than with wild type virus Ad300wt (Figures 6a and b and Supplementary Figure 2 in study III). This was an expected result, since 9HIF activity was shown to be low *in vitro* (Figure 1 in study III), reducing replication of Ad5/3-9HIF-Δ24-VEGFR-1-Ig and Ad5/3-9HIF-Δ24-E3. Also, VEGFR-1-Ig is not expected to add utility *in vitro*. Practically no effect of Ad5/3-9HIF-Δ24-VEGFR-1-Ig and Ad5/3-9HIF-Δ24-E3 was seen on fibroblasts, which underscores the high specificity for cancer cells of these viruses (Figure 6c in study III). The non-targeted viruses killed HUVECs very efficiently while Ad5/3-9HIF-Δ24-E3 had no effect (Figure 6d in study III) since HIF is not active in these cells under normoxic conditions (Calvani et al., 2006). However, Ad5/3-9HIF-Δ24-VEGFR-1-Ig caused about a 30% reduction in cell viability, suggesting that low levels of VEGFR-1-Ig are expressed from this virus even in the absence of oncolysis.

In a subcutaneous *in vivo* experiment, Ad5/3-9HIF-Δ24-E3 and Ad5/3-Δ24 showed the best antitumor effects with complete eradication of 38% of the tumors in both groups (Figure 7a in study III). This confirmed the high induction of HREs *in vivo* compared to *in vitro*. While also Ad5/3-9HIF-Δ24-VEGFR-1-Ig had antitumor efficacy, it was not more potent than Ad5/3-9HIF-Δ24-E3 and Ad5/3-Δ24, despite confirmed VEGFR-1-Ig expression (Figure 7b in study III). Reduction of tumor vasculature was also seen (Figure 7c in study III). A reason for Ad5/3-9HIF-Δ24-VEGFR-1-Ig being less effective than Ad5/3-9HIF-Δ24-E3 in this model might be collapse of vasculature due to VEGFR-1-Ig compromising intratumoral dissemination and/or vascular reinfection, as suggested before (Guse et al., 2007). Moreover, the antiangiogenic effect might result in larger necrotic parts within the tumor, which would not decrease tumor size over the course of such a short animal experiment. Furthermore, necrosis and hypoxia might compromise viral dissemination (Heldin et al., 2004).

Because subcutaneous tumors may not be a clinicinally meaningful representation of metastatic renal cell cancer, we set up a model of intraperitoneally disseminated kidney cancer with SN12L1-luc cells. Ad5/3-9HIF-Δ24-VEGFR-1-Ig treated mice survived significantly longer than any other group suggesting that in this model the antiangiogenic effect was useful (Figure 8 in study III). One reason for the superior antitumor efficacy in this model might be that in the intraperitoneal scenario, virus can spread within the tumor better through non-vascular routes as compared to the subcutaneous model.

In conclusion, our data shows that triple targeting, including dual-level transcriptional targeting and capsid modification can increase the selectivity, and antiangiogenic arming can improve antitumor efficacy of oncolytic adenoviruses for renal cell cancer. Such a virus might therefore be a promising agent for patients with kidney cancer resistant to all other available treatments.

4.7 Identification of breast cancer initiating cells in pleural effusions from breast cancer patients (IV)

Cancer stem cells might play an important role in the relapse of breast cancer following conventional treatment and could be one of the reasons for the incurable nature of metastatic breast cancer. These cancer stem cells are thought to be found in the $CD44^{+}CD24^{-/low}$ side population present in many breast cancers (Al-Hajj et al., 2003). Therefore, we sought to characterize breast cancer initiating $CD44^{+}CD24^{-/low}$ cells from pleural effusions of breast cancer patients.

Seven out of thirteen pleural effusion samples that we obtained contained a $CD44^{+}CD24^{-/low}$ subpopulation, which typically comprised 20–50% of the sample (Figure 1 and Supplementary Figure S1 in study IV). A feature suggested for both tumor and normal tissue stem cells is the membrane transporter–mediated capacity for Hoechst 33342 exclusion, which might serve as a useful surrogate marker for resistance to lipophilic anticancer drugs (Dean et al., 2005). $CD44^{+}CD24^{-/low}$ sorting enriched this subpopulation from 1% (±1.1%) to 7% (±2.1%) ($p = 0.0001$, Figure 2a and b in study IV). To further evaluate the possible stem cell characteristics of the $CD44^{+}CD24^{-/low}$ population, expression of *oct4* and *sox2* was studied by means of a semi-quantitative reverse transcriptase polymerase chain reaction analysis (real-time-PCR) (Nichols et al., 1998; Zhao et al., 2004). Surprisingly, we found an unequal amplification of β-actin, which had been included as a control for the amount of total RNA. This had been reported before (Cowan et al., 1999; Lee et al., 2003) and thus β-actin may not be the optimal control to use when analyzing putative stem cells. Nevertheless, since we only wanted to gain qualitative information about *oct4* and *sox2* expression, the non-reliability of β-actin did not interfere with our interpretation of the results. All samples were positive for *oct4* and *sox2*, suggesting that when a $CD44^{+}CD24^{-/low}$ population is present, it contains stem cell-like cells (Figure 2c in study IV). Oct4 and CD44 protein expression was confirmed by immunofluorescence staining (Figure 2d in study IV).

In summary, we confirmed that $CD44^{+}CD24^{-/low}$ cells can be purified from pleural effusions of breast cancer patients and that these cells exhibit stem cell properties. However, further studies are needed to investigate if true stem cells reside in this population.

4.8 Capsid modified oncolytic adenoviruses efficiently kill $CD44^{+}CD24^{-/low}$ breast cancer initiating cells (IV)

Next we were wondering whether breast cancer initiating $CD44^{+}CD24^{-/low}$ cells, thought to be resistant to conventional cancer treatments, could be killed with oncolytic adenoviruses.

Ad5/3-Δ24 and Ad5.pK7-Δ24 resulted in efficient cell killing of unsorted cells as well as $CD44^{+}CD24^{-/low}$ cells (Figure 3 and Supplementary Figure 3 in study IV). Ad5-Δ24RGD and wild type Ad5 (Ad300wt) were less potent suggesting that 5/3 and pK7 capsid modification could be useful for increasing antitumor efficacy. To confirm that capsid modified oncolytic viruses can completely eradicate the $CD44^{+}CD24^{-/low}$ population, trypan blue staining was performed (Figure 3e in study IV). Approximately 85% of the uninfected cells were alive after 8 days, whereas none of the cells infected with Ad5/3-Δ24 and Ad5.pk7-Δ24 survived.

In conclusion, we showed that oncolytic adenoviruses efficiently kill $CD44^{+}CD24^{-/low}$ cells which are thought to harbor the true cancer stem cell population. Moreover, adenoviral capsid modifications were able to significantly increase the oncolytic potency on this cell population.

4.9 Oncolytic adenoviruses exhibit antitumor efficacy against tumors established with $CD44^+CD24^{-/low}$ breast cancer initiating cells (IV)

Based on the *in vitro* data described in chapter 4.8, we hypothesized that capsid modified oncolytic adenoviruses would be able to inhibit growth of tumors induced with $CD44^+CD24^{-/low}$ cells.

Unsorted or $CD44^+CD24^{-/low}$ cells from a breast cancer patient formed tumors in all non-obese diabetic/severe combined immunodeficient mice by day 95. The first tumors in mice injected with $CD24^+$ cells appeared on day 113 (Figure 4 in study IV), confirming previous data that sorting for $CD44^+CD24^{-/low}$ selects for a population capable of forming tumors more rapidly. When cells were infected with Ad5/3-Δ24 prior to injection, no tumors grew (Figure 4 in study IV).

$CD44^+/CD24^{-/low}$ JIMT-1 cells (a low passage pleural effusion explant) formed tumors in the mammary fat pads of non-obese diabetic/severe combined immunodeficient. However, when cells were pretreated with Ad5/3-Δ24, no tumors formed (Figure 4 in study IV). To analyze the effect of Ad5/3-Δ24 on orthotopic pre-terminal $CD44^+/CD24^{-/low}$–derived disease, tumors were allowed to grow as large as animal regulations allowed. Then, intratumoral virus injections were performed, and abrogation of tumor growth was seen (Figure 4b in study IV). In a larger subsequent experiment, Ad5/3-Δ24 and Ad5.pK7-Δ24 resulted in significant antitumor efficacy versus mock (both $p < 0.0001$, Figure 5 in study IV).

Initially, 10% of JIMT-1 cells were $CD44^+CD24^{-/low}$. After sorting, 100% of cells injected into mice were $CD44^+CD24^{-/low}$, but when tumors were removed on day 48, the $CD44^+CD24^{-/low}$ cell proportion had returned to 11%. After virus injections, 6.4% (Ad300wt), 5.0% (Ad5/3-Δ24), 1.1% (Ad5.pK7-Δ24), and 3.8% (Ad5-Δ24RGD) of cells were of $CD44^+CD24^{-/low}$ type (**Figure 8A**). 14% (±3.4%) versus 3.0% (±0.9%) of cells in the $CD44^+CD24^{-/low}$ population of untreated versus treated tumors were capable of Hoechst exclusion ($p < 0.001$, **Figure 8B**).

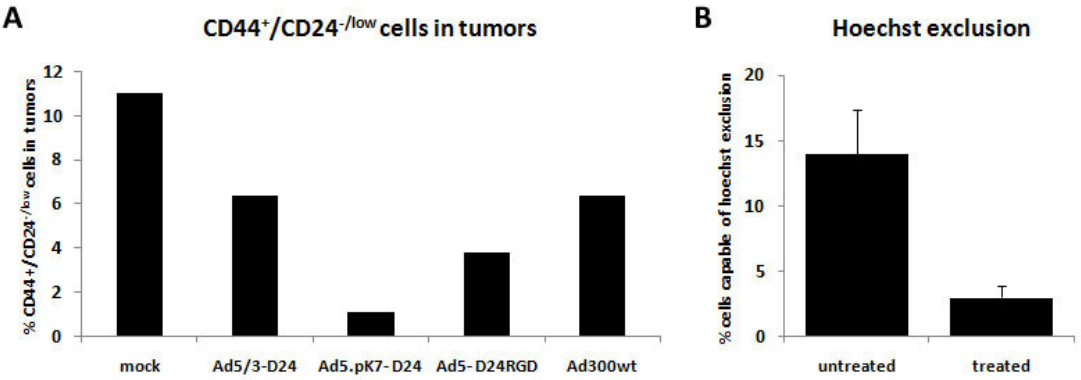

Figure 8: Stem cell properties of cells from virus treated tumors

$CD44^{+}/CD24^{-/low}$ cell sorting with untreated and virus treated tumors (**A**). Hoechst exclusion of $CD44^{+}/CD24^{-/low}$ cells from untreated and virus treated tumors (**B**).

In conclusion, we showed that capsid modified oncolytic adenoviruses are able to efficiently kill breast cancer initiating cells leading to significant antitumor efficacy. Promising preclinical data had been obtained earlier using capsid modified adenoviruses in breast cancer cell lines, clinical specimens, and orthotopic animal models of both locally advanced and metastatic breast cancer (Ranki et al., 2007a; Ranki et al., 2007b). Therefore, these viruses may be effective in the killing of both “differentiated” and “tumor initiating” breast cancer cells, which might be useful in treating patients suffering from incurable metastatic breast cancer.

5 SUMMARY AND CONCLUSIONS

The overall goal of this thesis was to improve treatment options for kidney and breast cancer using oncolytic adenoviruses.

An *in vivo* imaging system for following virus replication of oncolytic adenoviruses was successfully set up in study I and used in study II. This system might be useful for replacing or complementing tedious *ex vivo* assays with tissue samples and could reduce the number of required test animals because the killing of animals is not required.

Genetically engineered capsid modifications proved to be useful for transductional targeting to renal cell cancer tissue and for increasing the antitumor potency of oncolytic adenoviruses (study II). However, combining oncolytic adenoviruses with the antiangiogenic antibody did not result in improved antitumor efficacy in an intraperitoneal mouse model of disseminated kidney cancer. In this setting the combination therapy might not be beneficial because antiangiogenic treatment results in reduced tumor vascularity, which might compromise viral spread. Furthermore, the timing might not have been optimal. Instead of the simultaneous therapy used here, a consecutive therapy approach could be more useful so as to gain the full therapeutic effect of each drug without the risk of antagonism.

We set up a dual luciferase imaging system for simultaneous imaging of tumors and viruses *in vivo*. In an evaluation of hypoxia response elements we identified a renal cell cancer specific promoter using the novel dual luciferase imaging system. Based on these data and the results obtained in study II, we generated an infectivity enhanced, transductionally and transcriptionally targeted, antiangiogenic oncolytic adenovirus (Ad5/3-9HIF-Δ24-VEGFR-1-Ig) for kidney cancer treatment. Although, the data of study II did not support the combination of oncolytic virus therapy with antiangiogenic approaches for kidney cancer treatment, we believed that the situation could be different for an oncolytic virus that itself expresses an antiangiogenic molecule. Virus mediated expression of an antiangiogenic molecule will result

in high local concentration in the tumor and low systemic levels, thereby minimizing toxicity. The high local concentration within the tumor might result in necrosis inside the tumor, whereas a systemically administered antiangiogenic molecule might predominantly inhibit the vasculature from the outside of the tumor. Therefore, viral reinfection of the tumor might not be as much inhibited by an antiangiogenically armed adenovirus. Unfortunately, in a subcutaneous kidney cancer model Ad5/3-9HIF-Δ24-VEGFR-1-Ig did not result in smaller tumor sizes compared to the unarmed control virus. However, we found markedly decreased numbers of blood vessels in tumors treated with the antiangiogenic virus that might have led to larger areas of necrosis, which unfortunately does not necessarily result in smaller tumor sizes. It seemed that in an intraperitoneal model of kidney cancer representing a clinically more relevant model, Ad5/3-9HIF-Δ24-VEGFR-1-Ig could exhibit its potential much better since significantly prolonged survival compared to all other groups was observed.

Cancer stem cells might be key players in the relapse of tumors and in the formation of metastases. We found that growth of tumors induced with putative actual cancer stem cells, could be inhibited with capsid modified adenoviruses. Therefore, oncolytic adenovirus therapy might be useful for breast cancer treatment especially with regard to prevention of relapse and metastasis.

In conclusion, the studies in this thesis contribute to the understanding of how oncolytic adenoviruses can be improved to become viable treatment options for cancer. In particular, we provide tools for development and evaluation of oncolytic viruses and we present for the first time that combining three targeting moieties with an arming approach in one oncolytic virus is feasible and results in an anticancer agent with improved properties. Furthermore, evidence that oncolytic viruses might be useful for killing tumor initiating cells is provided.

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