APPROACHES FOR IMPROVING THE SAFETY AND EFFICACY OF ADENOVIRAL GENE THERAPY

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"The whole of science is nothing more than a refinement of everyday thinking."

Albert Einstein

To my parents

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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. Diaconu I, Denby L, Pesonen S, Cerullo V, Bauerschmitz GJ, Guse K, Rajecki M, Dias JD, Taari K, Kanerva A, Baker AH and Hemminki A.
Serotype chimeric and Fiber-Mutated Adenovirus Ad5/19p-HIT for Targeting Renal Cancer and Untargeting the Liver.
Human Gene Ther. 2009 Jun; 20:611-620.

II. Diaconu I, Cerullo V, Escutenaire S, Kanerva A, Bauerschmitz GJ, Hernandez-Alcoceba R, Pesonen S and Hemminki A.
Human adenovirus replication in immunocompetent Syrian hamsters can be attenuated with chlorpromazine or cidofovir
J Gene Med. 2010 May;12(5):435-45.

III. Guse K, Diaconu I, Rajecki M, Sloniecka M, Hakkarainen T, Ristimäki A Kanerva A, Pesonen S, Hemminki A.
Ad5/3-9HIF-Δ24-VEGFR-1-Ig, an infectivity enhanced, dual-targeted and antiangiogenic oncolytic adenovirus for kidney cancer treatment.
Gene Ther. 2009 Aug; 16(8):1009-20.

IV. Diaconu I, Cerullo V, Ugolini M, Escutenaire S, Loskog A, Eliopoulos A, Kanerva A, Pesonen S*, Hemminki A*.
Immune response is an important determinant of the anti-tumor effect of an oncolytic adenovirus coding for CD40L.
Manuscript

ii. Abbreviations

Ad	adenovirus
ADP	adenovirus death protein
bp	base pair
CAR	coxsackie-adenovirus receptor
CD	cytosine deaminase
Cox-2	cyclooxygenase-2
CBGr	click beetle green
CBr	click beetle red
CEA	carcinoembryonic antigen
CMV	cytomegalovirus
CR	constant region
CRAd	conditionally replicating adenovirus
CTL	cytotoxic T-lymphocytes
DMEM	Dulbecco's Modified Eagle's medium
DNA	deoxyribonucleic acid
FACS	fluorescence activated cell sorting
FC	fluoro cytosine
FCS	fetal calf serum
FU	fluoro uracil
GCV	ganciclovir
GFP	green fluorescent protein
GMCSF	granulocyte macrophage colony stimulating factor
h	hour
HCC	hepatocellular carcinoma
HEK	human embryonic kidney
HRE	hypoxia response elements
HSV-TK	herpes simplex thymidine kinase
hTERT	human telomerase reverse transcriptase
IFN	interferon
Ig	immunoglobulin
i.ha.	intrahepatic artery
IL	interleukin
i.p.	intraperitoneal
i.t.	intratumoral
ITR	inverted terminal repeat
i.v.	intravenous
Jak/STAT	Janus kinase/ Signal Transducers and Activators of Transcription
LacZ	β-galactosidase
luc	luciferase
MAPK	mitogen activated protein kinase
MAV	mouse adenovirus
MHC	major histocompatibility complex

MOI	multiplicity of infection
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium);CellTiter96 AQ_{ueous} One Solution-Cell Proliferation Assay
NF-κB	nuclear factor κB
NK	natural killer cells
NOD-LRR	nucleotide binding oligomerization domain/leucine-rich repeat
oct4	octamer-4
PAMP	pathogen-associated molecular patterns
PCR	polymerase chain reaction
pfu	plaque forming unit
pK	polylysine
PKR	protein kinase RNA-activated
PRR	pattern-recognition receptors
PSA	prostate specific antigen
Rb	retinoblastoma
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
RPMI	cell culture media developed at Roswell Park Memorial Institute
SEAP	secreted alkaline phosphatase
s.c.	subcutaneous
SCCHN	squamous cell carcinoma of the head and neck
SCID	severe combined immune deficiency
sox2	sex determining region Y box 2
Th	T helper cell
TK	thymidine kinase
TLR	toll like receptor
TNF	tumor necrosis factor
TP	terminal protein
TCID50	tissue culture infective dose 50
VA-RNAs	viral associated RNA
VEGF	vascular endothelial growth factor
vp	virus particle

iii. Abstract

Cancer is a devastating disease with poor prognosis and no curative treatment, when widely metastatic. Conventional therapies, such as chemotherapy and radiotherapy, have efficacy but are not curative and systemic toxicity can be considerable. Almost all cancers are caused due to abnormalities in the genetic material of the transformed cells.

Cancer gene therapy has emerged as a new treatment option, and past decades brought new insights in developing new therapeutic drugs for curing cancer. Oncolytic viruses constitute a novel therapeutic approach given their capacity to replicate in and kill specifically tumor cells as well as reaching tumor distant metastasis. As with any new therapy, safety issues need to be considered. Adenoviral therapy has proved good safety and efficacy in preclinical and clinical set up. Increasingly effective agents are developed and, consequently, replication associated side-effects remain a concern. There is still a lack of understanding to the full realization of efficacy and safety of these anticancer agents.

Adenoviral gene therapy has been suggested to cause liver toxicity. This study shows that new developed transductional targeted adenoviruses, in particular Ad5/19p-HIT, can be redirected towards kidney while adenovirus uptake by liver is minimal. Moreover, low liver transduction resulted in a favorable tumor to liver ratio of virus load.

Further, we established a new immunocompetent animal model – Syrian hamsters. Wild type adenovirus 5 was found to replicate in Hap-T1 hamster tumors and normal tissues. In general, hamster cell lines were found semi-permissive; however, some of them are nearly as permissive as human cells lines, for human adenovirus and exhibited sustained adenovirus replication. There are no antiviral drugs available to inhibit adenovirus replication. In our study, chlorpromazine and cidofovir efficiently abrogated virus replication *in vitro* and showed significant reduction *in vivo* in tumors and liver.

Once safety concerns were addressed together with the new given antiviral treatment options, we further improved oncolytic adenoviruses for better tumor penetration, local amplification and host system modulation. We analyzed two different hypoxia response elements, and found 9HIF to be a good candidate for kidney tumor transcriptional targeted therapy. Further, we created Ad5/3-9HIF-Δ24-VEGFR-1-Ig,

oncolytic adenovirus for improved infectivity and antiangiogenic effect for treatment of renal cancer. This virus exhibited increased anti-tumor effect and specific replication in kidney cancer cells.

The key player for good efficacy of oncolytic virotherapy is the host immune response. Thus, we engineered a triple targeted adenovirus Ad5/3-hTERT-E1A-hCD40L, which would lead to tumor elimination due to tumor-specific oncolysis and apoptosis together with an anti-tumor immune response prompted by the immunomodulatory molecule. This oncolytic adenovirus exhibited a potent oncolytic effect *in vitro* and *in vivo* together with induction of apoptosis. The immunostimulatory molecule, CD40L expressed by adenoviruses, mediated tumor regression by induction of innate and adaptive immune responses.

In conclusion, the results presented in this thesis constitute advances in our understanding of oncolytic virotherapy by successful tumor targeting, antiviral treatment options as a safety switch in case of replication associated side-effects, and modulation of the host immune system towards tumor elimination.

PART B

1 REVIEW OF THE LITERATURE

1.1 Introduction

Cancer is a major public health concern worldwide with an estimated 10.9 million new cases and 6.7 million deaths in 2002 (Parkin et al., 2005). Even though, overall cancer death rates in 2004 compared with 1990 in men and 1991 in women decreased by 18.4% and 10.5% respectively (Jemal et al., 2008), it is estimated that by 2020 cancer incidence will double if preventive measures are not taken (Eaton, 2003). Cancer still remains the second leading cause of death in developed countries and the fourth most common worldwide.

Standard treatment options for cancer can be divided in four main categories: surgery, chemotherapy, radiotherapy and biological therapy but nevertheless there is still no cure for widely metastatic cancer.

- Surgery – is usually performed for localized tumors, and aims for resection of tumors and as much as cancerous tissue possible. It has been the most common treatment option until radiation therapy has been introduced.
- Radiation therapy – uses ionizing radiation to control the tumors. Cancer cells are killed by damaging their DNA and making them unable to multiply.
- Chemotherapy – uses drugs to eliminate the cancer cells. These drugs are effective mainly on cells with a high rate of multiplication. Unfortunately not only cancer cells have this ability, though this treatment option can cause adverse side-effects.
- Biological therapy or targeted therapy – uses drugs that target characteristics of the cancer cells. This targeted therapy blocks biological activities through which the cancer cells can grow and spread.

As stated above, these treatment options are not curative for advanced metastatic cancer. Cancer is a disease caused by mutations in genes either inherited from our parents or even more often these changes occur during our life time. Therefore, researchers strongly believe that gene therapy might be the treatment option for cancer.

1.2 Cancer gene therapy

The emerging field of cancer gene therapy has gained a lot of interest in past decades. The use of viruses as anti-cancer treatments has been a therapeutic approach for almost 100 years. First report dates from 1912, when a patient with cervical cancer received a rabbies vaccination and tumor regression was observed (DePage, 1912). Following this, a wide range of viruses, including adenovirus (Ad), Bunyamwera, coxsackie, dengue, feline panleukemia, Ilheus, measles, mumps, Newcastle disease, vaccinia and West Nile virus, were tested to show their oncolytic potency in animals and humans (Asada, 1974; Gross, 1971; Huebner et al., 1956; Reichard et al., 1992; Southam and Moore, 1951), and a variety of clinical trials were performed as shown in Figure 1. Cancer gene therapy approaches can be classified in three main categories: immunotherapy, oncolytic virotherapy and gene transfer. In this context, new approaches have been developed for targeting of cancer cells, cancer vasculature, the immune system and the bone marrow.

There are multiple strategies to replace or repair the targeted genes:

- Insert healthy genes in place of abnormal or missing ones: e.g. most of the cancers have defective p53 gene which can be replaced/modified turning a cancer cell to apoptosis
- Ablate the functions of oncogenes which result in end of division of abnormal cells and limit the growth of the tumors
- Introduce genes which make cancer cells more susceptible to chemotherapy and radiotherapy
- Use immunostimulatory molecules or add genes to the immune system cells for better recognition of cancer cells
- Inactivate genes responsible for angiogenesis, which is essential for tumors to grow
- Use ‘suicide genes’ where a pro-drug is given to the patient and is reversed in the toxic form by the converting pro-drug enzyme produced by the cancer cells. Ultimately, neighboring cells are also killed by the so called bystander effect.

Between 1989 and 2009, around 1400 gene therapy clinical trials have been approved as reported in the Gene Therapy Clinical Trials Worldwide (provided by the Journal of Gene Medicine). Out of these, 70% are for cancer treatment and among them, 280 clinical trials worldwide were started for adenoviral cancer gene therapy.

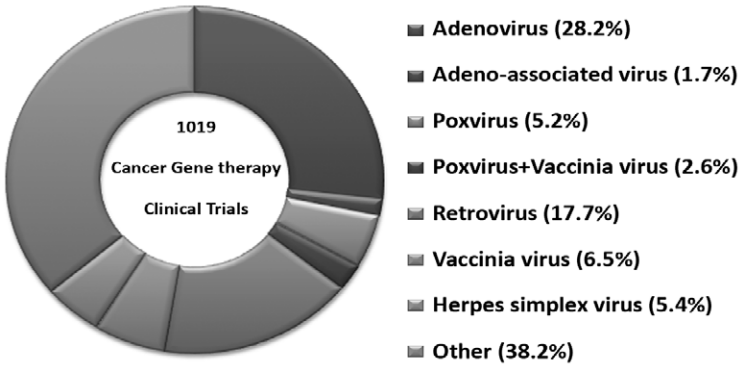

Figure 1 Cancer Gene Therapy trials reported till the end of 2009 in http://www.wiley.co.uk/genetherapy/clinical/ database.

1.3 Adenoviruses

Adenoviruses have potential as vectors for gene therapy because they can be easily genetically altered *in vitro* using recombinant DNA techniques. During the past 20 years, Ads were intensively studied and have become the most commonly used gene transfer vectors in the field of gene therapy. Adenoviral vectors are an attractive vehicle for gene transfer *in vitro* and *in vivo* due to easy production of high-titer stocks (up to 10^{13} pfu/ml), remarkable efficiency of each step in the adenovirus cell/nucleus entry process and low pathogenicity in humans. In addition, Ads are able to transduce both dividing and non-dividing cells, but are mostly incapable of genome integration into host cell chromosomes and the viral genome does not undergo rearrangement at a high rate. However, as disadvantages for using adenoviruses, we have three main challenges, such as the expression is transient (viral DNA does not integrate), the viral proteins can be expressed in the host and systemic delivery is hampered by the host immune system. Wild-type adenoviruses can cause mild upper respiratory tract infections but are not a major concern in healthy individuals. Therefore, Ad-based vectors have evolved as an

efficient tool for cancer treatment being successfully utilized in many clinical trials with variable but encouraging results.

1.3.1. Adenovirus structure and life-cycle

Ads were discovered in early 1950 and first isolated from human adenoids in 1953 (Rowe et al., 1953). Since then, 51 human serotypes of the adenoviridae family have been identified and divided into 4 genera (Aviadenovirus, Atadenovirus, Mastadenovirus, Siadenovirus) and 6 species (A-F) (Burmeister et al., 2004; Davison et al., 2003; de Jong et al., 2008; Li and Wadell, 1999). Ads have been shown to be responsible for a variety of illnesses including upper respiratory disease, epidemic conjunctivitis and infantile gastroenteritis (Berk, 2007).

Ads are nonenveloped, icosahedral particles, approximately 90nm in diameter, with fibers projecting from the vertices of the icosahedron. The virions contain 87% protein, 13% DNA and trace amounts of carbohydrate but no lipids (Rux and Burnett, 2004). Electron microscopy and X ray crystallography investigations revealed that the icosahedral shape contains 20 triangular surfaces and 12 vertices. The capsid of the virion is composed of 252 capsomere subunits: 240 hexons and 12 pentons surrounding a DNA-protein core complex. Each hexon is surrounded by 6 neighbouring subunits, while each penton is enclosed by 5 neighbouring subunits and has a fiber projecting from its vertex (Figure 2). The protein content of the capsid consists of three major capsid proteins (hexon (720 molecules/virion), penton base (60 molecules/virion) and knobbed fiber (36molecules/virion) (Zhang and Imperiale, 2003), four minor capsid proteins (VI, VIII, IX and IIIa) and four core proteins (Terminal protein, Protein Mu, VII and V) (Figure 2) (Berk, 2007; Nemerow et al., 2009).

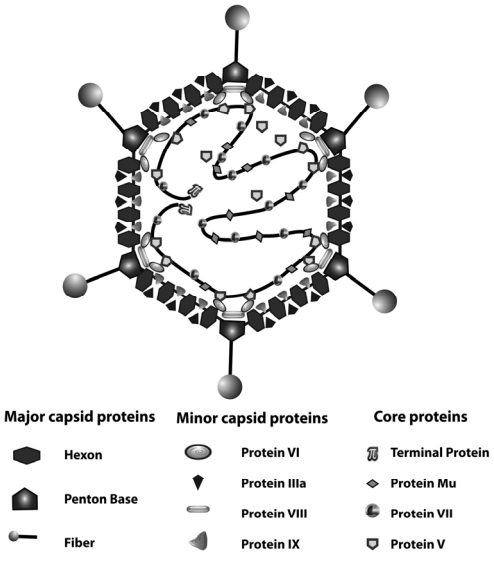

Figure 2 Schematic diagram of the Ad virion summarizing the location of the eleven structural proteins

The complete sequence of at least eight human and fifteen nonhuman Ads have been identified including Ad5 determined by Chroboczek and colleagues (Chroboczek et al., 1992) (Fig. 3).

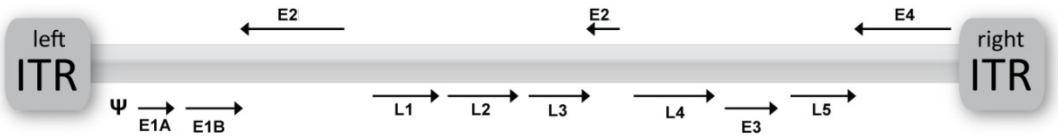

Figure 3 Schematic representation of Ad genome with early genes (E1-E4), late genes (L1-L5) and ψ representing the replication start point-adapted from (Berk, 2007).

The virus genome is a linear, double-stranded DNA with a terminal protein (TP) covalently attached to the 5' termini (Rekosh et al., 1977) which has inverted terminal repeats (ITRs). It is typically around 36 kbp in length and the ITR's sequences are around 100-140 bp at each end. Furthermore, the genome contains 5 early transcription units (E1A, E1B, E2, E3 and E4), three delayed early units (IX, IVa2 and E2 late) and one major late unit that is processed to generate 5 families of late RNAs (L1-L5). Adenovirus genome also contains viral associated RNAs (VA-RNAs) which play a critical role in regulating the translation process (Thimmappaya et al., 1982). It has been shown that, with the exception of E4 (Leppard, 1997), each early and late transcription unit encodes a series of polypeptides with related functions. E1A proteins are known to activate transcription and trigger entry into the S phase of the cell cycle, which renders the cell more susceptible to viral replication (Berk, 2007). Two E1B proteins interact with E1A gene products to induce cell growth (Berk, 2007). Three E2 proteins are reported to function in DNA replication (Berk, 2007), while E3 proteins mostly play a role in modulation of the antiviral host response to adenoviruses and are therefore dispensable for *in vitro* replication (Wold et al., 1999). One of the E3 proteins facilitates efficient progeny virus release by late cytolysis of infected cells and has been named adenovirus death protein (ADP) (Tollefson et al., 1996). In addition, another protein of the E3, 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 cytotoxic T lymphocytes (CTLs) (Kaplan et al., 1999). Late proteins are either capsid components, or proteins involved in capsid assembly (Berk, 2007).

The adenovirus life cycle mainly takes place in two phases: the early phase, which lasts for 5 to 6 hours and includes adsorption, penetration, movement of uncoated virions, transport to the nucleus and production of early genes and the second phase which starts by expression of late genes and assembly of the virus progeny as exemplified in Figure 4. Typically this cycle is completed in 24 to 36 hours.

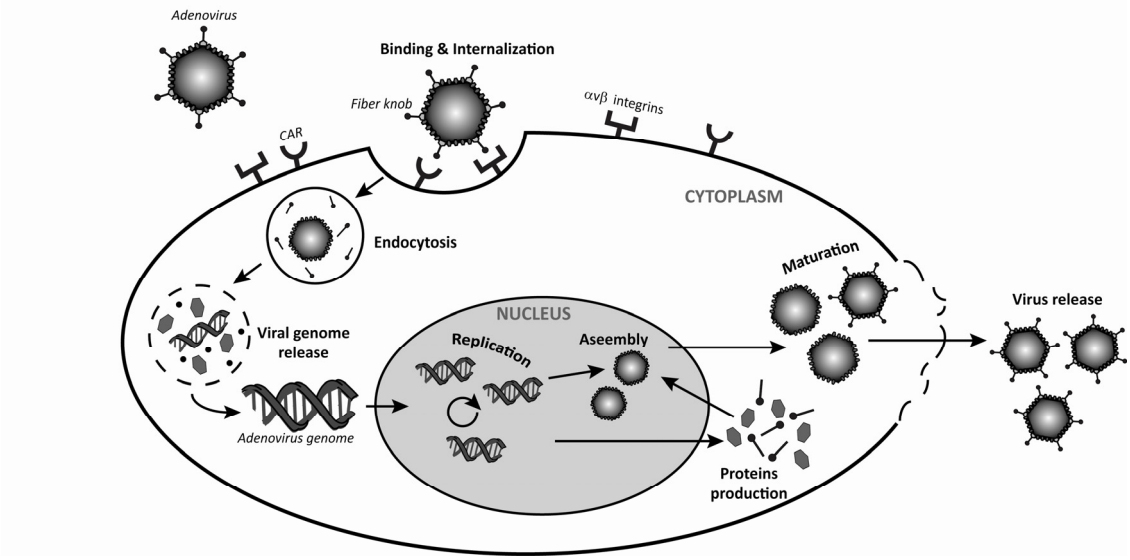

Figure 4: Adenovirus life-cycle - adapted from (Hakkarainen, 2005)

The first step in adenoviral infection is initiated with the binding of the adenovirus fiber knob to a high affinity cell surface receptor. Most adenovirus species, subgroups A and C-F, have been shown to utilize the coxsackie- and adenovirus receptor (CAR) (Bergelson et al., 1997; Roelvink et al., 1999). The initial virus binding is followed by receptor-mediated endocytosis in clathrin-coated pits, which is mediated by interactions between an arginine-glycine-aspartic acid (RGD) motif within the viral penton base and cellular $\alpha_v\beta$ integrins (Wickham et al., 1994; Wickham et al., 1993). Once internalized, the pH drops within the endosome resulting in a conformational change of the capsid structure. Endosome membrane is disrupted and the virus particle attaches to the nuclear pore complex of the nucleus. After the capsid attaches to the nuclear pore

complex, the viral DNA is injected into the nucleus (Berk, 2007). The process of early gene transcription is initiated with the production of the viral E1A transactivator and has three major consequences. First, cells enter the S phase of the cell cycle to replicate the DNA. This is achieved through a number of processes including the release of E2F upon E1A binding to the Rb tumor suppressor, inhibition of the p53 tumor suppressor by E1B-55K and direct blockage of apoptosis by the Bcl-2 homologue E1B-19K. Second, is the inhibition of cellular antiviral responses which includes the retention of major histocompatibility complex I (MHC I) molecules in the endoplasmic reticulum by E3-gp19K in order to suppress CTL-mediated cell death. The third main consequence is the synthesis of gene products needed for viral DNA replication (Berk, 2007). Following synthesis of the early gene products, DNA replication occurs within the nucleus and concomitantly the delayed early IX and IVa2 genes are transcribed. Translation of late RNA species leads to production of capsid proteins within the cytoplasm followed by their translocation to the nucleus. This ultimately results in genome packaging into assembled capsids, which are not released until the cell is lysed. Cell lysis requires disruption of intermediate filaments, such as vimentin and cytokeratin, which leads to collapse and rupture of the cell (Belin and Boulanger, 1987; Chen et al., 1993).

Once released from the cell, Ad can maintain a long term association with its host. Ads encode several gene products responsible for the evasion of the immune system and persistence in the human body. E1A gene products counteract the immune response by three mechanisms (Hayder and Mullbacher, 1996), such as: block IFN gene activation (Routes et al., 1996), interfere with IL-6 at two levels (transcription of IL-6 gene and transduction of IL-6 signal) (Janaswami et al., 1992; Takeda et al., 1994) and interrupt the process of TNF-induced cell death (Eckner et al., 1994; Hamel et al., 1993). E1B-19k gene is also able to counteract the TNF-induced cell death mechanism (White et al., 1992). Adenoviruses also encode two VA RNAs proteins which modulate the IFN response. VA-RNA_1 binds to inactive protein kinase RNA-activated (PKR) preventing it from binding to dsRNA and subsequent IFN production. VA-RNAs also inhibit the RNA interference process (Berk, 2007). The E3 gene products are responsible for the inhibition of cellular antiviral responses. E3-gp19K induces the retention of major histocompatibility complex

class I molecules in the endoplasmic reticulum in order to suppress cytotoxic T-lymphocyte-mediated cell death (McSharry et al., 2008).

1.3.2. Development of targeted adenoviruses

Ads as gene transfer vectors are currently the preferred tool for cancer gene therapy mostly because of their high capacity of gene expression *in vivo*. Ads as vectors can be classified in two groups: replication deficient and replication competent.

Replication deficient Ads lack one or more essential viral genes and they are used mostly as gene transfer vectors (Immonen et al., 2004; Merritt et al., 2001). In 'first generation' Ads, usually the E1A gene is deleted and the transgenes of interest are inserted. Given this, virus propagation must occur in specific cell lines, such as HEK 293 (Graham et al., 1977) or 911 (Fallaux et al., 1996), which express E1A gene necessary for viral replication. These viruses are fully competent to enter most human cells and express the transgenes *in vitro* and *in vivo.* These Ads can also have the E3 gene deleted, which does not affect the growth of the vector and, moreover, allows insertion of bigger transgenes into E1 region, since only up to 105% of the genome can be packaged into new virions (Berk, 2007). Further modifications of the Ad genome led to the development of 'second-generation' recombinant Ads. These vectors typically carry deletions in E2 or E4 in addition to deleted E1 and E3 regions and allow increased cloning capacity. 'Second-generation' vectors offer reduced viral gene expression and also block viral replication in transduced tissue. Overall, this renders these vectors less immunogenic than 'first generation' Ads (Shen, 2007). Later, Ad vectors lacking all viral genes were constructed to further decrease the vectors immunogenicity (Fisher et al., 1996; Kochanek et al., 1996). These vectors, so called 'gutless' or helper-dependent, retain the cis-acting sequences such as the inverted terminal repeats (ITR) at each end of the genome and also the packaging sequence at the left end, which is required for the replication and packaging of the newly formed virions.

Replication competent Ads, 'oncolytic adenoviruses', are mainly used for cancer gene therapy. These viruses kill cancer cells as part of their natural life cycle, lyse the cells and subsequent infect the neighboring ones. Oncolytic virus selectivity for tumors can occur due to two mechanisms: either at the level of infection or replication. To increase the selectivity of these vectors for cancer cells, specific promoters are inserted driving the

genes responsible for replication. To enhance replication and to target these viruses to tumor cells we can delete the genes responsible for viral replication in normal cells and retain the ones responsible for viral replication in cancer cells with deregulated cell cycle.

1.3.3 Tumor targeted adenoviruses

The perfect vector system to use for gene therapy would be a vector, which administered by a noninvasive route would target cells specifically within the target tissue and would produce therapeutic amount of transgene with the desired regulation. Tumors are heterogeneous, thus specific tumor targeting can be achieved using three different strategies: 1) transductional targeting, 2) transcriptional targeting and 3) generation of new conditionally replicating viruses.

1.3.3.1 Transductional targeting to cancer cells

The effectiveness of gene therapy is governed mainly by the ability of the vector to be delivered to the relevant tissue and, once there, to express the gene product in appropriate quantities (Russell, 2000). Recombinant Ad5 is the most commonly used vector for gene therapy. Several strategies have been employed for targeting adenoviruses including: modification of the knob domain by adding different peptides into the c-terminus or HI-loop, pseudotyping with fibers derived from other Ad serotypes for binding other receptors than CAR (Bauerschmitz et al., 2006; Kanerva et al., 2002a; Kanerva et al., 2002b; Kanerva et al., 2003; Kangasniemi et al., 2006; Ranki et al., 2007a; Ranki et al., 2007b; Sarkioja et al., 2006) and chemical Ad5 capsid modification. For the first approach, several modalities have been employed (Mizuguchi and Hayakawa, 2004) to insert peptides into the HI loop of the fiber knob. These include those discovered by phage display library to show high affinity for vascular endothelial cells (Havenga et al., 2001; Havenga et al., 2002; Nicklin et al., 2000), cancer cells (Nicklin et al., 2003), RGD in HI loop (Bauerschmitz et al., 2002; Dmitriev et al., 1998), transferrin receptors (Xia et al., 2000), vascular smooth muscle cells (Work et al., 2004) and kidney derived peptides (Denby et al., 2004; Denby et al., 2007).

Second approach has featured pseudotyping fibers from all known adenovirus subgroups in the context of an Ad5 capsid. For example, altered vector tropism was reported by substitution of the Ad5 fiber protein into that of Ad3 which was first reported by Krasnykh and colleagues (Krasnykh et al., 1996). These approaches of

swapping the Ad5 fiber with fibers or fiber knobs of other adenovirus serotypes (Ad3, Ad7, Ad11, Ad16, Ad17, Ad35) and creating "pseudotyped" hybrid vectors, succeeded in changing the tropism of Ad5 to receptors expressed on tumor cells more than CAR (Mizuguchi and Hayakawa, 2004). Most of the reported studies have utilized cell lines or primary patient samples *in vitro*, or intratumoral (i.t.) administration *in vivo*, while data on systemic delivery has been scarce.

More importantly, several recent publications suggest that the biodistribution of Ad, following intravenous (i.v.) administration, is not determined by the fiber knob. While knob modification may be able to enhance tumor transduction, it has not reduced uptake by non-target organs such as the liver. Instead, the fiber shaft may play a key role in determining biodistribution (Bayo-Puxan et al., 2006; Haviv et al., 2002; Kanerva et al., 2002b; Ranki et al., 2007b; Sarkioja et al., 2006; Shayakhmetov et al., 2002). For example, Ad5 pseudotyped with the Ad35 fiber (including shaft) or interaction of Ad5 hexon with blood factors demonstrated less accumulation of the virus into the liver compared to Ad5 following i.v. administration (Shayakhmetov et al., 2002; Waddington et al., 2008). Also, mutation of the KKTK region of the fiber shaft has been reported to alter virus biodistribution (Alemany and Curiel, 2001; Bayo-Puxan et al., 2006). Therefore, approaches that have switched the complete fiber (instead of just the knob) or mutated relevant regions of the shaft may be appealing for influencing the biodistribution of systemically delivered virus.

1.3.3.2 Transcriptional targeting to cancer cells

Using transcriptional targeting strategies for genetically engineered Ads, gene expression is controlled by a tumor-tissue specific promoter. Two main strategies for tumor-selective adenoviral replication have been evaluated: first, deletions in the Ad genes responsible for replication in normal cells but dispensable in cancer cells, and second, viral genes responsible of replication placed under tumor tissue specific promoters.

Infectivity enhanced and highly effective viruses can cause toxicity at high doses. As a result, improvements in selectivity may reduce the side-effects. In this regard, utilization of tumor specific promoters, such as: the human telomerase reverse transcriptase promoter (hTERT) (Ito et al., 2006; Takakura et al., 2010; Yokoyama et al., 2008), the α–

fetoprotein promoter (Hallenbeck et al., 1999), the prostate-specific antigen (PSA) promoter (Rodriguez et al., 1997), a mucin-like DF3 antigen promoter (Kurihara et al., 2000), cyclooxygenase-2 (COX-2) for gastrointestinal cancer (Yamamoto et al., 2001), carcinoembryonic antigen (CEA) for colorectal liver metastases (Brand et al., 1998), human glandular kallikrein 2 for prostate cancer (Xie et al., 2001) and hypoxia response elements (HREs) for kidney cancer (Binley et al., 2003) may help to reduce the adverse effects of oncolytic viral therapy. Furthermore, we can insert microRNA's targets in the untranslated region of the E1A gene to specifically target liver hepatocytes. This modification showed significant decrease of replication of the vector in hepatocytes without altering the replication of the vector in the other cells (Ylosmaki et al., 2008).

1.3.3.3 Conditionally replicating adenoviruses for cancer therapy

The first oncolytic adenovirus, named dl1520, has been described by Barker and Berk in 1987. This replication competent adenovirus known as ONYX-015 is an Ad2/5 chimera, which lacks functional E1B-55K (Bischoff et al., 1996). E1B-55K gene binds and inactivates p53 in infected cells resulting in induction of S-phase, which is required for effective virus replication (Yew and Berk, 1992). Theoretically, this virus should replicate only in cells where p53 is not functional, which is the case of most human cancers (Ries et al., 2000). Other E1B-modified oncolytic adenoviruses were generated by deletion of both genes, E1B-55K and E1B-19K, which additionally target Rb negative cancers (Duque et al., 1999). The replication efficiency of this E1B-55K mutant seldom reaches the rate of replication of the wild type adenovirus (Howe et al., 2000). This may be explained by the other function of E1B-55K, as a mRNA transporter, which might result in inefficient replication of ONYX-015 (Dix et al., 2001).

Two conserved regions in E1A, constant region 1 (CR1) and 2 (CR2), are essential for binding of Rb protein, which favors E2F release and induction of S-phase. These regions have higher affinity than the normal Rb-E2F binding which occurs normally in cells. Deletions in CR1 and CR2 regions lead to defective Rb binding in normal cells resulting in cell cycle arrest and no S-phase induction - necessary for virus replication. On the contrary, cancer cells are defective on Rb pathway and allow virus replication to occur. These CR1-deleted mutants are barely selective and viral replication is attenuated (Heise and Kirn, 2000).

In contrast, a single 24bp deletion in CR2 preserves the oncolytic activity in Rb negative tumor cells and makes these vectors's replication deficient in normal cells (Fueyo et al., 2000; Heise and Kirn, 2000). Since many cancer types have E2F overexpression, it has been thought that E2F would be a perfect candidate as a promoter for the E1A. In this regard, Johnson and colleagues constructed an oncolytic virus ONYX-411 in which both E1 and E4 are driven by the E2F-1 promoter and, in addition, E1A has a deletion into the CR2 region (Johnson et al., 2002). This virus retained the oncolytic potency in cancer cells at the same levels with a wild-type both *in vitro* and *in vivo* - following systemic administration. In summary, these conditionally replicating adenoviruses are designed to replicate in, and subsequently kill only cancer cells without affecting normal cells.

1.4 Clinical trials with oncolytic adenoviruses

Clinical trials and treatments with oncolytic and non-replicating adenoviruses are dating from 1950's; however, there are no conclusive results from early clinical trials. Only during the last decades, clinical trials with oncolytic viruses elucidated the efficacy of this virotherapy. In 1996, a phase I clinical trial was initiated with the direct injection of dl1520 (see 1.3.3.3.) for head and neck cancers (Ganly et al., 2000). In this trial, 14% of patients showed tumor regression rates of >50%. During the following years this vector was used under the name ONYX-015 in a total of 18 clinical trials (Phase I and II) with almost 300 treated patients (Alemany, 2007; Yu and Fang, 2007). Better results were noticed when the virus was used in combination with cisplatin and 5-fluorouracil (5-FU) in a Phase II trial (Khuri et al., 2000), as about 65% of treated patients with head and neck tumors had objective responses. The first oncolytic adenovirus that reached completed Phase III trial was H101 (similar to dl1520, but also deleted for E3). This oncolytic Ad showed 79% response rate in the combination with chemotherapy versus 40% in the chemotherapy only group (Xia et al., 2004).

Table 1 shows a selection of the above described clinical trials and others, using oncolytic adenoviruses.

Adenovirus	Tumor-specificity	Phase	Cancer	Route of admin.	Responses/ total patients	Reference
dl1520 (ONYX-015)	E1B-55 kDa-deletion	I	Head & Neck	i.t.	2/22	(Ganly et al., 2000)
dl1520 (ONYX-015)	E1B-55 kDa-deletion	II	Head & Neck	i.t.	4/30	(Nemunaitis et al., 2001b)
dl1520 (ONYX-015) chemotherapy	E1B-55 kDa-deletion	II	Head & Neck	i.t.	19/30	(Khuri et al., 2000)
dl1520 (ONYX-015)	E1B-55 kDa-deletion	I	Pancreatic	i.t.	0/23	(Mulvihill et al., 2001)
dl1520 (ONYX-015)	E1B-55 kDa-deletion	I	Ovarian	i.p.	0/16	(Vasey et al., 2002)
dl1520 (ONYX-015)	E1B-55 kDa-deletion	I	Metastatic lung	i.v.	0/10	(Nemunaitis et al., 2001a)
dl1520 (ONYX-015)	E1B-55kD deletion	I	HCC	i.v., i.t.	1/5	(Habib et al., 2002)
ONYX-015 + 5-FU + leucovorin	E1B-55kD deletion	I	Colorectal cancer	i.ha.	1/11	(Reid et al., 2001)
ONYX-015 + etarnercept	E1B-55kD deletion	I	Advanced cancers	i.v.	0/9	(Nemunaitis et al., 2007)
Ad5-CD/TKrep + GCV/5-FU + radiation	E1B-55kD deletion + TK/CD transgene	I	Prostate cancer	i.t.	15/15	(Freytag et al., 2003)
ONYX-015 + 5-FU	E1B-55kD deletion	I-II	HCC and colorectal	i.t., i.ha., i.v.	3/16	(Habib et al., 2001)
dl1520 (ONYX-015)	E1B-55 kDa-deletion	II	Colorectal	i.v.	0/18	(Hamid et al., 2003)
dl1520 (ONYX-015)	E1B-55 kDa-deletion	I	Glioma	i.t.	3/24	(Chiocca et al., 2004)
CV787 (CG7870)	E1A under probasin and E1B under PSA promoter	I-II	Prostate	i.v.	0/23	(Small et al., 2006)
CV706	E1A expression under PSA promoter	II	Prostate	i.t.	5/20	(DeWeese et al., 2001)
H101	E1B-55 kDa-deletion	I-II	Multiple	i.t.	3/15, 14/46	(Yu and Fang, 2007)

H101+ chemotherapy	E1B-55 kDa-deletion	III	Head & Neck	i.t.	41/52	Yu and Fang, 2007)
H101 + cisplatin/adriamycin + 5-FU	E1B-55kD deletion	III	SCCHN	i.t.	71/160	(Xia et al., 2004)
ONYX-015 + MAP chemotherapy	E1B-55kD deletion	I-II	Sarcoma	i.t.	1/6	(Galanis et al., 2005)
ONYX-015 + gemcitabine	E1B-55kD deletion	I-II	Pancreatic cancer	i.t.	2/21	(Hecht et al., 2003)

1.5 Efficacy of adenoviral gene therapy

Efficacy data of adenoviral gene therapy has been scarce since the primarily end point of the clinical trials has been the safety. Still, a plethora of efficacy data was reported in animal models (Bauerschmitz et al., 2002; Kanerva et al., 2002a; Kanerva et al., 2003; Kangasniemi et al., 2006; Raki et al., 2007; Ranki et al., 2007b). Preclinical efficacy of adenoviral gene therapy shows promising results and tumors were eradicated following i.t. administration of the oncolytic adenovirus (Bischoff et al., 1996; Cerullo et al., 2010; Heise and Kirn, 2000). As depicted in Table 1, adenoviral gene therapy alone has minimal effect, but in combination with chemotherapy or radiotherapy, the efficacy of the treatment is increased. Best results are reported for ONYX-15, as mentioned above in clinical trials chapter. Moreover, another version of this virus, H101, gained marketing approval for cancer treatment in China (Yu and Fang, 2007), and is intended for i.t. injection of head and neck cancers or other accessible solid tumors in combination with chemotherapy. More recently, new era of oncolytic adenoviruses, Ad5/3-Cox2L-Δ24, ICOVIR 7, Ad5/3-Δ24-GMCSF and Ad5-Δ24-GMCSF were tested in an advanced therapy access program (ATAP). The results showed low toxicity of the vectors and no severe side-effects. Additionally, objective responses were noticed for almost 60% of patients in these studies as follow: 11/18 for Ad5/3-Cox2L-Δ24 (Pesonen et al., 2010); 9/17 for ICOVIR-7 (Nokisalmi et al., 2010); 8/12 for Ad5/3-Δ24-GMCSF (Koski et al., 2010) and 8/16 for Ad5-Δ24-GMCSF (Cerullo et al., 2010).

1.6 Safety considerations for adenoviral gene therapy

As for any new treatment option, safety concerns need to be addressed. Ads commonly cause respiratory diseases, but may also cause illness, such as gastroenteritis and conjunctivitis. Even though Ads based on serotype 5 have proved efficient *in vitro* and *in vivo* in preclinical studies and safe in patients, their therapeutic effect as single agents therapy is still uncertain (Hermiston, 2006). The main safety concerns for adenoviral gene therapy are: 1) liver toxicity, 2) host immune response and 3) lack of antiviral treatment in case of uncontrolled virus spread in the body.

1.6.1 Liver toxicity

Adenoviral tissue tropism differs among the serotypes. It is well established that following i.v. administration of Ads either replication deficient or competent, liver is sequestering a big part from the input dose. Liver toxicity started to become a real concern, especially after a patient died due to increased liver enzymes and cytokine storm produced following intra-hepatic administration (Raper et al., 2003). The route of administration plays a critical role in virus biodistribution and toxicity. Following i.v. administration of the virus, kupffer cells, the macrophages of the liver, are the main cells taking up the virus, which leads to necrosis of these cells. In preclinical studies in mice it was shown that a second dose of virus administration can transduce the liver better. This was observed due to no responsiveness of kupffer cells saturated from the initial dose (Manickan et al., 2006). Moreover, Waddington and colleagues showed that liver transduction is mediated through the interaction of adenoviral hexon protein with the blood coagulation factor X (Waddington et al., 2008).

Successful approaches to overcome liver tropism of adenoviral gene therapy include: serotype switching, warfarin treatment and coating Ad5 with high molecular weight polyethylene glycol (Wong et al., 2010). There is no clear evidence that adenoviral gene therapy could induce liver toxicity in humans. Therefore, adenoviral liver tropism is still subject of debate as mouse liver is known to uptake human adenoviruses more efficiently than other mammals studied (Yamamoto and Curiel, 2010).

1.6.2 Host immune response

Adenoviruses elicit a strong immune response along with increased transduction and replication inside the tumors. This can be a disadvantage for adenoviral therapy, leading to rapid clearance of the virus and preventing virus replication and spreading inside the tumor (Prestwich et al., 2009). New strategies for engineering adenoviral vectors have been employed to overcome these immunological barriers (Cerullo et al., 2010; Koski et al., 2010; Loskog et al., 2005). Host defense mechanisms towards adenoviruses can be classified in innate and adaptive immune responses. For the adaptive immunity, the host has four options to respond to Ad: 1) cellular immune responses mediated by T cells; 2) humoral response orchestrated by B-cells and leading to the production of neutralizing antibodies; 3) production of interferons (IFNs) to ablate the intracellular activities of the invading virus; and 4) induction of apoptosis by switching into proapoptotic proteins.

1.6.2.1 Innate immune responses

The innate immune response is the host's first line of defense. Ads induce the innate responses immediately after infection (Raper et al., 2003; Zhang et al., 2001). The induction of the innate immune response following adenovirus infection has been well studied both *in vitro* and *in vivo* (Cerullo et al., 2007; Muruve et al., 2004; Tuve et al., 2009). It became of great interest, in particular because of the one and only lethal adverse effect reported with Ad and thought to be due to innate immune response, which provoked cytokine storm, intravascular coagulopathy and multiorgan failure (Raper et al., 2003). Even though many improvements have been made to understand the mechanisms of interaction of viruses with the innate immune system, still little is known. The innate immune response is the major player for the clearance of adenovirus from the body (Lenaerts et al., 2008). Host cells have a range of strategies to overcome any danger signal by releasing specific cytokines and chemokines, leading to recruitment of neutrophils responsible for the inflammatory response (Muruve et al., 1999). Neutrophils recruited at the site of infection produce cytokines which lead to amplification of the antiviral immune cascade. At the same time, recruitment of macrophages and natural killer (NK) cells and activation of complement are important factors for the clearing of the adenovirus (Worgall et al., 1997). Macrophages, and specially monocytes, are phagocytes which release antiviral cytokines and effectively

present antigens necessary for induction of adaptive immune response (Guidotti and Chisari, 2001). On the other hand, NK cells spontaneously kill MHC-I deficient tumor cells (Whiteside and Herberman, 1995). They mediate the cytotoxicity via perforin and induction of different cytokines. As described by Smyth and colleagues, NK cells are highly responsive to many cytokines such as IL-2, IL-12, IL-15 and IFNs, and they increase their cytolytic, secretory, proliferative and anti tumor activities (Smyth et al., 2001). More recently, an increasing body of evidence is pointing out the importance of the Toll-like receptor family (TLRs) as a major factor in modulating the innate immune response towards adenoviruses (Cerullo et al., 2007). TLRs interact with various viral components triggering part of the immune response to adenoviral vectors. TLR9, an endosomal receptor, is activated by double strand DNA (dsDNA). This receptor is able to sense viral infection at cellular levels and triggers cytokines expression as a response (Cerullo et al., 2007). In addition, TLR2, expressed on cell membrane, is also able to sense a viral infection eliciting part of the characteristic immune response to the adenovirus (Suzuki et al., 2010). Another function of the innate immune system is recognition of structures or products known as pathogen-associated molecular patterns (PAMPs) through a set of receptors called pattern-recognition receptors (PRRs) (Akira et al., 2006). The best studied receptors from this family are TLRs and NOD-LRR (nucleotide binding oligomerization domain/leucine-rich repeat) (Huang and Yang, 2009). These ubiquitous receptors are particularly abundant on dendritic cells and macrophages. This recognition triggers a series of events that finally eradicate viral infection. A principal mechanism for this is mediated through Nf-κB activation which signals via mitogen activated protein kinase (MAPK) pathway and results in transcription of different chemokines and cytokines of the host cell (Ferreira et al., 1999; Girardin et al., 2002; Inohara and Nunez, 2003). Finally, the activation of complement is also an important innate defense mechanism of the host to enhance viral clearance. Appledorn and colleagues demonstrated that complement C3 knock-out mice have a reduced cytokine production upon stimulation with adenovirus (Appledorn et al., 2008).

1.6.2.2 Adaptive immune responses

During the last decade, scientists have discovered new receptors of the innate immune system that can shape the adaptive immune response. Still, there is no such

distinct line between innate and adaptive immunity. These two processes are cross-linked and cannot exist separately. Adaptive immunity is a complex process orchestrating different mechanisms including: cellular immune responses, humoral responses, the role of IFNs in bridging the innate with adaptive response and induction of apoptosis mediated by effector cells.

1.6.2.2.1 Cellular immune response

Cellular immune response against tumors is orchestrated by T cells, and is a balance between induction of anti-tumor response and clearance of virus itself. Several studies demonstrated that cellular immune response towards virus elimination is mainly T cell mediated along with induction of the humoral response and production of IFNs (Russell, 2000; Schagen et al., 2004). T cell mediated response involves both cytotoxic CD8+ and helper CD4+ cells. After the uptake of adenovirus, viral proteins and transgenes are expressed, processed into small oligopeptides and presented on the cell surface. These antigens are recognized by CTLs in a complex with class I proteins of the MHC on the surface of the cell. The binding of CD8+ T cells to this peptide-MHC complex I leads to formation of specific CTLs towards Ad or transgene product (LacZ for instance) (Schagen et al., 2004). Further, the cellular immune response is engaged by CD4+ T helper (Th) cells primarily belonging to Th1 subset (Yang and Wilson, 1995; Yang et al., 1995). These CD4+ cells, in contrast of CD8+ cells, are activated by antigens from the input virions. These antigens are presented through the MHC class II molecules on the surface of the antigen presenting cells. Activated CD4+ cells start to produce IL-2 and IFN- γ (Maraskovsky et al., 1989). These cytokines belonging to Th1 subset, further on induce CD8+ cells differentiation into cytotoxic CD8+ cells (CTLs) (Wille et al., 1989). It has been also suggested that activated CD4+ cells can destroy Ad-transduced cells themselves (Yang and Wilson, 1995).

1.6.2.2.2 Humoral response

Besides cellular immune response, another immune adaptive mechanism towards adenovirus is the humoral response. The humoral response is represented by the production of antibodies targeted towards any pathogen incorporated by cells. In case of adenoviral infection, these antibodies are mainly targeted towards adenoviral capsid proteins (Gahery-Segard et al., 1998; Willcox and Mautner, 1976). These antibodies do

not contribute to virus elimination (Yang et al., 1996) but they prevent adenovirus binding to cells and promote opsonization by macrophages (Schagen et al., 2004). Pre-existing immunity towards wild-type adenovirus occurs in most of the patients. Given this, the humoral immune responses are of importance for planning the dose, route of administration and target tissue.

Humoral response depends on B cell capacity of recognizing viral antigens and producing immunoglobulins. This recognition process is mediated through CD4+ helper cells (Yang et al., 1996). They release immunoglobulins into plasma which specifically recognize the antigens. This process starts with the binding of adenovirus particles to the surface of immunoglobulin of B cells (Schagen et al., 2004). After internalization and processing of the virus, the antigens are exposed on the surface of B-cells through MHC class II molecules (Paul and Seder, 1994). The complex formed can be recognized by the activated T helper cells of the Th2 subset. These activated CD4+ cells start to produce cytokines like IL-4, IL-5, IL-6 and IL-10 which induce B cell transformation into plasma cells (Paul and Seder, 1994). Further on, the plasma cells secrete antibodies which are against adenovirus capsid. Even though it was mentioned that Th1 subset can also induce a small humoral response, this is more involved in antibody-isotype switching (Boom et al., 1988). In conclusion, Th2 cells control the production of Ab isotypes IgG1, IgG2b, IgA and IgE mediated by cytokines like IL-4 while Th1 cells control the switch to IgG2a or Ig3 as a response to IFN-γ secretion (Finkelman et al., 1990; Germann et al., 1995; Schagen et al., 2004).

1.6.2.2.3 Interferons

Interferons are divided in two classes: type I with IFN-α and IFN-β and type 2 with IFN-γ. Interferons are thought to be the bridge between innate and adaptive response to adenoviral infection. They are released very early after virus infection and present certain cell specificity. Type I interferons are thought to play a critical role in both innate and adaptive responses, while type II, IFN-γ, mostly acts for adaptive immune response (Goodbourn et al., 2000). IFN-γ is crucial for many events that occur in tumors, including up-regulation of pathogen recognition, antigen processing and presentation, regulation of the antiviral state, inhibition of cellular proliferation and induction of apoptosis, immunomodulation, and leukocyte trafficking (Schroder et al., 2004). The mechanism of

action for IFNs is mediated through Jak/STAT pathway (Look et al., 1998). Interferons bind to cellular receptors which leads to formation of STAT complexes (Paulson et al., 1999). These complexes are transferred to the nucleus where they bind to interferon-response elements of the cellular DNA and inhibit the intracellular activities of the invading virus (Randall and Goodbourn, 2008).

1.6.2.2.4 Apoptosis

Another strategy for the human body to overcome the viral infection is induction of apoptosis in infected cells. A major player in this process is p53 tumor suppressor protein. This protein regulates the transcription of specific genes which are involved in cell cycle arrest and apoptosis. However, adenoviral gene E1B-19k gene can counteract the proapoptotic effect of the p53 (Han et al., 1996).

Another mechanism for inducing apoptosis is complemented by TNF-α production (Elkon et al., 1997). This cytokine is immediately secreted by macrophages and leukocytes as a response to viral infection. TNF-α plays an important role in virus clearance from the body through direct induction of caspase pathway (Russell, 2000).

Additionally, Fas and Fas-ligand are also involved in the induction of apoptosis. These proteins are reported to be the major mediators in adenovirus elimination from the liver (Chirmule et al., 1999). Adenoviral proteins encoded in the E3 region cause Fas to be removed from the cell surface and degraded. FIP-3 protein gets activated and blocks the NF-κB release, and the proapoptotic pathway is inhibited (Li et al., 1999). Moreover, E1A can induce direct apoptosis mediated through caspase-8 pathway, independent of p53 presence (Putzer et al., 2000).

1.6.3 Antiviral treatment

Wild type adenovirus infections can cause severe or even lethal infections, especially in immunocompromised patients (Claas et al., 2005; Leen et al., 2006). Development of more effective and more potent viruses raised concerns of uncontrolled replication of these vectors once administered in the body. So far, there is no approved treatment for adenoviral infections.

Several classes of nucleoside (e.g. ribavirin) and nucleotide (e.g. cidofovir, adefovir, tenofovir) analogues have been tested *in vitro* (Naesens et al., 2005). Morfin and colleagues have demonstrated *in vitro* that ribavirin's efficacy is specific for species C Ads

(Morfin et al., 2005). Another proposed drug, cidofovir, is by far the most tested drug for anti-adenoviral treatment. Cidofovir (Vistide as commercial name) showed inhibition of adenoviral replication for all serotypes tested (Lenaerts et al., 2008). The selectivity of this drug is due to its higher affinity for viral DNA polymerase if compared to cellular DNA polymerases. The acyclic nucleoside phosphonate compounds get phosphorylated through cellular kinases and further serve as alternate substrates for viral DNA polymerases. New derivatives of these compounds showed potent activity against DNA viruses including Ads, poxviruses and herpes viruses (Lenaerts et al., 2008).

Acyclic nucleoside analogues such as acyclovir or gancyclovir have been proposed as alternative antiviral drugs. Only ganciclovir showed modest efficacy as reported by Raki and colleagues (Raki et al., 2007). Nucleoside and nucleotide analogues have been proposed as potential therapeutic agents but clinical data with these compounds is still subject of debate (Lenaerts et al., 2008). Ribavirin treatment was successful in some studies (Liles et al., 1993; McCarthy et al., 1995) while the lack of efficacy has been reported elsewhere (Ljungman, 2004). Other antiviral approaches suggest targeting the entry of the virus in the cells or altering some mechanisms involved in packaging and assembly of the vector. In this regard, $NMSO_3$, sulfatic sialic acid, was found to inhibit cellular binding of several Ads (Kaneko et al., 2001). Chlorpromazine was also suggested because of its mechanism of action on clathrin coated pits assembly (Wang et al., 1993). This drug has been used for decades as antipsychotic in the clinic (Lehman et al., 2004). Moreover, Kanerva and colleagues found reduced replication of Ad *in vitro* in cell lines and liver explants (Kanerva et al., 2007). However, these results could not be confirmed *in vivo* due to lack of a good animal model (Kanerva et al., 2007).

It is well known that human Ads do not replicate in small animal models like mice or rats, which are often used for assessing biodistribution, efficacy and toxicity of the adenovirotherapy. Lenaerts and colleagues suggested the use of non-human Ads, such as MAV-1 (mouse adenovirus), as an alternative for assessing the efficacy of antiviral drugs (Lenaerts et al., 2008). They tested the antiviral effect of cidofovir in immunodeficient mice treated with MAV-1 and noticed a delay in progression of the disease, but could not prevent the fatal MAV-1 induced disease. The study concluded that immune system plays

a critical role in adenoviral clearance from the body, and the need for an immune competent, syngeneic animal model is obvious (Lenaerts et al., 2008).

Recently, Syrian hamsters have been proposed as a promising animal model for studying replication, toxicity, biodistribution and anti-tumor activity of adenoviruses (Bortolanza et al., 2007; Thomas et al., 2006; Thomas et al., 2008; Toth et al., 2008). However, even though Toth and colleagues (Toth et al., 2008) showed abrogation of virus replication in immunosuppressed animals with a cidofovir analog, it has not been previously demonstrated that adenovirus replication can be significantly reduced in immune competent animals.

1.7 Future directions: arming oncolytic adenoviruses for improving the efficacy

Although many techniques have been employed to genetically engineer adenoviruses, there are still no curative vectors available. Enhancement of the replication and selectivity of adenoviruses towards cancer cells transformed these vectors into 'safe machineries'; however, it might hamper their overall oncolytic effect as anti-cancer drugs. Efficacy of adenoviruses can be further improved by insertion of transgene cassettes, such as VEGF, hCD40L, HSV-TK, GMCSF into the viral backbone. The purpose of these transgenes is to enhance the elimination of cancer cells (Alemany, 2007). Arming oncolytic adenoviruses improves the potency of these vectors by combining their intrinsic oncolytic potency with their ability to deliver tumor-specific transgenes (Liu and Kirn, 2008).

1.7.3 Antiangiogenic gene therapy

Angiogenesis is essential for tumor progression and metastasis. Tumors require new blood-vessel formation to grow and spread. Angiogenesis is coordinated by the balance between the two factors: pro-angiogenic and anti-angiogenic. When this balance is shifted towards pro-angiogenic factors, tumors start to grow beyond $1mm^3$. VEGF and its downstream pathway have a critical role in regulation of angiogenesis. Also angiopoietins, Notch pathway and integrin pathways are involved (Azam et al., 2010). VEGF is by far the most studied angiogenic factor, and a plethora of drugs have been

developed to target it. Many cancer cell lines secrete VEGF *in vitro* and also *in vivo* if cells are injected in animals. In addition many human carcinoma types express VEGF (Ferrara, 2004). Antiangiogenic agents have been shown to alter different stages of angiogenesis. Up to date, we have commercially approved antiangiogenic drugs with proved clinical benefit: bevacizumab, sorafenib, sunitinib and thalidomide (Escudier et al., 2007a; Escudier et al., 2007b; Rini et al., 2008; Stadler, 2005). These agents can be used alone or in combination with chemotherapy. Combination therapy showed more stringent results with the exception of renal cell carcinomas where the agents alone exhibit 30% to 40% improved progression free-survival (Azam et al., 2010).

The use of an armed oncolytic Ad for local expression of antiangiogenic compounds might decrease the systemic exposure/toxicity, while allowing high concentration of the agent in the tumor area. Therefore, this might be an efficient and safe approach for further investigation. Moreover, if these new vectors are administered systemically, they will also engage the normal tissue to express these agents (Wadhwa et al., 2002). Antiangiogenic therapeutic strategies showed good efficacy and safety in preclinical and clinical studies (Escudier et al., 2007a; Escudier et al., 2007b; Rini et al., 2008; Stadler, 2005; Yoo et al., 2007; Zhang et al., 2005). Treatment responses were different between the tumors types studied, perhaps due to different mechanisms of action involved. It is clear that improvements in engineering new vectors along with a better understanding of the mechanisms will facilitate the use of antiangiogenic agents in clinical trials.

1.7.2 Suicide gene therapy

Suicide genes encode an enzyme which will convert a prodrug into its cytotoxic compound inducing cell death. Approaches for suicide gene therapy started with the use of adenoviruses coding for herpes simplex virus thymidine kinase (HSV-TK) gene in combination with the use of the prodrug ganciclovir (GCV) (Wong et al., 2010). GCV gets phosphorylated by HSV-TK, and induces single-strand breaks which lead to cell death. This active metabolite can spread in the tumor mass causing the bystander effect. It has been shown in preclinical studies that Ads coding HSV-TK gene in combination with GCV increased anti-tumor efficacy and survival (Nanda et al., 2001; Raki et al., 2007). However, the efficacy of this strategy might be hampered by the direct effect of GCV on adenovirus replication (Hakkarainen et al., 2006; Raki et al., 2007).

Another strategy for suicide gene therapy is using cytosine deaminase (CD) gene. This gene, when expressed in the cells, converts the nontoxic compound 5-fluoro-cytosine (5FC) to the cytotoxic 5FU (Chalikonda et al., 2008; Foloppe et al., 2008). Oncolytic adenoviruses armed with CD have shown increased anti-tumor efficacy in several different cancer models (Ichikawa et al., 2000; Liu and Deisseroth, 2006). Both strategies described above, HSV-TK and CD, were used alone or in combination with chemotherapy *in vitro* and *in vivo* showing increased anti-tumor effect (Dias et al., 2010; Raki et al., 2007).

Arming oncolytic adenoviruses with suicide genes will exhibit increased anti-tumor effect by combining the effect of oncolytic replication and local prodrug activity. This treatment strategy has been already tested in clinical trials. Currently, one phase III trial for prostate cancer is ongoing using CD/TK fusion gene and ADP protein expressed by an Ad5 based adenovirus in combination with radiotherapy (Wong et al., 2010).

1.7.3 Immunotherapy

The immune system uses a wide plethora of mechanisms in response to adenoviral gene therapy. Efficacy of oncolytic virotherapy is hampered by the immediate innate immune response which leads to rapid clearance of the virus and also due to the adaptive immune response which induces long term immunity against the vector (Cerullo et al., 2010; Tuve et al., 2009). The innate immune response towards viruses can be modulated using cyclophosphamide, an alkylating agent used in cancer treatment (Berd and Mastrangelo, 1988). Cyclophosphamide treatment has been shown to enhance viral replication due to reduction of neutralizing antibodies and reduction of T regulatory cells (Rollinghoff et al., 1977). Moreover, it has been also shown that cyclophosphamide treatment increases viral replication and oncolysis in Syrian hamsters (Thomas et al., 2008).

Recently, many attempts have been made to circumvent the immune response using armed oncolytic viruses. These approaches are aiming for increased viral replication and enhanced anti-tumor activity. Oncolytic adenoviruses expressing IL2, B7-1 or IL-4 cytokines showed increased anti-tumor effect in immunocompetent murine model (Lee et al., 2006; Post et al., 2007). Zhang and colleagues also reported a pronounced anti tumor effect *in vitro* in different carcinoma cell lines and inhibition of tumor growth *in*

vivo using a modified oncolytic adenovirus armed with IL-24 cytokine (Zhang et al., 2009). Further, in a more comprehensive study, Cerullo and colleagues showed increased anti-tumor efficacy of an oncolytic adenovirus expressing GM-CSF both in preclinical and clinical studies. Preclinical data in immunocompetent Syrian hamsters showed eradication of syngeneic hamster tumors and after challenge with the same cell line complete rejection of the tumors was seen. Moreover, tumor specific immune response was demonstrated in patients treated with Ad5-D24-GMCSF (Cerullo et al., 2010).

Oncolytic adenovirus expressing the chemokine RANTES showed recruitment of DCs, NK cells and macrophages at the tumor site, and engagement of CTLs and NK responses promoting tumor regression (Lapteva et al., 2009).

More recently, oncolytic adenoviruses have been designed to express immunomodulatory molecules, such as CD40L (Gomes et al., 2009). Extensive studies in the past were using vectors expressing CD40L for better antigen presentation (Crystal, 1999). Recently, it has been shown that an oncolytic Ad expressing CD40L induced increased anti-tumor activity correlated with cell cycle blockade and induction of apoptosis (Gomes et al., 2009).

Altogether, these studies clearly demonstrate the importance of the immune system in the context of oncolytic adenoviral therapy. Immune modulation combined with oncolytic adenoviruses may help to enhance the initial phases of viral replication inside the tumors and to induce a long-lasting immunity in case of relapsed tumors.

2 AIMS OF THE THESIS

1. To reduce liver tropism and target kidney moieties using capsid modified adenoviruses in the context of systemic delivery.

2. To set up an immunocompetent animal model permissive for adenovirus replication- Syrian hamster model and to evaluate potential antiviral activity of chlorpromazine and cidofovir in this new animal model.

3. To evaluate tissue specific promoters for renal cell cancer and to generate a targeted and armed oncolytic adenovirus for enhanced selectivity and improved anti-tumor efficacy in kidney cancer models.

4. To generate a transcriptionally and transductionally targeted and armed oncolytic adenovirus with CD40L to potentiate the anti-tumor effect due to immune response prompted by the immunomodulating molecule.

3 MATERIALS AND METHODS

3.1 Cell lines (I, II, III, IV)

Characteristics of the cell lines used in the studies are described in Table 1.

Table 1: List of human, hamster and mouse cell lines used in the studies

Cell line name	Description	Used in
293	Human transformed embryonic kidney cells	II, III, IV
911	Human transformed embryonic retinoblasts	II
A549	Human lung adenocarcinoma	II, III, IV
786-O	Human renal cell adenocarcinoma	I, II
786-O-CBGr	Human renal cell adenocarcinoma stably transfected with click beetle green luciferase	II
ACHN	Human renal cell adenocarcinoma	I, II
Caki-2	Human renal cell carcinoma	I, II
769-P	Human renal cell adenocarcinoma	I, II
Sv7tert	Human renal cell carcinoma	I, II
SN12C	Human renal cell carcinoma	II
SN12L1	Human renal cell carcinoma	II
SN12L1-luc	Human renal cell carcinoma stably transfected with firefly luciferase	II
FHS173WE	Human fibroblasts	II
HUVEC	Human umbilical vein endothelial cells	II
Hap-T1	Hamster pancreatic carcinoma	III
H2T	Hamster pancreatic carcinoma	III
DDT1-MF2	Hamster leiomyosarcoma	III
HaK	Hamster kidney derived	III
EJ	Human bladder carcinoma	IV
MB49	Mouse bladder carcinoma	IV
RAMOS-BLUE	Human B-cell cell line	IV

All cell lines were maintained in the conditions recommended by the manufacturer.

3.2 Human specimens

Fresh normal and cancerous tumor samples were obtained with signed informed consent and ethical committee permission from a patient undergoing surgery at Helsinki University Central Hospital - 62 year old female with clear cell carcinoma of kidney, G3, pT3a. The complete kidney was removed and therefore normal kidney tissue could be

obtained from the same patient. Sample processing was performed within one hour from surgery.

3.3 Adenoviruses

Replication deficient viruses and replication competent viruses were amplified on 293 and A549 cells, respectively, and purified on double cesium chloride gradients following standard protocols (Luo et al., 2007). Presence of inserted genes and absence of wild-type virus was confirmed by PCR and sequencing. Virus particle (vp) concentrations were assessed by measuring absorbance at 260nm and plaque forming unit titers were determined with standard $TCID_{50}$ assay on 293 cells.

3.3.1 Replication deficient adenoviruses (I, II, III, IV)

Generation, characterization and main features of the replication-deficient adenoviruses used in these studies are described in Table 2 and in more detail in studies I, II, III and IV.

Table 2: List of replication deficient adenoviruses

Virus name	E1 *	Fiber	Used in	Reference
Ad5LacZ	LacZ	Wild type serotype 5	I	(Yotnda et al., 2004)
Ad5/19p	LacZ	5/19p serotype chimerism	I	(Denby et al., 2007)
Ad5/19p-HIT	LacZ	5/19p serotype chimerism HITSLLS inserted in the HI loop	I	(Denby et al., 2007)
Ad5/19p-HTT	LacZ	5/19p serotype chimerism HTTHREP inserted in the HI loop	I	(Denby et al., 2007)
Ad5/19p-APA	LacZ	5/19p serotype chimerism APASLYN inserted in the HI loop	I	(Denby et al., 2007)
Ad5luc1	Luciferase	Wild type serotype 5	II, III	(Kanerva et al., 2002a)
Ad5/3luc1	Luciferase	5/3 serotype chimerism	II, IV	(Kanerva et al., 2002a)
Ad5-9HIF-luc	Luciferase 9HIF promoter	Wild type serotype 5	II	(Guse et al., 2009)
Ad5-OB36-luc	Luciferase OB36 promoter	Wild type serotype 5	II	(Guse et al., 2009)

Ad5/3-CMV-hCD40L	hCD40L	Wild type serotype 5	IV	Study IV
Ad5/3-CMV-mCD40L	mCD40L	Wild type serotype 5	IV	Study IV

* The marker genes and transgenes 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.

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

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

Table 3: 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	II, III	ATCC[1]
Ad5/3-Δ24	24 bp deletion[2]	Wild type	5/3 serotype chimerism	II	(Kanerva et al., 2003)
Ad5/3-9HIF-Δ24-E3	9HIF promoter and 24 bp deletion[2]	Wild type	5/3 serotype chimerism	II	(Guse et al., 2009)
Ad5/3-9HIF-Δ24-VEGFR-1-Ig	9HIF promoter and 24 bp deletion[2]	VEGFR-1-Ig	5/3 serotype chimerism	II	(Guse et al., 2009)
Ad5/3-hTERT-E1A	hTERT promoter	Δgp19k	5/3 serotype chimerism	IV	(Bauerschmitz et al., 2006)
Ad5/3-hTERT-hCD40L	hTERT promoter	hCD40L	5/3 serotype chimerism	IV	Study IV

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

3.3.3 Construction of Ad5/3-CMV-hCD40L, Ad5/3-CMV-mCD40L and Ad5/3-hTERT-hCD40L (IV)

Ad5/3-hTERT-E1A-hCD40L was generated and amplified using standard adenovirus preparation techniques (Bauerschmitz et al., 2006; Kanerva and Hemminki, 2004; Kanerva et al., 2002b; Volk et al., 2003). Briefly, human CD40L cDNA, kind gift from Prof Eliopoulos, was amplified with specific primers featuring insertion of specific restriction sites SunI/MunI. hCD40L was then subcloned into pTHSN plasmid and subsequently recombined with an pAd5/3-hTERT-E1A (Bauerschmitz et al., 2008; Volk et al., 2003)

rescue plasmid to generate pAd5/3-hTERT-E1A-hCD40L. This plasmid was linearized with PacI and transfected into A549 cells for amplification and rescue.

All phases of the cloning were confirmed with PCR and multiple restriction digestions. Virus production was performed on A549 cells to avoid risk of wild type recombination. hCD40L is under the E3 promoter, which results in replication associate transgene expression.

For construction of non-replicating adenoviruses, expression cassettes with either hCD40L or mCD40L were inserted into the multiple cloning site of pShuttle-CMV plasmid (Stratagene, La Jolla, CA, USA). Shuttle plasmids were recombined with pAdeasy-1.5/3 plasmid (Stratagene, La Jolla, CA, USA), which carries the whole adenovirus genome, and resulting rescue plasmids were transfected to 293 cells to generate Ad5/3-CMV-hCD40L and Ad5/3-CMV-mCD40L.

3.4 *In vitro* studies

3.4.1 Gene transfer assays (I, II, III)

Cell line cells were plated in 24 well plates in triplicates and infected with viruses for 30 minutes in 200 µL of growth medium with 2% FCS. Cells were washed once and complete medium was added. After 24 hours incubation at 37^0C, β-galactosidase (gal) (Galacto Light Plus, Tropix, Bedford, MA) or luciferase (E1500, Promega, WI, USA) assays were performed according to the manufacturer instructions.

For gene transfer assays in study II, cells were plated in 24 well plates in triplicates and 8 hours later were incubated with/without drugs for 18 hours. Cells were infected afterwards with 500 VP/cell +/- drugs for 30 minutes, washed once with PBS and 10% complete medium +/- drugs was added. Cells were incubated at 37° C for 24 hours. After the incubation, cell lysates were analyzed as mentioned above.

Human specimens were minced and washed twice. Samples were resuspended in 2% RPMI and then infected with 5000 VP/cell and β -gal assay was performed as described above.

3.4.2 Cytotoxicity assays - MTS (II, III, IV)

Cells on 96-well plates were infected with indicated viruses at different concentrations (0.1, 1, 10, 100, 1000 VP/cell) in growth medium containing 2% FCS. One hour later, cells were washed and incubated in growth medium containing 5% FCS for 4 to 8 days. Cell viability was then analyzed using MTS assay (Cell Titer 96 AQueous One Solution Proliferation Assay, Promega).

3.4.3 Western blot (III)

Cells were infected with 10 vp per cell, medium was changed after 1 h and cells were incubated for 72 h. 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.

3.4.4 Quantitative real-time polymerase chain reaction (qPCR) (I, II)

DNA was extracted from samples using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). A quantification standard curve was generated and samples were analyzed using SYBR green for study I and Taqman probes for study II. Primers, probes and reaction conditions are described more in detail in study I and study II, respectively.

3.4.5 Quantification of infectious particles of tissue samples (II, III)

Selected organs including tumors were collected and stored at -80^{0}C. The net weight of solid tissues was determined, and tissues were homogenized in growth media without supplements, freeze-thawed, and supernatant was analyzed to determine the plaque forming units (pfu) by $TCID_{50}$ assay on 293 cells. Results were normalized to the net weight of the tumors and organs.

3.4.6 Replication assay *in vitro* (II)

Cell line monolayers were preincubated for 1h with chlorpromazine 0.1µg/ml, cidofovir 5µg/ml, or cytosine arabinoside 0.05µg/ml or growth medium (mock) and then infected with Ad300wt (10 vp/cell) which was added on supernatant. Infection media was replaced by fresh growth medium ± drugs 1.5 hours later. At indicated time points, cells and supernatant were frozen. Replication was analyzed after three freeze/thaw cycles. The number of infectious particles (pfu) in supernatant was titered on 293 cells by $TCID_{50}$ assay using the following formula $T= 10^{1 + d(S-0.5)}$ and transformed to pfu = $T/10^{0.7}$

(AdEasy protocol: Quantum Biotechnology; Qbiogen, Carlsbad, CA). To compare virus burst, infection and subsequent replication over time, A549 and Hap-T1 cells were plated in 96 well plates (as in the standard $TCID_{50}$ assay) and infected with Ad300wt at dilutions from $1:10^7$-$1:10^{14}$. Virus titers were calculated after 10 days and transformed to pfu values as described above.

3.4.7 Flow-cytometry (IV)

Human embryonic kidney 293 cells were infected with viruses expressing hCD40L. Twenty four hours after infection, cells were stained with hCD40L-FITC antibody for 30 minutes and Flow Cytometry analysis was performed on Becton Dickinson instrument (BDLSR).

3.4.8 Functionality assays for hCD40L (IV)

Cell line A549 monolayers ($5x10^6$ cells/T25 flask) were infected with 1000 vp/cell of Ad5/3-hTERT-E1A-hCD40L or Ad5/3-hTERT-E1A and one flask not infected (mock). Supernatant was collected 48h following infection and filtered with 0.02µm filters (Whatman 6809-1002, Maidstone, England). EJ cell line monolayers were transfected with the plasmid pNiFty-Luc (InvivoGen) and cultured overnight. Supernatant collected from A549 monolayers was added on top of the EJ transfected cells and cultured for 12 hours and 1µg/ml hCD40L protein (Abcam) was used as positive control for the assay. Cells were lysed and luciferase activity was measured according to the manufacturer's manual (Luciferase Assay System, Promega, Madison, WI). Ramos-Blue cell line, a human B-lymphocyte cell line which stably expresses an NF-κB/AP-1-inducible SEAP reporter gene was stimulated with the same supernatant collected from A549 infected cells and cells producing SEAP in the supernatant were monitored and quantified using the QUANTI-Blue assay reagent (InvivoGen, San Diego, CA, USA).

3.4.9 Immunofluorescence and immunohistochemistry staining (I, II, IV)

Table 4: List of antibodies and conditions used in the studies

Antibody	Dilution	Catalog number	Company	Detection	Used in
anti-beta-galactosidase	1:200	AB1211	Chemicon International Inc	LSAB2 System-HRP [1]	I
anti-hexon	1:100	MA1-82982	ABR-Affinity BioReagents	Power Vision kit [2]	II

anti-Von Willebrand Factor	1:200	A0082	DakoCytomation	Alexa Fluor 594 [3]	II
FITC Mouse Anti-human CD40L	1:5	555699	BD Pharmigen	FITC	IV
FITC Mouse IgG1	1:5	555909	BD Pharmigen	FITC	IV
Anti human CD40	1:100	VP-C349	Vector Laboratories	LSAB2 System-HRP [1]	IV
Rabbit Anti-active Caspase-3	1:100	559565	BD Pharmigen	LSAB2 System-HRP [1]	IV
Rabbit Anti-mouse F4/80	1:100	14-4801	ebioscience	LSAB2 System-HRP [1]	IV
Rat Anti-mouse CD45	1:100	550539	BD Pharmigen	IHC Select kit	IV
Rat Anti-mouse CD19	1:50	14-0193	ebioscience	IHC Select kit	IV
Rat Anti-mouse CD4	1:50	14-0041	ebioscience	IHC Select kit	IV
Rat Anti-mouse CD8	1:100	14-0083	ebioscience	IHC Select kit	IV

[1] kit purchased from DakoCytomation, Carpinteria, CA, USA (K0673)
[2] PowerVision Poly-HRP-antimouse/rabbit/rat (ImmunoVision Technologies Co., Brisbane, CA 94005,USA)
[3] secondary antibody Molecular Probes, Invitrogen (dilution used 1:250)
[4] IHC Select kit (DAB150-RT, Millipore, MA, USA)

Tissues fixed in 4% formalin for paraffin blocks or frozen tissues embedded in Tissue Tek OCT (Sakura, Torrance, CA, USA) were made. Tissue sections of 4µm thickness were prepared and incubated with primary antibody at dilutions mentioned in Table 4 for 1 hour at room temperature. Further, sections were incubated according to manufacturer instructions with the detection kits as described in Table 4. Sections were counterstained with hematoxyline and dehydrated in ethanol, clarified in xylene and sealed with Canada balsam. For the immunofluorescence staining, sections were fixed in 4% paraformaldehyde and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Pictures at magnifications of 10x, 20x, 40x, 63x and 100x were taken with an Axioplan2 microscope (Carl Zeiss) equipped with Axiocam (Zeiss).

3.4.10 LacZ staining (I)

Whole mount tissues were fixed in fixing solution (25%glutaraldehyde, 100mM EGTA pH7.3, 1M $MgCl_2$, 0.1M phosphate buffer pH7.3, Sigma Aldrich) and stained with X-gal staining solution (1mg/ml X-Gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, Sigma Aldrich). Whole mount pictures were analyzed at 1.1 magnification by Leica MZFLIII microscope equipped

with ColorView Soft Imaging system. Further, tissues were fixed in 4% paraformaldehyde and embedded in paraffin blocks. Tissue sections of 4µm thickness were prepared and counterstained with eosin, dehydrated in ethanol, clarified in xylene and sealed with Canada balsam. Pictures at magnifications of 10x, 20x, 40x, 63x and 100x were taken with Axioplan2 microscope (Carl Zeiss) equipped with Axiocam (Zeiss).

3.5 *In vivo* studies

All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice were purchased from Taconic (Ejby, Denmark) at the age of 4-5 weeks and quarantined for at least one week. Syrian (Golden) hamsters (Mesocricetus auratus) were obtained from Taconic (Ejby, Denmark) at 4 to 5 weeks of age and quarantined at least for 1 week prior to the study. Health status of the mice and hamsters was frequently monitored and soon as any sign of excess pain or distress was evident they were killed.

3.5.1 Animal models in study I

Subcutaneous (s.c.) ACHN tumors were induced in Nude and Nod-Scid mice in both flanks. Virus injections were performed on three consecutive days (days 1-3) i.t. with 10^9 VP. Intravenous injections with $5x10^{10}$ VP were performed on day 2. Mice were euthanized 24 hours after the last i.t. injection and/or 48 hours after the i.v. injection and selected organs including tumors were collected and stored at -80^0C. Tissue samples were homogenized and lysed with Cell Culture Lysis Buffer, freeze-thawed, and supernatant was analyzed for LacZ activity with Galacto Light Plus (Tropix, Bedford, MA) as described earlier (Kangasniemi et al., 2006).

Peritoneal metastatic disease was established in SCID mice by i.p. injection of 10^7 ACHN cells per mouse. After 28 days mice received i.p a dose of $5x10^9$ VP. Mice were euthanized 48 hours later. Selected organs and tumors were collected, stored at -80^0C and analyzed as above. Results were normalized to protein content of the organs by DC Protein Assay (Bio-Rad, Hercules, Manassas, CA, USA).

SCID mice bearing i.p. or s.c. tumors were injected i.v. with $5x10^{10}$ VP. 48 hours after the i.v. injection selected organs including tumors were collected and stained either for LacZ activity by X-gal staining or by immunohistochemistry for LacZ.

3.5.2 Animal models in study II

3.5.2.1 Intratumoral replication and biodistribution of oncolytic adenovirus

Hamsters were injected s.c. in four different sites with 10^7 cells/site for all four different cell lines (HaP-T1, H2T, HaK and DDT1-MF2) and injected i.t. with Ad300wt at a dose of $5x10^{10}$ vp/tumor. Hamsters were killed at four different time points: 30 minutes, 24, 48, and 72h after the virus injection and selected organs including tumors were collected and stored at -80^0C. The net weight of solid tissues was determined, and tissues were homogenized in 0% growth media, freeze-thawed, and supernatant was analyzed to determine the plaque forming units (pfu) by $TCID_{50}$ assay on 293 cells. Results were normalized to the net weight of the tumors and organs.

3.5.2.2 Treatment with drugs *in vivo*

Hamsters were injected s.c. in four different sites with 10^7 Hap-T1cells/site. When tumors reached the volume of ca. $80mm^3$ hamsters were randomized into groups and injected with $5x10^9$ VP/tumor Ad300wt and 30 minutes later drugs were injected i.p. The animals were killed at four different time points: 24, 48, 72 and 96h after the i.t. injection and selected organs including tumors were collected and stored at -80° C. Tissue samples were analyzed as described above and virion production is reported by pfu normalized to protein content.

3.5.3 Animal models in study III

3.5.3.1 Luciferase activity experiment

Nude mice were injected s.c. with $5x10^6$ 786-O cells and injected i.t. with $3x10^8$ vp. Two days later, mice were imaged and then killed. Tumors were collected and analyzed for luciferase expression.

For the i.p. models, tumors were induced with 10^7 786-O or 786-O-CBGr cells and after 20 days mice were imaged and 10^8 VP was administered i.p. Two days later mice were imaged again for tumor and virus location. Mice were then killed; livers were collected and analyzed for luciferase expression.

3.5.3.2 Bioluminescence imaging

Mice were injected i.p. with 4.5 mg of D-Luciferin (Promega, Madison, WI) diluted in 100 µl 0% RPMI 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).

3.5.3.3 Subcutaneous tumor growth inhibition experiment

Nude mice with s.c. induced tumors with $5x10^6$ 786-O cells were injected i.t. with viruses at a dose of 10^8 VP. 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). On day 17, mice were killed and tumors were collected and prepared for immunofluorescence staining.

3.5.3.4 Survival experiment

SCID mice injected i.p. with 10^7 SN12L1-luc cells were injected i.p. with virus at a dose of $5x10^8$ vp on day 10 after cell injection. Mice were monitored for survival and imaged on day 9, 18, 25 and 32 after cell injection.

3.5.4 Animal models in study IV

3.5.4.1 Tumor growth follow-up

For the immune deficient models 10^6 A549 or EJ cells were injected subcutaneously into flanks of Nude mice (n=5mice/group). When tumors reached the size of approximately 5 x 5mm, virus was injected i.t., at a dose of 10^8VP/tumor for three times (days 0, 2 and 4). For the immunocompetent model $5x10^5$ MB49 cells were injected subcutaneously on shaved flanks of C57BL/6 mice (n=7mice/group). Virus was injected three times i.t. at the dose of $3x10^8$ vp/tumor on days 0,2 and 4, when tumors reached the size of approximately 5 x 5 mm. Tumor growth was followed and organs/tumors were collected at the end of the experiments. Tissues were embedded in paraffin and histology and immunohistochemistry were performed. Spleens were minced and splenocytes were cultured in 10% DMEM supplemented with 1% L-glutamine and

penicillin/streptomycin. Supernatants were collected at 24, 48 and 72 hours and analyzed for cytokines by FACS Array.

3.5.4.2 Elisa

Tumor bearing mice, nude mice with A549 tumors and C57BL/6 mice with MB49 tumors were injected i.t. with either, 10^8VP/tumor of Ad5/3-hTERT-E1A-hCD40L and Ad5/3-CMV-hCD40L, or $3x10^8$VP/tumor of Ad5/3-CMV-mCD40L on days 0, 2 and 4. Blood samples were taken on days 4, 8 and 12 after first virus injection. hCD40L and mCD40L concentration in the serum was determined with Human CD40 Ligand Elisa kit (ELH-CD40L-001, RayBiotech Inc, Norcross GA, USA**)** and Mouse sCD40L Elisa kit (BMS6010, Bender Medsytems, Austria) according to the manufacturer's protocol.

3.5.4.3 FACS-Array

FACS array was performed for collected blood serum and supernatant from cultured splenocytes and IL-6, TNF, IL-12, IFNγ and RANTES were analyzed according to manufacturer protocol (BD Cytometric Bead Array Mouse Flex Sets, BD Biosciences Pharmingen Franklin Lakes, NJ).

3.6 Statistics for studies I-IV

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* experiments 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 pair wise comparison of groups.

4 RESULTS AND DISCUSSION

The scope of this thesis is to address two issues of adenoviral gene therapy treatment: 1) safety and 2) efficacy.

First, studies I and II are investigating more the safety of adenoviral gene therapy and also giving treatment options in case of adenoviral replication induced side-effects:

- Limit liver toxicity by genetically engineering new vectors to retarget other organs
- Developing new animal models – Syrian hamsters- for assessing safety of oncolytic therapy
- Prove the efficiency of antiviral drugs in syngeneic immune competent animal models

Second, studies III and IV reveal two new approaches for targeted oncolytic adenovirus therapy with preclinical and clinical applications:

- Promoter evaluation for increased tumor transcriptional targeting
- Arming oncolytic virus with antiangiogenic transgene
- Evaluating the efficacy of replication deficient and replication competent adenoviruses coding for immunomodulatory molecules
- Immune response towards adenoviruses coding for hCD40L and mCD40L in different animal models

4.1 Genetically modified adenoviruses limit liver toxicity and enhance transduction of kidney targeted moieties (I)

Genetically modified adenoviruses have emerged as promising therapeutic agents for advanced cancers refractory to other available treatments. Following intravenous administration, the predominant site of sequestration of Ad5 based Ads is the liver, with significant hepatocyte transduction. Liver tropism of Ad5 started to raise concerns when one patient died in 1999 following administration of the vector into the hepatic artery. Moreover, extensive study by Waddington and colleagues showed direct binding of Ad5 to coagulation factor X via its hexon protein (Waddington et al., 2008). This provided a new rational for studying different Ad serotypes or Ad5 based chimeric adenoviruses with a modified tropism. Examples of the latter approach are e.g. fiber modifications (Mizuguchi and Hayakawa, 2004).

In this regard, previous report from Denby and colleagues identified Ad19p as a useful tool for targeting kidneys in rats (Denby et al., 2004; Denby et al., 2007). Moreover, the chimeric viruses Ad5/19p were further modified with rat kidney homing peptides inserted into the HI loop of the fiber: Ad5/19p - HTTHREP (Ad5/19p-HTT); Ad5/19p - HITSLLS (Ad5/19p-HIT) and Ad5/19p - APASLYN (Ad5/19p-APA). They presented a better rat kidney transduction without increased toxicity in the targeted organ. In study I, we analyzed the modified adenoviruses in the context of liver detargeting associated with kidney targeting in different mice models. First, *in vitro* studies showed that these vectors, tested in six different kidney cell lines and human ex-vivo kidney explants, did not improve the transduction, with the exception of ACHN cell line (Figure 1 and 2 study I). The absence of improved kidney transduction might result from a lack of stroma and tumor vascularization that are important mediators for virus transduction. Nevertheless, an increased transduction of the fiber modified adenoviruses was observed in s.c. or i.p. induced kidney tumors (Figure 3 and 4 study I). Confirming the results from Denby and colleagues according to which fiber modified adenoviruses can transduce kidney tumors more efficiently than Ad5 in orthotopic mice models, our study focused next on evaluating liver tropism of these vectors. The best candidate proved to

be Ad5/19p-HIT which exhibited the highest transduction of mouse kidneys, as shown in Figure 4 (study I). In s.c. or i.p. induced tumors, injected either i.t, i.p, or i.v, liver tropism was significantly downregulated. Our results are in agreement with those reported by Shayakhmetov et al who showed decreased accumulation in the liver of the pseudotyped Ad5/Ad35 virus, speculating that reduced interaction with CAR, coagulation factor IX and protein C4 might play a critical role in the detargeting effect (Shayakhmetov et al., 2002). It is also known that Ad19p uses sialic acids as the primary receptor (Arnberg et al., 2000a; Arnberg et al., 2000b; Burmeister et al., 2004). Moreover, Denby and colleagues showed reduced affinity of Ad5/19p modified vectors with factor X (Denby et al., 2007). Complete understanding of the mechanism reducing liver tropism of these fiber modified adenoviruses would require further studies. Additionally, this study demonstrates that tumor to liver transduction ratio is increased following intravenous or intraperitoneal administration of the viruses (Figure 6 study I).

In conclusion, our study provides one more evidence for safety of adenoviral treatment. By modifying adenoviral genome, we improved viral transduction in targeted tissues and reduced liver uptake of the vector. Altogether, modified adenoviruses are promising tools for systemic and local delivery, in particular for human kidney tumors.

4.2 Syrian hamsters as a new immunocompetent animal model for assessing adenoviral replication (II)

The safety and efficacy of oncolytic adenoviruses was widely assessed and discussed in many preclinical and clinical studies (Khuri et al., 2000; Xia et al., 2004; Yu and Fang, 2007). Still, most metastatic tumors have no curative treatment option to date. First generation adenoviruses showed modest anti-tumor effect. The tumor microenvironment together with the immune system exerts an antiviral effect leading to rapid virus clearance. More insights into the mechanism of action of Ads and the obstacles to virus delivery to the tumor site provided the scientific rationale for engineering more potent oncolytic adenoviruses. More potent oncolytic adenoviruses might imply more toxic agents. Human adenoviruses do not replicate productively in

murine tissues (Blair et al., 1989; Ying et al., 2009). *In vivo* analysis of adenoviral replication has been hampered by the lack of a permissive immune competent animal model. Recently, Syrian hamsters have been suggested as a suitable animal model for studying adenovirus replication (Bortolanza et al., 2007; Thomas et al., 2006; Thomas et al., 2007). Furthermore, as hamster cell lines have been developed, syngeneic tumors can be grown and tumor-host immune responses have been addressed in immune competent environment. In this study, a new syngeneic Syrian hamster model was established for assessing permissivity of wild-type adenovirus replication. First, hamster carcinoma cell lines, Hap-T1, DDT1-MF2, HaK and H2T, were transduced and killed by human adenoviruses. This was in accordance with previous studies (Thomas et al., 2006). Hap-T1, a hamster pancreatic cell line was the most permissive cell line for studying adenoviruses (Figure 1, study II). Hap-T1 cell line was also compared in different assays with human lung carcinoma cell line A549. In terms of transduction and replication, the hamster cell line was as permissive as the human cell line in terms of effective transduction and virion production (Figure 3, study II). Further, syngeneic tumors were grown in hamsters and tumor growth rates were compared. Hap-T1 cell line exhibited the best result, inducing 0.5 cm^3 tumors in approximately one week, followed by the other cell lines which developed tumors within five weeks. The most permissive tumors for adenovirus replication proved to be Hap-T1 with a 24-fold increase in virion production between 24 and 48 hours following i.t. administration. Taken together, these data showed that Hap-T1 syngeneic tumors in Syrian hamster are a useful model for assessing adenoviral replication and its side-effects. Likewise, the same model was used to assess anti-tumor immune response using an oncolytic adenovirus armed with the immunostimulatory cytokine GM-CSF (see chapter 1.6.3.) and showed a complete rejection of the tumors after rechallenge in the group injected with this virus (Cerullo et al., 2010). In conclusion, this model can be used for characterizing both viral replication (study II) and host immune response.

4.3 Antiviral treatment with chlorpromazine and cidofovir in case of oncolytic virotherapy induced side-effects (II)

Concerns have been raised about the toxicity of oncolytic viruses. Specifically, immune compromised patients are at risk for uncontrolled viral replication, leading to severe and even fatal side-effects (Claas et al., 2005; Fischer, 2008; Raper et al., 2003). There have been many attempts (see chapter 1.5.3) to establish antiviral treatments but so far no curative antiviral drugs have been on the market.

In this study, we propose the use of chlorpromazine and cidofovir to inhibit adenovirus replication. Chlorpromazine, widely used as an antipsychotic drug, was previously reported by Kanerva and colleagues to inhibit adenovirus replication *in vitro* and in *ex-vivo* human liver explants (Kanerva et al., 2007). The mechanism of action of chlorpromazine was reported in 1993 by Wang and colleagues to interfere in the clathrin coated pit assembly at the cell surface (Wang et al., 1993). Adenoviruses are binding the cells through the interaction of the fiber knob with CAR. The initial binding is followed by receptor-mediated endocytosis in clathrin-coated pits. Here, we analyzed the effect of chlorpromazine both: *in vitro* and *in vivo*. That is, the transduction of the adenovirus in hamster pancreatic carcinoma cell line Hap-T1 was not hampered by the treatment with chlorpromazine (Figure 5, study II). In contrast, when viral replication was assessed in this cell line, the virion production was inhibited 25-fold compared with non-treated cells. A possible explanation for this effect could be that the mechanism of entry and action of the adenovirus in hamster cells is not known or other functions of chlorpromazine might be involved (Day and Dimattina, 1977). Further investigations should be performed in this regard. *In vivo,* the drug had a limited inhibition effect on viral replication in the liver but more stringent effect in the tumor (Figure 6 study II).

Cidofovir, an acyclic nucleoside phosphonate analogue, is by far the most widely used antiviral drug (Baba et al., 1987; de Oliveira et al., 1996; Gordon et al., 1991; Hartline et al., 2005). This compound acts as a chain terminator during DNA replication and is effective as antiviral agent for all human adenovirus serotypes tested (see chapter 1.5.3) (Lenaerts et al., 2008). Recently, Toth and colleagues reported that CMX001 (an analog of

cidofovir) significantly reduces adenoviral replication in immune suppressed Syrian hamsters. In our study, we first analyzed the effect of cidofovir on adenovirus transduction and replication *in vitro*. As expected, this drug did not influence adenovirus transduction (Figure 5 study II) meanwhile the virion production was 368-fold downregulated (Figure 4 study II). When we tested cidofovir *in vivo,* in a syngeneic immune competent Syrian hamster model, the antiviral effect was confirmed in both tumor and liver (Figure 6 study II).

In conclusion, both antiviral drugs proposed in this study, chlorpromazine and cidofovir exert an inhibitory effect on adenovirus replication. Since no significant effect was seen in transgene expression, the mechanisms prompted by these drugs could be downstream the nuclear delivery. Altogether, chlorpromazine and cidofovir could be useful for oncolytic gene therapy by offering a tool to control and reduce the side-effects associated with replication.

4.4 Promoter evaluation for increased transcriptional targeting (III)

In this study (Study III) we focused our research on investigating new transcriptionally targeted adenoviruses for renal cancer treatment. Renal cancer is one of the most refractory diseases to conventional therapies, such as chemotherapy and radiotherapy (Godley and Kim, 2002; Longo et al., 2007). As reviewed in chapter 1.3.3.2, many strategies have been employed for transcriptional targeting of Ads to tumor cells. Most of the cancers are defective in p16/Rb or p53 pathways, but in the case of renal cancers the main tumor suppressor gene that plays a critical role in tumor development is Von-Hippel-Lindau gene (VHL) (Shuin et al., 1994). The most studied function of this gene is the regulation of the transcription factor - hypoxia inducible factor (HIF) (Kaelin, 2004). This transcription factor is the key regulator for induction of genes which regulate adaptation and survival of cells from normoxic to hypoxic conditions (Wang et al., 1995). This heterodimer binds to specific DNA sequences, HREs, further inducing the regulation of important angiogenic factors, such as VEGF or other mitogenic factors (TGFα, TGFβ,

cyclin D, etc). HIF is highly expressed in renal carcinomas due to hypoxic conditions and defective VHL pathway in these tumors. Thus, engineering vectors using HIF promoter would increase the efficiency of adenoviral therapy for renal cancer. In this study, we analyzed the activity of two HREs promoters regulating luciferase expression: 9HIF and OB36. Replication deficient adenoviruses Ad5-9HIF-luc and Ad5-OB36-luc were constructed and compared with Ad5-CMV-luc (Ad5Luc1), which has a strong expression of luciferase due to a CMV driving promoter. Under normoxic conditions *in vitro*, the OB36 promoter exerted a stronger effect than 9HIF promoter, and both promoters induced the expression of higher levels of luciferase when compared with Ad5Luc1 (Figure 1 a-d study III). In contrast, when renal cancer cell lines were under hypoxic conditions, 9HIF was stronger than OB36 in two out of three cell lines. As in normoxic conditions, both promoters induced higher expression of luciferase compared with Ad5Luc1 (Figure 1 e-f study III). In conclusion, *in vitro* results showed modest activity of 9HIF promoter. In an s.c. renal cancer model, both promoters exerted potent activity driving expression of higher levels of luciferase when compared with luciferase expression induced by the CMV promoter (Figure 2 study III). In an i.p. kidney tumor model, Ad5-9HIF-Luc presented specific luciferase expression in the tumors without expression in the liver (Figure 4a, b study III). Taken together, these results demonstrate efficient induction of HREs elements *in vivo*. In conclusion, 9HIF promoter was identified as a good candidate for kidney tumor transcriptional targeted therapy.

4.5 Improved anti-tumor effect with an oncolytic virus armed with antiangiogenic molecule (III)

In this study we constructed a triple targeted oncolytic adenovirus as treatment option for renal cell cancer. We propose the use of Ad5/3-9HIF-Δ24-VEGFR-1-Ig, based on previous transduction studies and promoter evaluation described above, for improved infectivity and antiangiogenic effect in the treatment of renal cancer. It is well known that tumor progression and metastasis require persistent blood supply (Ferrara, 2004). Kidney tumors, in particular, are highly vascularized and defective for VHL/HIF pathways

(Fukata et al., 2005; Kim and Kaelin, 2004). The key player in regulating angiogenesis is VEGF, which was found to be expressed by most tumors including kidney tumors (Nicol et al., 1997). There are two main receptors for VEGF: fms-like-tyrosine-kinase receptor (flt-1 or VEGFR-1) and kinase domain region receptor (KDR or VEGFR-2) (Ferrara, 2004). Already in 1993, Kim et al showed that anti-VEGF antibodies can inhibit the growth of several tumor cell lines in mice (Kim et al., 1993). The fusion protein VEGFR-1-Ig, used in this study to arm the oncolytic adenovirus, was previously reported to induce vascular endothelial cell mitogenesis (Kendall et al., 1996; Olofsson et al., 1998). This virus also features the Ad5/3 chimeric capsid for enhanced transduction, 9HIF controlling E1A for more efficient transcriptional targeting and Δ24bp deletion in the E1A gene for more specific viral replication in tumor cells, as described in chapter 1.3.3.3. (Figure 5a study III). We also constructed Ad5/3-9HIF-Δ24-E3 as control adenovirus. This virus has all the features of the Ad5/3-9HIF-Δ24-VEGFR-1-Ig except the antiangiogenic molecule expressed from the E3 region.

Analysis of the oncolytic potency showed lower cytopathic effect in the case of Ad5/3-9HIF-Δ24-VEGFR-1-Ig. The oncolytic potency of the virus was the same as the isogenic control Ad5/3-9HIF-Δ24-E3. This virus was less potent than Ad5/3-Δ24 but usually more potent than Ad300wt (Figure 6a, b; Supplementary Figure 2 in study III). This effect might be explained by the previously observed lower activity of 9HIF promoter *in vitro* in normoxic conditions (Figure 1 study III). Also, VEGFR-1-Ig expressed by infected cells (Figure 5b, study III) is not expected to present any anti-tumor effect *in vitro*. The absence of lytic effect in normal fibroblasts, infected with Ad5/3-9HIF-Δ24-VEGFR-1-Ig or Ad5/3-9HIF-Δ24-E3 confirms the high specificity of these viruses for cancer cells (Figure 6c study III). A 100% cell killing was observed in HUVEC cells infected with Ad5/3-Δ24 or Ad300wt viruses. In contrast, Ad5/3-9HIF-Δ24-E3 did not exert any lytic effect since HIF is not active in these cells in normoxic conditions. Ad5/3-9HIF-Δ24-VEGFR-1-Ig showed 30% killing effect possibly related to a minimal expression of VEGFR-1-Ig.

Further, we analyzed the anti-tumor efficacy and the effect of the transgene in two different animal models. First, in an s.c. kidney cancer model, viruses were injected i.t. and Ad5/3-9HIF-Δ24-E3 and Ad5/3-Δ24 treatment resulted in complete eradication of 38% of the tumors (Figure 7 study III). Ad5/3-9HIF-Δ24-VEGFR-1-Ig was not as effective as

the two previous viruses despite the expression of VEGFR-1-Ig (Figure 7b study III). An explanation for this minimal effect could be the collapse of the vasculature and increased necrosis inside the tumor caused by VEGFR-1-Ig expression. In addition, necrosis and hypoxic conditions have been shown to inhibit viral dissemination (Heldin et al., 2004). Therefore, when we analyzed the tumors, we observed a profound inhibition of vascularization in the group treated with Ad5/3-9HIF-Δ24-VEGFR-1-Ig. Together with low vascularization we noticed increased hexon protein expression in the tumors.

Intraperitoneally disseminated renal cancer induced with SN12L1-luc cells might exemplify better the conditions favorable for the use of Ad5/3-9HIF-Δ24-VEGFR-1-Ig. In this model, mice treated with Ad5/3-9HIF-Δ24-VEGFR-1-Ig showed a significant increase in survival when compared with the other groups (Figure 8 study III). A possible explanation of this increased survival might be the different vascularization of these tumors which might be more susceptible to anti-angiogenic therapy.

Altogether, the results show the efficacy of Ad5/3-9HIF-Δ24-VEGFR-1-Ig, in the context of preclinical studies and suggest the virus as a good candidate for the treatment of patients with renal cell cancer. One concern for this treatment approach might be to the safety of VEGFR-1-Ig due to some reports of potential toxicity of sFlt (Mahasreshti et al., 2003). On the other hand, promising safety and efficacy results from a clinical trial with a related antiangiogenic molecule, VEGF-trap, have already been generated (Riely and Miller, 2007).

4.6 Development/characterization and efficacy of new immunotherapy agents (IV)

Immunotherapy has gained a lot of interest in the past years. Many studies show growing body evidence that the immune system might be the key player in tumor growth, survival or eradication. These studies also made immunotherapy more appealing for future developments in cancer gene therapy (Prestwich et al., 2008). The immune system in cancer patients fails to respond mainly because of the immunosuppressive

environment in the tumor and surrounding tissue, and lack of tumor antigen presentation.

As discussed in previous studies (study III and chapters 1.3.3.2 and 1.3.3.3), developing new transductionally/transcriptionally targeted oncolytic adenoviruses could be a useful approach for tumor clearance and increased survival. In the present study, a new oncolytic adenovirus, Ad5/3-hTERT-E1A-hCD40L, is proposed for cancer treatment.

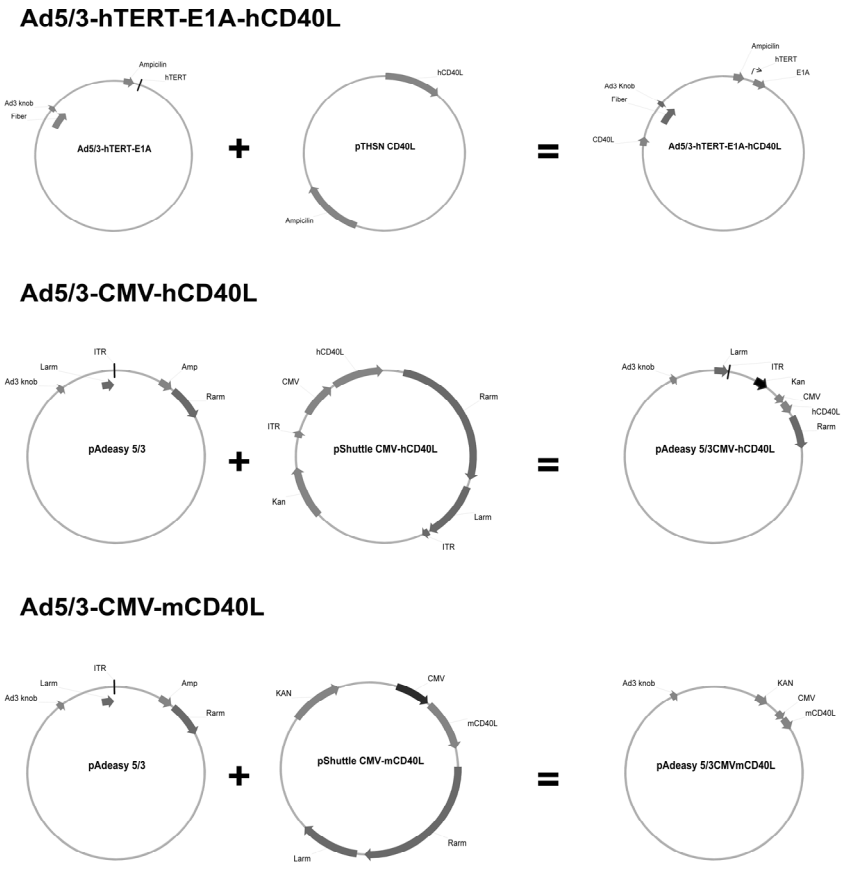

Figure 5 Cloning strategies for replication competent and replication deficient adenoviruses coding for CD40L

This virus features three genetic modifications: a serotype switching with Ad3 knob for improved tumor transduction, insertion of hTERT promoter driving E1A gene for tumor selectivity and insertion of an hCD40L encoding sequence for potentiating the anti-tumor immune response combined with apoptosis (Figure5, Figure 1, study IV). hTERT has been reported to be active preferentially in cells with a high division rate, which is the main feature of all cancer cells. Oncolytic adenoviruses driven by this promoter also showed good safety data (Takakura et al., 1999; Yokoyama et al., 2008).

CD40L also known as CD154 is predominantly expressed on $CD4^+$ T-cells and binds to its receptor CD40 present on the membrane of antigen-presenting cells (APCs) (Grewal and Flavell, 1998). This interaction leads to activation of both innate and adaptive immune response. Binding of CD40L to CD40 expressed by macrophages and dendritic cells (DC) leads to antigen presentation and cytokine production (van Kooten and Banchereau, 2000). The CD40L-CD40 interaction also provides costimulatory signals which trigger T cells expansion (Grewal and Flavell, 1998) and increase IL-12 production necessary for the engagement of cytotoxic T cells in the anti-tumor immune response (Loskog et al., 2005; Mackey et al., 1998). Similarly, CD40L was shown *in vitro* and *in vivo* to induce apoptosis (Fernandes et al., 2009; Loskog et al., 2004; Loskog et al., 2005).

Given these effects, we hypothesized that Ad5/3-hTERT-E1A-hCD40L oncolytic adenovirus could lead to tumor elimination due to tumor-specific oncolysis and apoptosis together with an anti-tumor immune response prompted by the immunomodulatory molecule. For better confirmation of CD40L effects, two replication deficient viruses coding for hCD40L and mouse CD40L were constructed: Ad5/3-CMV-hCD40L and Ad5/3-CMV-mCD40L (Figure 5, Figure 1 study IV). The three cloned viruses expressed the transgenes *in vitro* and *in vivo*. *In vitro*, hCD40L was expressed at the same rate with both replication deficient and competent adenovirus. The experiment was performed on 293 cell line which expresses E1A gene enabling the non-oncolytic virus to replicate at a similar rate as the oncolytic virus. Another reason for the equal expression might be the strong expression of the transgene due to the CMV promoter used in the non-replicating virus (Figure 1B study IV). We also noticed efficient expression of the transgenes *in vivo* (Figure 1C study IV). Analysis of hCD40L expression in serum samples of nude mice revealed a higher concentration of the protein in the group treated with the replication deficient adenovirus. mCD40L protein was also detected in the serum of injected immunocompetent C57BL/6 mice (Figure 1C study IV). Further, we investigated whether hCD40L encoded by oncolytic adenovirus is functional. Two assays were performed in this regard, measuring the potency of NF-κB activation. Both showed increased activity of the expressed protein (Figure 1D study IV).

Human CD40L is known to induce apoptosis in the presence of its CD40 receptor. We analyzed two different cell lines, EJ which is positive for CD40 receptor, and A549 which

is negative for the receptor. Ad5/3-hTERT-E1A-hCD40L oncolytic virus exerted a more potent killing effect on the EJ cells expressing CD40. Meanwhile Ad5/3-hTERT-E1A, the control isogenic virus, was more potent in A549 cell line (Figure 2A-D study IV). We confirmed the apoptotic effect induced by CD40L in the presence of CD40 receptor also *in vivo*. A replication deficient adenovirus Ad5/3-CMV-hCD40L was administered in tumors induced with either EJ or A549 cell line. In A549 tumors (negative for CD40 receptor) the virus did not have any anti-tumor effect. In mice bearing EJ tumors (positive for CD40 receptor), the virus resulted in a significant decrease in tumor growth (Figure 3A, B study IV). In both tumor models, the oncolytic virus Ad5/3-hTERT-E1A-hCD40L induced the same significant decrease in tumor growth as its control isogenic virus Ad5/3-hTERT-E1A (Figure 3 C, D study IV). To better understand this phenomenon, tumor tissues were analyzed at the end of the experiment for assessing caspase-3 activity as a marker for apoptosis. Immunohistochemistry analysis revealed increased caspase 3 activity in the group treated with Ad5/3-hTERT-E1A-hCD40L when compared with the control groups: Ad5/3-hTERT-E1A and Ad5/3-CMV-hCD40L (Figure 4 study IV). To conclude, our new developed oncolytic adenovirus exhibits a potent oncolytic effect *in vitro* and *in vivo* and induces apoptosis.

4.7 Immune responses induced by CD40L protein in a syngeneic immunocompetent animal model

In order to assess the immune response induced by CD40L protein, we needed an immunocompetent animal model. As previously discussed, human adenoviruses do not replicate in mouse tissue. In addition hCD40L was previously shown to be inactive in mice (Spriggs et al., 1992). As a result, we engineered a replication deficient adenovirus coding for mCD40L with the same capsid modification Ad5/3 used for the other viruses.

In a syngeneic mouse model, s.c. tumors were induced with the MB49 bladder carcinoma cell line and were injected with either Ad5/3-CMV-mCD40L or the control virus Ad5/3-CMV-Luc1 (Ad5/3Luc1). There was a significant decrease in tumor growth ($p=0.002$) in the group injected with Ad5/3-CMV-mCD40L when compared with

Ad5/3Luc1 treated group (Figure 5A study IV). Tumors were collected and immunohistochemistry was performed for caspase-3 activity. While Ad5/3Luc1 exerted a minimal effect, we noticed an increase of caspase-3 activity in tumors treated with the virus expressing mCD40L (Figure 5B study IV).

As previously discussed in chapter 1.5.2, the mechanisms of host defence are mediated mainly by the innate and adaptive immune responses. In this regard, we analyzed tumor tissues and serum collected from mice and supernatant from cultured splenocytes to demonstrate the effect of CD40L protein on both innate and adaptive immune responses.

Tissues analyzed by immunohistochemistry for macrophage marker F4/80, leucocytes antigen CD45 and B-cell CD19+ expression revealed higher expression of these immune modulating factors in the tumors treated with Ad5/3-CMV-mCD40L (Figure 6B study IV). In addition, mCD40L expressed protein induced the production of cytokines and chemokines such as RANTES and TNF-α (Figure 6A study IV). It is known that adenovirus *per se* triggers a strong innate immune response, but the levels of cytokines and chemokines induced after infection were significantly higher with Ad5/3-CMV-mCD40L virus than Ad5Luc1 virus (Figure 6A study IV). In mice treated with the virus coding for CD40L, we observed increased levels of IL-12 which is an important mediator of the adaptive immune response. IL-12 further stimulated IFN-γ production which resulted in T-cell priming and stimulation. To assess the toxicity of these adenoviruses, IL-6 was measured and no significant difference was seen between the groups. Regarding the adaptive immune response, higher levels of IFN-γ production were noticed in the group treated with Ad5/3-CMV-mCD40L. Moreover, immunohistochemistry staining revealed a high T cell infiltration (CD3 positive) in the tumors. While there was no difference for the expression of T helper- $CD4^+$ cells, the number of cytotoxic $CD8^+$ T cells was nevertheless increased (Figure 6C study IV). Based on these results, immunotherapy using CD40L protein is a tantalizing therapy approach and could be successfully used in the clinic. Above all, Ad5/3-hTERT-E1A-hCD40L has already proven its safety and efficacy in a few patients treated in an advanced therapy access program (Pesonen et al data unpublished).

5 SUMMARY AND CONCLUSIONS

The goal of this thesis was to assess the safety profile of adenoviral therapy and increase the efficacy of this approach using genetically engineering oncolytic adenoviruses.

The suggested liver toxicity, imperfect animal models and lack of antiviral treatments are different pitfalls in adenovirus gene therapy that we addressed in the present thesis.

Many studies have shown that adenoviruses can be modified to target different tissues. Although, liver tropism of adenovirus in humans is still a subject of debate, developing adenoviruses which untarget the liver and are redirected to preferred tissues is of particular interest. In this study, we used a chimeric Ad5/19p-HIT adenovirus which targets receptors different from the Ad5 receptor CAR. The peptide inserted into the HI loop augmented the retargeting of this vector towards kidney moieties. Following either intravenous or intraperitoneal administration of this adenovirus, kidney tumors and normal tissues were better transduced compared with the control virus. In addition, the natural tropism of adenovirus for the liver was ablated in all orthotopic animal models, independently of the route of administration of the vector. In conclusion, adenoviruses can be modified specifically to target kidney moieties and untarget the liver.

Furthermore, mouse tissues are known not to be permissive for adenoviral replication. Here, we established a new syngeneic immunocompetent animal model – Syrian hamsters with pancreatic induced tumors. Wild-type Ad5 efficiently transduced and killed all hamster cell lines *in vitro* and exhibited sustained replication in tumors and different normal tissues *in vivo*. The results also suggest that while hamster cell lines in general are permissive for human adenovirus type 5, replication and subsequent cytotoxicity is variable. Nevertheless, this study confers the “best available” animal model for assessing adenovirus replication and its associated side-effects. This animal model was further used to show inhibition of adenovirus replication by antiviral drugs such as chlorpromazine and cidofovir. Oncolytic virotherapy has shown promise as effective cancer treatment, but only limited efficacy was noticed in clinical settings. On the other hand, more effective and potent viruses may also lead to uncontrolled

replication. There are no available antiviral treatment options in case of replication associated side-effects. Based on our results, chlorpromazine and cidofovir could be good candidates to inhibit adenoviral replication. Both drugs ablated viral replication *in vitro* and exhibited a significant reduction of adenovirus replication in tumors and liver normal tissue of hamsters. Clinical data may ultimately define the effect of these drugs on adenovirus replication.

Our other studies focused on arming oncolytic adenoviruses for improving their efficacy.

The key factor in regulating angiogenesis is VEGF and is by far the most studied angiogenic factor. We generated an infectivity enhanced, transductionally and transcriptionally targeted, antiangiogenic oncolytic adenovirus Ad5/3-9HIF-Δ24-VEGFR-1-Ig. In an orthotopic subcutaneous induced tumor model, the virus exhibited a modest anti-tumor effect. The local expression of antiangiogenic molecule resulted in a significant decrease of blood vessels number. The latter effect might have induced necrosis in the tumor. On the other hand, this effect did not result in significant tumor regression. In an intraperitoneal tumor model, more closely related to clinical set up, Ad5/3-9HIF-Δ24-VEGFR-1-Ig treatment resulted in increased survival compared with the other treated groups. Given the modest effect of this approach, I further generated a more potent oncolytic adenovirus: Ad5/3-hTERT-E1A-hCD40L. While Ad5/3-9HIF-Δ24-VEGFR-1-Ig was generated for enhanced tumor targeting and local anti-tumor effect due to antiangiogenic molecule, Ad5/3-hTERT-E1A-hCD40L targets the tumor more effectively and is augmented by insertion of the immunostimulatory molecule CD40L. Besides local apoptotic effects, CD40L has an important role in modulating the anti-tumor immune responses. Immunotherapy is thought to be the answer for cancer treatment since scientists discovered that the host immune response is a major player in tumor clearance. In this regard, Ad5/3-hTERT-E1A-hCD40L exerted the same oncolytic effect as control virus when used in nude mice which lack effective immune function. Despite this, local apoptotic events were evident in the tumors treated with the adenoviruses coding for CD40L. In a syngeneic animal model, adenovirus coding for CD40L molecule also successfully engaged innate and adaptive immune responses inducing significant tumor regression.

To summarize, the studies from this thesis offer new cancer treatment options using armed oncolytic adenoviruses. First, the safety profile of adenoviral gene therapy was assessed; using capsid modified adenoviruses enabled to limit liver toxicity and increase kidney targeting. Second, a new immunocompetent animal model, Syrian hamster, was developed. Third, antiviral treatment options are also suggested as a safety switch in case of replication associated side-effects. Further, the potency of oncolytic viruses was increased by arming them with antiangiogenic molecules, which resulted in an increased survival of the animals. Finally, new immunotherapy agent, Ad5/3-hTERT-E1A-hCD40L, was generated and assessed and could translate into successful clinical approach.

These studies could contribute to the emergence of successful clinical embodiments of cancer gene therapy with oncolytic adenoviruses and thus increase the treatment options of patients with currently incurable cancer.

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Sincerely

Iulia

7 REFERENCES

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783-801.

Alemany, R. (2007). Cancer selective adenoviruses. Mol Aspects Med *28*, 42-58.

Alemany, R., and Curiel, D.T. (2001). CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther *8*, 1347-1353.

Appledorn, D.M., McBride, A., Seregin, S., Scott, J.M., Schuldt, N., Kiang, A., Godbehere, S., and Amalfitano, A. (2008). Complex interactions with several arms of the complement system dictate innate and humoral immunity to adenoviral vectors. Gene Ther *15*, 1606-1617.

Arnberg, N., Edlund, K., Kidd, A.H., and Wadell, G. (2000a). Adenovirus type 37 uses sialic acid as a cellular receptor. J Virol *74*, 42-48.

Arnberg, N., Kidd, A.H., Edlund, K., Olfat, F., and Wadell, G. (2000b). Initial interactions of subgenus D adenoviruses with A549 cellular receptors: sialic acid versus alpha(v) integrins. J Virol *74*, 7691-7693.

Asada, T. (1974). Treatment of human cancer with mumps virus. Cancer *34*, 1907-1928.

Azam, F., Mehta, S., and Harris, A.L. (2010). Mechanisms of resistance to antiangiogenesis therapy. Eur J Cancer *46*, 1323-1332.

Baba, M., Mori, S., Shigeta, S., and De Clercq, E. (1987). Selective inhibitory effect of (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and 2'-nor-cyclic GMP on adenovirus replication *in vitro*. Antimicrob Agents Chemother *31*, 337-339.

Bauerschmitz, G.J., Guse, K., Kanerva, A., Menzel, A., Herrmann, I., Desmond, R.A., Yamamoto, M., Nettelbeck, D.M., Hakkarainen, T., Dall, P.*, et al.* (2006). Triple-targeted oncolytic adenoviruses featuring the cox2 promoter, E1A transcomplementation, and serotype chimerism for enhanced selectivity for ovarian cancer cells. Mol Ther *14*, 164-174.

Bauerschmitz, G.J., Lam, J.T., Kanerva, A., Suzuki, K., Nettelbeck, D.M., Dmitriev, I., Krasnykh, V., Mikheeva, G.V., Barnes, M.N., Alvarez, R.D.*, et al.* (2002). Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. Cancer Res *62*, 1266-1270.

Bauerschmitz, G.J., Ranki, T., Kangasniemi, L., Ribacka, C., Eriksson, M., Porten, M., Herrmann, I., Ristimaki, A., Virkkunen, P., Tarkkanen, M.*, et al.* (2008). Tissue-specific promoters active in CD44+CD24-/low breast cancer cells. Cancer Res *68*, 5533-5539.

Bayo-Puxan, N., Cascallo, M., Gros, A., Huch, M., Fillat, C., and Alemany, R. (2006). Role of the putative heparan sulfate glycosaminoglycan-binding site of the adenovirus type 5 fiber shaft on liver detargeting and knob-mediated retargeting. J Gen Virol *87*, 2487-2495.

Belin, M.T., and Boulanger, P. (1987). Processing of vimentin occurs during the early stages of adenovirus infection. J Virol *61*, 2559-2566.

Bennett, E.M., Bennink, J.R., Yewdell, J.W., and Brodsky, F.M. (1999). Cutting edge: adenovirus E19 has two mechanisms for affecting class I MHC expression. J Immunol *162*, 5049-5052.

Berd, D., and Mastrangelo, M.J. (1988). Effect of low dose cyclophosphamide on the immune system of cancer patients: depletion of CD4+, 2H4+ suppressor-inducer T-cells. Cancer Res *48*, 1671-1675.

Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., and Finberg, R.W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science *275*, 1320-1323.

Berk, A.J. (2007). Adenoviridae: the viruses and their replication. Fields Virology, 5th edition *In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.)*, 2355-2394

Binley, K., Askham, Z., Martin, L., Spearman, H., Day, D., Kingsman, S., and Naylor, S. (2003). Hypoxia-mediated tumour targeting. Gene Ther *10*, 540-549.

Bischoff, J.R., Kirn, D.H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J.A., Sampson-Johannes, A., Fattaey, A*., et al.* (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science *274*, 373-376.

Blair, G.E., Dixon, S.C., Griffiths, S.A., and Zajdel, M.E. (1989). Restricted replication of human adenovirus type 5 in mouse cell lines. Virus Res *14*, 339-346.

Boom, W.H., Liano, D., and Abbas, A.K. (1988). Heterogeneity of helper/inducer T lymphocytes. II. Effects of interleukin 4- and interleukin 2-producing T cell clones on resting B lymphocytes. J Exp Med *167*, 1350-1363.

Bortolanza, S., Alzuguren, P., Bunuales, M., Qian, C., Prieto, J., and Hernandez-Alcoceba, R. (2007). Human adenovirus replicates in immunocompetent models of pancreatic cancer in Syrian hamsters. Hum Gene Ther *18*, 681-690.

Brand, K., Loser, P., Arnold, W., Bartels, T., and Strauss, M. (1998). Tumor cell-specific transgene expression prevents liver toxicity of the adeno-HSVtk/GCV approach. Gene Ther *5*, 1363-1371.

Burmeister, W.P., Guilligay, D., Cusack, S., Wadell, G., and Arnberg, N. (2004). Crystal structure of species D adenovirus fiber knobs and their sialic acid binding sites. J Virol *78*, 7727-7736.

Cerullo, V., Pesonen, S., Diaconu, I., Escutenaire, S., Arstila, P.T., Ugolini, M., Nokisalmi, P., Raki, M., Laasonen, L., Sarkioja, M*., et al.* (2010). Oncolytic adenovirus coding for granulocyte macrophage colony-stimulating factor induces antitumoral immunity in cancer patients. Cancer Res *70*, 4297-4309.

Cerullo, V., Seiler, M.P., Mane, V., Brunetti-Pierri, N., Clarke, C., Bertin, T.K., Rodgers, J.R., and Lee, B. (2007). Toll-like receptor 9 triggers an innate immune response to helper-dependent adenoviral vectors. Mol Ther *15*, 378-385.

Chalikonda, S., Kivlen, M.H., O'Malley, M.E., Eric Dong, X.D., McCart, J.A., Gorry, M.C., Yin, X.Y., Brown, C.K., Zeh, H.J., 3rd, Guo, Z.S*., et al.* (2008). Oncolytic virotherapy for ovarian carcinomatosis using a replication-selective vaccinia virus armed with a yeast cytosine deaminase gene. Cancer Gene Ther *15*, 115-125.

Chen, P.H., Ornelles, D.A., and Shenk, T. (1993). The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. J Virol *67*, 3507-3514.

Chiocca, E.A., Abbed, K.M., Tatter, S., Louis, D.N., Hochberg, F.H., Barker, F., Kracher, J., Grossman, S.A., Fisher, J.D., Carson, K*., et al.* (2004). A phase I open-label, dose-escalation, multi-institutional trial of injection with an E1B-Attenuated adenovirus, ONYX-015, into the peritumoral region of recurrent malignant gliomas, in the adjuvant setting. Mol Ther *10*, 958-966.

Chirmule, N., Moscioni, A.D., Qian, Y., Qian, R., Chen, Y., and Wilson, J.M. (1999). Fas-Fas ligand interactions play a major role in effector functions of cytotoxic T lymphocytes after adenovirus vector-mediated gene transfer. Hum Gene Ther *10*, 259-269.

Chroboczek, J., Bieber, F., and Jacrot, B. (1992). The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. Virology *186*, 280-285.

Claas, E.C., Schilham, M.W., de Brouwer, C.S., Hubacek, P., Echavarria, M., Lankester, A.C., van Tol, M.J., and Kroes, A.C. (2005). Internally controlled real-time PCR monitoring of adenovirus DNA load in serum or plasma of transplant recipients. J Clin Microbiol *43*, 1738-1744.

Crystal, R.G. (1999). *In vivo* and ex vivo gene therapy strategies to treat tumors using adenovirus gene transfer vectors. Cancer Chemother Pharmacol *43 Suppl*, S90-99.

Davison, A.J., Benko, M., and Harrach, B. (2003). Genetic content and evolution of adenoviruses. J Gen Virol *84*, 2895-2908.

Day, R.S., 3rd, and Dimattina, M. (1977). Photodynamic action of chlorpromazine on adenovirus 5:repairable damage and single strand breaks. Chem Biol Interact *17*, 89-97.

de Jong, J.C., Osterhaus, A.D., Jones, M.S., and Harrach, B. (2008). Human adenovirus type 52: a type 41 in disguise? J Virol *82*, 3809; author reply 3809-3810.

de Oliveira, C.B., Stevenson, D., LaBree, L., McDonnell, P.J., and Trousdale, M.D. (1996). Evaluation of Cidofovir (HPMPC, GS-504) against adenovirus type 5 infection *in vitro* and in a New Zealand rabbit ocular model. Antiviral Res *31*, 165-172.

Denby, L., Work, L.M., Graham, D., Hsu, C., von Seggern, D.J., Nicklin, S.A., and Baker, A.H. (2004). Adenoviral serotype 5 vectors pseudotyped with fibers from subgroup D show modified tropism *in vitro* and *in vivo*. Hum Gene Ther *15*, 1054-1064.

Denby, L., Work, L.M., Seggern, D.J., Wu, E., McVey, J.H., Nicklin, S.A., and Baker, A.H. (2007). Development of renal-targeted vectors through combined *in vivo* phage display and capsid engineering of adenoviral fibers from serotype 19p. Mol Ther *15*, 1647-1654.

DePage, N.G. (1912). Sulla scomparsa di un enorme canero vegetante del callo dell'utero senza cura chirurgica. Ginecologia, 82-88.

DeWeese, T.L., van der Poel, H., Li, S., Mikhak, B., Drew, R., Goemann, M., Hamper, U., DeJong, R., Detorie, N., Rodriguez, R.*, et al.* (2001). A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. Cancer Res *61*, 7464-7472.

Dias, J.D., Liikanen, I., Guse, K., Foloppe, J., Sloniecka, M., Diaconu, I., Rantanen, V., Eriksson, M., Hakkarainen, T., Lusky, M.*, et al.* (2010). Targeted chemotherapy for head and neck cancer with a chimeric oncolytic adenovirus coding for bifunctional suicide protein FCU1. Clin Cancer Res *16*, 2540-2549.

Dix, B.R., Edwards, S.J., and Braithwaite, A.W. (2001). Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway? J Virol *75*, 5443-5447.

Dmitriev, I., Krasnykh, V., Miller, C.R., Wang, M., Kashentseva, E., Mikheeva, G., Belousova, N., and Curiel, D.T. (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via

utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. J Virol *72*, 9706-9713.

Duque, P.M., Alonso, C., Sanchez-Prieto, R., Lleonart, M., Martinez, C., de Buitrago, G.G., Cano, A., Quintanilla, M., and Ramon y Cajal, S. (1999). Adenovirus lacking the 19-kDa and 55-kDa E1B genes exerts a marked cytotoxic effect in human malignant cells. Cancer Gene Ther *6*, 554-563.

Eaton, L. (2003). World cancer rates set to double by 2020. BMJ *326*, 728.

Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B., and Livingston, D.M. (1994). Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev *8*, 869-884.

Elkon, K.B., Liu, C.C., Gall, J.G., Trevejo, J., Marino, M.W., Abrahamsen, K.A., Song, X., Zhou, J.L., Old, L.J., Crystal, R.G.*, et al.* (1997). Tumor necrosis factor alpha plays a central role in immune-mediated clearance of adenoviral vectors. Proc Natl Acad Sci U S A *94*, 9814-9819.

Escudier, B., Eisen, T., Stadler, W.M., Szczylik, C., Oudard, S., Siebels, M., Negrier, S., Chevreau, C., Solska, E., Desai, A.A.*, et al.* (2007a). Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med *356*, 125-134.

Escudier, B., Pluzanska, A., Koralewski, P., Ravaud, A., Bracarda, S., Szczylik, C., Chevreau, C., Filipek, M., Melichar, B., Bajetta, E.*, et al.* (2007b). Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. Lancet *370*, 2103-2111.

Fallaux, F.J., Kranenburg, O., Cramer, S.J., Houweling, A., Van Ormondt, H., Hoeben, R.C., and Van Der Eb, A.J. (1996). Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum Gene Ther *7*, 215-222.

Fernandes, M.S., Gomes, E.M., Butcher, L.D., Hernandez-Alcoceba, R., Chang, D., Kansopon, J., Newman, J., Stone, M.J., and Tong, A.W. (2009). Growth inhibition of human multiple myeloma cells by an oncolytic adenovirus carrying the CD40 ligand transgene. Clin Cancer Res *15*, 4847-4856.

Ferrara, N. (2004). Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev *25*, 581-611.

Ferreira, V., Sidenius, N., Tarantino, N., Hubert, P., Chatenoud, L., Blasi, F., and Korner, M. (1999). *In vivo* inhibition of NF-kappa B in T-lineage cells leads to a dramatic decrease in cell proliferation and cytokine production and to increased cell apoptosis in response to mitogenic stimuli, but not to abnormal thymopoiesis. J Immunol *162*, 6442-6450.

Finkelman, F.D., Holmes, J., Katona, I.M., Urban, J.F., Jr., Beckmann, M.P., Park, L.S., Schooley, K.A., Coffman, R.L., Mosmann, T.R., and Paul, W.E. (1990). Lymphokine control of *in vivo* immunoglobulin isotype selection. Annu Rev Immunol *8*, 303-333.

Fischer, S.A. (2008). Emerging viruses in transplantation: there is more to infection after transplant than CMV and EBV. Transplantation *86*, 1327-1339.

Foloppe, J., Kintz, J., Futin, N., Findeli, A., Cordier, P., Schlesinger, Y., Hoffmann, C., Tosch, C., Balloul, J.M., and Erbs, P. (2008). Targeted delivery of a suicide gene to human colorectal tumors by a conditionally replicating vaccinia virus. Gene Ther *15*, 1361-1371.

Freytag, S.O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D.G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M*., et al.* (2003). Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. Cancer Res *63*, 7497-7506.

Fueyo, J., Gomez-Manzano, C., Alemany, R., Lee, P.S., McDonnell, T.J., Mitlianga, P., Shi, Y.X., Levin, V.A., Yung, W.K., and Kyritsis, A.P. (2000). A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect *in vivo*. Oncogene *19*, 2-12.

Fukata, S., Inoue, K., Kamada, M., Kawada, C., Furihata, M., Ohtsuki, Y., and Shuin, T. (2005). Levels of angiogenesis and expression of angiogenesis-related genes are prognostic for organ-specific metastasis of renal cell carcinoma. Cancer *103*, 931-942.

Gahery-Segard, H., Farace, F., Godfrin, D., Gaston, J., Lengagne, R., Tursz, T., Boulanger, P., and Guillet, J.G. (1998). Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. J Virol *72*, 2388-2397.

Galanis, E., Okuno, S.H., Nascimento, A.G., Lewis, B.D., Lee, R.A., Oliveira, A.M., Sloan, J.A., Atherton, P., Edmonson, J.H., Erlichman, C*., et al.* (2005). Phase I-II trial of ONYX-015 in combination with MAP chemotherapy in patients with advanced sarcomas. Gene Ther *12*, 437-445.

Ganly, I., Kirn, D., Eckhardt, G., Rodriguez, G.I., Soutar, D.S., Otto, R., Robertson, A.G., Park, O., Gulley, M.L., Heise, C*., et al.* (2000). A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. Clin Cancer Res *6*, 798-806.

Germann, T., Rude, E., and Schmitt, E. (1995). The influence of IL12 on the development of Th1 and Th2 cells and its adjuvant effect for humoral immune responses. Res Immunol *146*, 481-486.

Girardin, S.E., Sansonetti, P.J., and Philpott, D.J. (2002). Intracellular vs extracellular recognition of pathogens--common concepts in mammals and flies. Trends Microbiol *10*, 193-199.

Godley, P., and Kim, S.W. (2002). Renal cell carcinoma. Curr Opin Oncol *14*, 280-285.

Gomes, E.M., Rodrigues, M.S., Phadke, A.P., Butcher, L.D., Starling, C., Chen, S., Chang, D., Hernandez-Alcoceba, R., Newman, J.T., Stone, M.J*., et al.* (2009). Antitumor activity of an oncolytic adenoviral-CD40 ligand (CD154) transgene construct in human breast cancer cells. Clin Cancer Res *15*, 1317-1325.

Goodbourn, S., Didcock, L., and Randall, R.E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J Gen Virol *81*, 2341-2364.

Goodrum, F.D., and Ornelles, D.A. (1999). Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication. J Virol *73*, 7474-7488.

Gordon, Y.J., Romanowski, E., Araullo-Cruz, T., Seaberg, L., Erzurum, S., Tolman, R., and De Clercq, E. (1991). Inhibitory effect of (S)-HPMPC, (S)-HPMPA, and 2'-nor-cyclic GMP on clinical ocular adenoviral isolates is serotype-dependent *in vitro*. Antiviral Res *16*, 11-16.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol *36*, 59-74.

Grewal, I.S., and Flavell, R.A. (1998). CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol *16*, 111-135.

Gross, S. (1971). Measles and leukaemia. Lancet *1*, 397-398.

Guidotti, L.G., and Chisari, F.V. (2001). Noncytolytic control of viral infections by the innate and adaptive immune response. Annu Rev Immunol *19*, 65-91.

Guse, K., Diaconu, I., Rajecki, M., Sloniecka, M., Hakkarainen, T., Ristimaki, A., Kanerva, A., Pesonen, S., and Hemminki, A. (2009). Ad5/3-9HIF-Delta24-VEGFR-1-Ig, an infectivity enhanced, dual-targeted and antiangiogenic oncolytic adenovirus for kidney cancer treatment. Gene Ther *16*, 1009-1020.

Habib, N., Salama, H., Abd El Latif Abu Median, A., Isac Anis, I., Abd Al Aziz, R.A., Sarraf, C., Mitry, R., Havlik, R., Seth, P., Hartwigsen, J.*, et al.* (2002). Clinical trial of E1B-deleted adenovirus (dl1520) gene therapy for hepatocellular carcinoma. Cancer Gene Ther *9*, 254-259.

Habib, N.A., Sarraf, C.E., Mitry, R.R., Havlik, R., Nicholls, J., Kelly, M., Vernon, C.C., Gueret-Wardle, D., El-Masry, R., Salama, H.*, et al.* (2001). E1B-deleted adenovirus (dl1520) gene therapy for patients with primary and secondary liver tumors. Hum Gene Ther *12*, 219-226.

Hakkarainen, T. (2005). Enhancement of cancer gene therapy with modified viral vectors and fusion genes. Gene Ther Mol Biol *9*, 153-168.

Hakkarainen, T., Hemminki, A., Curiel, D.T., and Wahlfors, J. (2006). A conditionally replicative adenovirus that codes for a TK-GFP fusion protein (Ad5Delta24TK-GFP) for evaluation of the potency of oncolytic virotherapy combined with molecular chemotherapy. Int J Mol Med *18*, 751-759.

Halbert, D.N., Cutt, J.R., and Shenk, T. (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J Virol *56*, 250-257.

Hallenbeck, P.L., Chang, Y.N., Hay, C., Golightly, D., Stewart, D., Lin, J., Phipps, S., and Chiang, Y.L. (1999). A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum Gene Ther *10*, 1721-1733.

Hamel, P.A., Phillips, R.A., Muncaster, M., and Gallie, B.L. (1993). Speculations on the roles of RB1 in tissue-specific differentiation, tumor initiation, and tumor progression. FASEB J *7*, 846-854.

Hamid, O., Varterasian, M.L., Wadler, S., Hecht, J.R., Benson, A., 3rd, Galanis, E., Uprichard, M., Omer, C., Bycott, P., Hackman, R.C.*, et al.* (2003). Phase II trial of intravenous CI-1042 in patients with metastatic colorectal cancer. J Clin Oncol *21*, 1498-1504.

Han, J., Sabbatini, P., Perez, D., Rao, L., Modha, D., and White, E. (1996). The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. Genes Dev *10*, 461-477.

Hartline, C.B., Gustin, K.M., Wan, W.B., Ciesla, S.L., Beadle, J.R., Hostetler, K.Y., and Kern, E.R. (2005). Ether lipid-ester prodrugs of acyclic nucleoside phosphonates: activity against adenovirus replication *in vitro*. J Infect Dis *191*, 396-399.

Havenga, M.J., Lemckert, A.A., Grimbergen, J.M., Vogels, R., Huisman, L.G., Valerio, D., Bout, A., and Quax, P.H. (2001). Improved adenovirus vectors for infection of cardiovascular tissues. J Virol *75*, 3335-3342.

Havenga, M.J., Lemckert, A.A., Ophorst, O.J., van Meijer, M., Germeraad, W.T., Grimbergen, J., van Den Doel, M.A., Vogels, R., van Deutekom, J., Janson, A.A.*, et al.* (2002). Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. J Virol *76*, 4612-4620.

Haviv, Y.S., Blackwell, J.L., Kanerva, A., Nagi, P., Krasnykh, V., Dmitriev, I., Wang, M., Naito, S., Lei, X., Hemminki, A.*, et al.* (2002). Adenoviral gene therapy for renal cancer requires retargeting to alternative cellular receptors. Cancer Res *62*, 4273-4281.

Hayder, H., and Mullbacher, A. (1996). Molecular basis of immune evasion strategies by adenoviruses. Immunol Cell Biol *74*, 504-512.

Hecht, J.R., Bedford, R., Abbruzzese, J.L., Lahoti, S., Reid, T.R., Soetikno, R.M., Kirn, D.H., and Freeman, S.M. (2003). A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. Clin Cancer Res *9*, 555-561.

Heise, C., and Kirn, D.H. (2000). Replication-selective adenoviruses as oncolytic agents. J Clin Invest *105*, 847-851.

Heldin, C.H., Rubin, K., Pietras, K., and Ostman, A. (2004). High interstitial fluid pressure - an obstacle in cancer therapy. Nat Rev Cancer *4*, 806-813.

Hermiston, T. (2006). A demand for next-generation oncolytic adenoviruses. Curr Opin Mol Ther *8*, 322-330.

Howe, J.A., Demers, G.W., Johnson, D.E., Neugebauer, S.E., Perry, S.T., Vaillancourt, M.T., and Faha, B. (2000). Evaluation of E1-mutant adenoviruses as conditionally replicating agents for cancer therapy. Mol Ther *2*, 485-495.

Huang, X., and Yang, Y. (2009). Innate immune recognition of viruses and viral vectors. Hum Gene Ther *20*, 293-301.

Huebner, R.J., Rowe, W.P., Schatten, W.E., Smith, R.R., and Thomas, L.B. (1956). Studies on the use of viruses in the treatment of carcinoma of the cervix. Cancer *9*, 1211-1218.

Ichikawa, T., Tamiya, T., Adachi, Y., Ono, Y., Matsumoto, K., Furuta, T., Yoshida, Y., Hamada, H., and Ohmoto, T. (2000). *In vivo* efficacy and toxicity of 5-fluorocytosine/cytosine deaminase gene therapy for malignant gliomas mediated by adenovirus. Cancer Gene Ther *7*, 74-82.

Immonen, A., Vapalahti, M., Tyynela, K., Hurskainen, H., Sandmair, A., Vanninen, R., Langford, G., Murray, N., and Yla-Herttuala, S. (2004). AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. Mol Ther *10*, 967-972.

Inohara, N., and Nunez, G. (2003). NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol *3*, 371-382.

Ito, H., Aoki, H., Kuhnel, F., Kondo, Y., Kubicka, S., Wirth, T., Iwado, E., Iwamaru, A., Fujiwara, K., Hess, K.R.*, et al.* (2006). Autophagic cell death of malignant glioma cells induced by a conditionally replicating adenovirus. J Natl Cancer Inst *98*, 625-636.

Janaswami, P.M., Kalvakolanu, D.V., Zhang, Y., and Sen, G.C. (1992). Transcriptional repression of interleukin-6 gene by adenoviral E1A proteins. J Biol Chem *267*, 24886-24891.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., and Thun, M.J. (2008). Cancer statistics, 2008. CA Cancer J Clin *58*, 71-96.

Johnson, L., Shen, A., Boyle, L., Kunich, J., Pandey, K., Lemmon, M., Hermiston, T., Giedlin, M., McCormick, F., and Fattaey, A. (2002). Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. Cancer Cell *1*, 325-337.

Kaelin, W.G., Jr. (2004). The von Hippel-Lindau tumor suppressor gene and kidney cancer. Clin Cancer Res *10*, 6290S-6295S.

Kaneko, H., Kato, K., Mori, S., and Shigeta, S. (2001). Antiviral activity of NMSO3 against adenovirus *in vitro*. Antiviral Res *52*, 281-288.

Kanerva, A., and Hemminki, A. (2004). Modified adenoviruses for cancer gene therapy. Int J Cancer *110*, 475-480.

Kanerva, A., Mikheeva, G.V., Krasnykh, V., Coolidge, C.J., Lam, J.T., Mahasreshti, P.J., Barker, S.D., Straughn, M., Barnes, M.N., Alvarez, R.D.*, et al.* (2002a). Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. Clin Cancer Res *8*, 275-280.

Kanerva, A., Raki, M., Ranki, T., Sarkioja, M., Koponen, J., Desmond, R.A., Helin, A., Stenman, U.H., Isoniemi, H., Hockerstedt, K.*, et al.* (2007). Chlorpromazine and apigenin reduce adenovirus replication and decrease replication associated toxicity. J Gene Med *9*, 3-9.

Kanerva, A., Wang, M., Bauerschmitz, G.J., Lam, J.T., Desmond, R.A., Bhoola, S.M., Barnes, M.N., Alvarez, R.D., Siegal, G.P., Curiel, D.T.*, et al.* (2002b). Gene transfer to ovarian cancer versus normal tissues with fiber-modified adenoviruses. Mol Ther *5*, 695-704.

Kanerva, A., Zinn, K.R., Chaudhuri, T.R., Lam, J.T., Suzuki, K., Uil, T.G., Hakkarainen, T., Bauerschmitz, G.J., Wang, M., Liu, B.*, et al.* (2003). Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus. Mol Ther *8*, 449-458.

Kangasniemi, L., Kiviluoto, T., Kanerva, A., Raki, M., Ranki, T., Sarkioja, M., Wu, H., Marini, F., Hockerstedt, K., Isoniemi, H.*, et al.* (2006). Infectivity-enhanced adenoviruses deliver efficacy in clinical samples and orthotopic models of disseminated gastric cancer. Clin Cancer Res *12*, 3137-3144.

Kaplan, J.M., Armentano, D., Scaria, A., Woodworth, L.A., Pennington, S.E., Wadsworth, S.C., Smith, A.E., and Gregory, R.J. (1999). Novel role for E4 region genes in protection of adenovirus vectors from lysis by cytotoxic T lymphocytes. J Virol *73*, 4489-4492.

Kendall, R.L., Wang, G., and Thomas, K.A. (1996). Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun *226*, 324-328.

Khuri, F.R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I.F., Romel, L., Gore, M., Ironside, J., MacDougall, R.H., Heise, C.*, et al.* (2000). a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nat Med *6*, 879-885.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature *362*, 841-844.

Kim, W.Y., and Kaelin, W.G. (2004). Role of VHL gene mutation in human cancer. J Clin Oncol *22*, 4991-5004.

Koski, A., Kangasniemi, L., Escutenaire, S., Pesonen, S., Cerullo, V., Diaconu, I., Nokisalmi, P., Raki, M., Rajecki, M., Guse, K.*, et al.* (2010). Treatment of Cancer Patients With a Serotype 5/3 Chimeric Oncolytic Adenovirus Expressing GMCSF. Mol Ther.

Kurihara, T., Brough, D.E., Kovesdi, I., and Kufe, D.W. (2000). Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. J Clin Invest *106*, 763-771.

Lapteva, N., Aldrich, M., Weksberg, D., Rollins, L., Goltsova, T., Chen, S.Y., and Huang, X.F. (2009). Targeting the intratumoral dendritic cells by the oncolytic adenoviral vaccine expressing RANTES elicits potent antitumor immunity. J Immunother *32*, 145-156.

Lee, Y.S., Kim, J.H., Choi, K.J., Choi, I.K., Kim, H., Cho, S., Cho, B.C., and Yun, C.O. (2006). Enhanced antitumor effect of oncolytic adenovirus expressing interleukin-12 and B7-1 in an immunocompetent murine model. Clin Cancer Res *12*, 5859-5868.

Leen, A.M., Bollard, C.M., Myers, G.D., and Rooney, C.M. (2006). Adenoviral infections in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant *12*, 243-251.

Lehman, A.F., Kreyenbuhl, J., Buchanan, R.W., Dickerson, F.B., Dixon, L.B., Goldberg, R., Green-Paden, L.D., Tenhula, W.N., Boerescu, D., Tek, C*., et al.* (2004). The Schizophrenia Patient Outcomes Research Team (PORT): updated treatment recommendations 2003. Schizophr Bull *30*, 193-217.

Lenaerts, L., De Clercq, E., and Naesens, L. (2008). Clinical features and treatment of adenovirus infections. Rev Med Virol *18*, 357-374.

Leppard, K.N. (1997). E4 gene function in adenovirus, adenovirus vector and adeno-associated virus infections. J Gen Virol *78 (Pt 9)*, 2131-2138.

Li, Q., and Wadell, G. (1999). Genetic variability of hexon loops 1 and 2 between seven genome types of adenovirus serotype 7. Arch Virol *144*, 1739-1749.

Li, Y., Kang, J., Friedman, J., Tarassishin, L., Ye, J., Kovalenko, A., Wallach, D., and Horwitz, M.S. (1999). Identification of a cell protein (FIP-3) as a modulator of NF-kappaB activity and as a target of an adenovirus inhibitor of tumor necrosis factor alpha-induced apoptosis. Proc Natl Acad Sci U S A *96*, 1042-1047.

Liles, W.C., Cushing, H., Holt, S., Bryan, C., and Hackman, R.C. (1993). Severe adenoviral nephritis following bone marrow transplantation: successful treatment with intravenous ribavirin. Bone Marrow Transplant *12*, 409-412.

Liu, T.C., and Kirn, D. (2008). Gene therapy progress and prospects cancer: oncolytic viruses. Gene Ther *15*, 877-884.

Liu, Y., and Deisseroth, A. (2006). Oncolytic adenoviral vector carrying the cytosine deaminase gene for melanoma gene therapy. Cancer Gene Ther *13*, 845-855.

Ljungman, P. (2004). Treatment of adenovirus infections in the immunocompromised host. Eur J Clin Microbiol Infect Dis *23*, 583-588.

Longo, R., D'Andrea, M.R., Sarmiento, R., Salerno, F., and Gasparini, G. (2007). Integrated therapy of kidney cancer. Ann Oncol *18 Suppl 6*, vi141-148.

Look, D.C., Roswit, W.T., Frick, A.G., Gris-Alevy, Y., Dickhaus, D.M., Walter, M.J., and Holtzman, M.J. (1998). Direct suppression of Stat1 function during adenoviral infection. Immunity *9*, 871-880.

Loskog, A., Dzojic, H., Vikman, S., Ninalga, C., Essand, M., Korsgren, O., and Totterman, T.H. (2004). Adenovirus CD40 ligand gene therapy counteracts immune escape mechanisms in the tumor Microenvironment. J Immunol *172*, 7200-7205.

Loskog, A.S., Fransson, M.E., and Totterman, T.T. (2005). AdCD40L gene therapy counteracts T regulatory cells and cures aggressive tumors in an orthotopic bladder cancer model. Clin Cancer Res *11*, 8816-8821.

Luo, J., Deng, Z.-L., Luo, X., Tang, N., Song, W.-X., Chen, J., Sharff, K.A., Luu, H.H., Haydon, R.C., K.W., K*., et al.* (2007). A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. Nat Protoc *2*, 1236-1247.

Mackey, M.F., Gunn, J.R., Maliszewsky, C., Kikutani, H., Noelle, R.J., and Barth, R.J., Jr. (1998). Dendritic cells require maturation via CD40 to generate protective antitumor immunity. J Immunol *161*, 2094-2098.

Mahasreshti, P.J., Kataram, M., Wang, M.H., Stockard, C.R., Grizzle, W.E., Carey, D., Siegal, G.P., Haisma, H.J., Alvarez, R.D., and Curiel, D.T. (2003). Intravenous delivery of adenovirus-mediated soluble FLT-1 results in liver toxicity. Clin Cancer Res *9*, 2701-2710.

Manickan, E., Smith, J.S., Tian, J., Eggerman, T.L., Lozier, J.N., Muller, J., and Byrnes, A.P. (2006). Rapid Kupffer cell death after intravenous injection of adenovirus vectors. Mol Ther *13*, 108-117.

Maraskovsky, E., Chen, W.F., and Shortman, K. (1989). IL-2 and IFN-gamma are two necessary lymphokines in the development of cytolytic T cells. J Immunol *143*, 1210-1214.

McCarthy, A.J., Bergin, M., De Silva, L.M., and Stevens, M. (1995). Intravenous ribavirin therapy for disseminated adenovirus infection. Pediatr Infect Dis J *14*, 1003-1004.

McSharry, B.P., Burgert, H.G., Owen, D.P., Stanton, R.J., Prod'homme, V., Sester, M., Koebernick, K., Groh, V., Spies, T., Cox, S*., et al.* (2008). Adenovirus E3/19K promotes evasion of NK cell recognition by intracellular sequestration of the NKG2D ligands major histocompatibility complex class I chain-related proteins A and B. J Virol *82*, 4585-4594.

Merritt, J.A., Roth, J.A., and Logothetis, C.J. (2001). Clinical evaluation of adenoviral-mediated p53 gene transfer: review of INGN 201 studies. Semin Oncol *28*, 105-114.

Mizuguchi, H., and Hayakawa, T. (2004). Targeted adenovirus vectors. Hum Gene Ther *15*, 1034-1044.

Morfin, F., Dupuis-Girod, S., Mundweiler, S., Falcon, D., Carrington, D., Sedlacek, P., Bierings, M., Cetkovsky, P., Kroes, A.C., van Tol, M.J*., et al.* (2005). *In vitro* susceptibility of adenovirus to antiviral drugs is species-dependent. Antivir Ther *10*, 225-229.

Mulvihill, S., Warren, R., Venook, A., Adler, A., Randlev, B., Heise, C., and Kirn, D. (2001). Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. Gene Ther *8*, 308-315.

Muruve, D.A., Barnes, M.J., Stillman, I.E., and Libermann, T.A. (1999). Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury *in vivo*. Hum Gene Ther *10*, 965-976.

Muruve, D.A., Cotter, M.J., Zaiss, A.K., White, L.R., Liu, Q., Chan, T., Clark, S.A., Ross, P.J., Meulenbroek, R.A., Maelandsmo, G.M*., et al.* (2004). Helper-dependent adenovirus vectors elicit intact innate but attenuated adaptive host immune responses *in vivo*. J Virol *78*, 5966-5972.

Naesens, L., Lenaerts, L., Andrei, G., Snoeck, R., Van Beers, D., Holy, A., Balzarini, J., and De Clercq, E. (2005). Antiadenovirus activities of several classes of nucleoside and nucleotide analogues. Antimicrob Agents Chemother *49*, 1010-1016.

Nanda, D., Vogels, R., Havenga, M., Avezaat, C.J., Bout, A., and Smitt, P.S. (2001). Treatment of malignant gliomas with a replicating adenoviral vector expressing herpes simplex virus-thymidine kinase. Cancer Res *61*, 8743-8750.

Nemerow, G.R., Pache, L., Reddy, V., and Stewart, P.L. (2009). Insights into adenovirus host cell interactions from structural studies. Virology *384*, 380-388.

Nemunaitis, J., Cunningham, C., Buchanan, A., Blackburn, A., Edelman, G., Maples, P., Netto, G., Tong, A., Randlev, B., Olson, S.*, et al.* (2001a). Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. Gene Ther *8*, 746-759.

Nemunaitis, J., Khuri, F., Ganly, I., Arseneau, J., Posner, M., Vokes, E., Kuhn, J., McCarty, T., Landers, S., Blackburn, A.*, et al.* (2001b). Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. J Clin Oncol *19*, 289-298.

Nemunaitis, J., Senzer, N., Sarmiento, S., Zhang, Y.A., Arzaga, R., Sands, B., Maples, P., and Tong, A.W. (2007). A phase I trial of intravenous infusion of ONYX-015 and enbrel in solid tumor patients. Cancer Gene Ther *14*, 885-893.

Nicklin, S.A., Dishart, K.L., Buening, H., Reynolds, P.N., Hallek, M., Nemerow, G.R., Von Seggern, D.J., and Baker, A.H. (2003). Transductional and transcriptional targeting of cancer cells using genetically engineered viral vectors. Cancer Lett *201*, 165-173.

Nicklin, S.A., White, S.J., Watkins, S.J., Hawkins, R.E., and Baker, A.H. (2000). Selective targeting of gene transfer to vascular endothelial cells by use of peptides isolated by phage display. Circulation *102*, 231-237.

Nicol, D., Hii, S.I., Walsh, M., Teh, B., Thompson, L., Kennett, C., and Gotley, D. (1997). Vascular endothelial growth factor expression is increased in renal cell carcinoma. J Urol *157*, 1482-1486.

Nokisalmi, P., Pesonen, S.*,* Escutenaire, S., Sarkioja, M., Raki, M., Cerullo, V., Laasonen, L., Alemany, R., Rojas, J., Cascallo, M.*, et al.* (2010). Oncolytic adenovirus ICOVIR-7 in patients with advanced and refractory solid tumors. Clin Cancer Res *16*, 3035-3043.

Olofsson, B., Korpelainen, E., Pepper, M.S., Mandriota, S.J., Aase, K., Kumar, V., Gunji, Y., Jeltsch, M.M., Shibuya, M., Alitalo, K.*, et al.* (1998). Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci U S A *95*, 11709-11714.

Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. (2005). Global cancer statistics, 2002. CA Cancer J Clin *55*, 74-108.

Paul, W.E., and Seder, R.A. (1994). Lymphocyte responses and cytokines. Cell *76*, 241-251.

Paulson, M., Pisharody, S., Pan, L., Guadagno, S., Mui, A.L., and Levy, D.E. (1999). Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. J Biol Chem *274*, 25343-25349.

Pesonen, S., Nokisalmi, P., Escutenaire, S., Sarkioja, M., Raki, M., Cerullo, V., Kangasniemi, L., Laasonen, L., Ribacka, C., Guse, K.*, et al.* (2010). Prolonged systemic circulation of chimeric oncolytic adenovirus Ad5/3-Cox2L-D24 in patients with metastatic and refractory solid tumors. Gene Ther *17*, 892-904.

Post, D.E., Sandberg, E.M., Kyle, M.M., Devi, N.S., Brat, D.J., Xu, Z., Tighiouart, M., and Van Meir, E.G. (2007). Targeted cancer gene therapy using a hypoxia inducible factor dependent oncolytic adenovirus armed with interleukin-4. Cancer Res *67*, 6872-6881.

Prestwich, R.J., Errington, F., Diaz, R.M., Pandha, H.S., Harrington, K.J., Melcher, A.A., and Vile, R.G. (2009). The case of oncolytic viruses versus the immune system: waiting on the judgment of Solomon. Hum Gene Ther *20*, 1119-1132.

Prestwich, R.J., Harrington, K.J., Pandha, H.S., Vile, R.G., Melcher, A.A., and Errington, F. (2008). Oncolytic viruses: a novel form of immunotherapy. Expert Rev Anticancer Ther *8*, 1581-1588.

Putzer, B.M., Stiewe, T., Parssanedjad, K., Rega, S., and Esche, H. (2000). E1A is sufficient by itself to induce apoptosis independent of p53 and other adenoviral gene products. Cell Death Differ *7*, 177-188.

Raki, M., Hakkarainen, T., Bauerschmitz, G.J., Sarkioja, M., Desmond, R.A., Kanerva, A., and Hemminki, A. (2007). Utility of TK/GCV in the context of highly effective oncolysis mediated by a serotype 3 receptor targeted oncolytic adenovirus. Gene Ther *14*, 1380-1388.

Randall, R.E., and Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol *89*, 1-47.

Ranki, T., Kanerva, A., Ristimaki, A., Hakkarainen, T., Sarkioja, M., Kangasniemi, L., Raki, M., Laakkonen, P., Goodison, S., and Hemminki, A. (2007a). A heparan sulfate-targeted conditionally replicative adenovirus, Ad5.pk7-Delta24, for the treatment of advanced breast cancer. Gene Ther *14*, 58-67.

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

Raper, S.E., Chirmule, N., Lee, F.S., Wivel, N.A., Bagg, A., Gao, G.P., Wilson, J.M., and Batshaw, M.L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab *80*, 148-158.

Reichard, K.W., Lorence, R.M., Cascino, C.J., Peeples, M.E., Walter, R.J., Fernando, M.B., Reyes, H.M., and Greager, J.A. (1992). Newcastle disease virus selectively kills human tumor cells. J Surg Res *52*, 448-453.

Reid, T., Galanis, E., Abbruzzese, J., Sze, D., Andrews, J., Romel, L., Hatfield, M., Rubin, J., and Kirn, D. (2001). Intra-arterial administration of a replication-selective adenovirus (dl1520) in patients with colorectal carcinoma metastatic to the liver: a phase I trial. Gene Ther *8*, 1618-1626.

Rekosh, D.M., Russell, W.C., Bellet, A.J., and Robinson, A.J. (1977). Identification of a protein linked to the ends of adenovirus DNA. Cell *11*, 283-295.

Riely, G.J., and Miller, V.A. (2007). Vascular endothelial growth factor trap in non small cell lung cancer. Clin Cancer Res *13*, s4623-4627.

Ries, S.J., Brandts, C.H., Chung, A.S., Biederer, C.H., Hann, B.C., Lipner, E.M., McCormick, F., and Korn, W.M. (2000). Loss of p14ARF in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). Nat Med *6*, 1128-1133.

Rini, B.I., Halabi, S., Rosenberg, J.E., Stadler, W.M., Vaena, D.A., Ou, S.S., Archer, L., Atkins, J.N., Picus, J., Czaykowski, P*., et al.* (2008). Bevacizumab plus interferon alfa compared with interferon alfa monotherapy in patients with metastatic renal cell carcinoma: CALGB 90206. J Clin Oncol *26*, 5422-5428.

Rodriguez, R., Schuur, E.R., Lim, H.Y., Henderson, G.A., Simons, J.W., and Henderson, D.R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res *57*, 2559-2563.

Roelvink, P.W., Mi Lee, G., Einfeld, D.A., Kovesdi, I., and Wickham, T.J. (1999). Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. Science *286*, 1568-1571.

Rollinghoff, M., Starzinski-Powitz, A., Pfizenmaier, K., and Wagner, H. (1977). Cyclophosphamide-sensitive T lymphocytes suppress the *in vivo* generation of antigen-specific cytotoxic T lymphocytes. J Exp Med *145*, 455-459.

Routes, J.M., Li, H., Bayley, S.T., Ryan, S., and Klemm, D.J. (1996). Inhibition of IFN-stimulated gene expression and IFN induction of cytolytic resistance to natural killer cell lysis correlate with E1A-p300 binding. J Immunol *156*, 1055-1061.

Rowe, W.P., Huebner, R.J., Gilmore, L.K., Parrott, R.H., and Ward, T.G. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med *84*, 570-573.

Russell, W.C. (2000). Update on adenovirus and its vectors. J Gen Virol *81*, 2573-2604.

Rux, J.J., and Burnett, R.M. (2004). Adenovirus structure. Hum Gene Ther *15*, 1167-1176.

Sarkioja, M., Kanerva, A., Salo, J., Kangasniemi, L., Eriksson, M., Raki, M., Ranki, T., Hakkarainen, T., and Hemminki, A. (2006). Noninvasive imaging for evaluation of the systemic delivery of capsid-modified adenoviruses in an orthotopic model of advanced lung cancer. Cancer *107*, 1578-1588.

Schagen, F.H., Ossevoort, M., Toes, R.E., and Hoeben, R.C. (2004). Immune responses against adenoviral vectors and their transgene products: a review of strategies for evasion. Crit Rev Oncol Hematol *50*, 51-70.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol *75*, 163-189.

Shayakhmetov, D.M., Li, Z.Y., Ni, S., and Lieber, A. (2002). Targeting of adenovirus vectors to tumor cells does not enable efficient transduction of breast cancer metastases. Cancer Res *62*, 1063-1068.

Shen, Y. (2007). Viral vectors and their applications. Fields Virology, 5th Edition *In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.)*, 539-564.

Shuin, T., Kondo, K., Torigoe, S., Kishida, T., Kubota, Y., Hosaka, M., Nagashima, Y., Kitamura, H., Latif, F., Zbar, B.*, et al.* (1994). Frequent somatic mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinomas. Cancer Res *54*, 2852-2855.

Small, E.J., Carducci, M.A., Burke, J.M., Rodriguez, R., Fong, L., van Ummersen, L., Yu, D.C., Aimi, J., Ando, D., Working, P.*, et al.* (2006). A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. Mol Ther *14*, 107-117.

Smyth, M.J., Godfrey, D.I., and Trapani, J.A. (2001). A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol *2*, 293-299.

Southam, C.M., and Moore, A.E. (1951). West Nile, Ilheus, and Bunyamwera virus infections in man. Am J Trop Med Hyg *31*, 724-741.

Spriggs, M.K., Armitage, R.J., Strockbine, L., Clifford, K.N., Macduff, B.M., Sato, T.A., Maliszewski, C.R., and Fanslow, W.C. (1992). Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. J Exp Med *176*, 1543-1550.

Stadler, W.M. (2005). Targeted agents for the treatment of advanced renal cell carcinoma. Cancer *104*, 2323-2333.

Suzuki, M., Cerullo, V., Bertin, T.K., Cela, R., Clarke, C., Guenther, M., Brunetti-Pierri, N., and Lee, B. (2010). MyD88-dependent silencing of transgene expression during the innate and adaptive immune response to helper-dependent adenovirus. Hum Gene Ther *21*, 325-336.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., and Inoue, M. (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res *59*, 551-557.

Takakura, M., Nakamura, M., Kyo, S., Hashimoto, M., Mori, N., Ikoma, T., Mizumoto, Y., Fujiwara, T., Urata, Y., and Inoue, M. (2010). Intraperitoneal administration of telomerase-specific oncolytic adenovirus sensitizes ovarian cancer cells to cisplatin and affects survival in a xenograft model with peritoneal dissemination. Cancer Gene Ther *17*, 11-19.

Takeda, T., Nakajima, K., Kojima, H., and Hirano, T. (1994). E1A repression of IL-6-induced gene activation by blocking the assembly of IL-6 response element binding complexes. J Immunol *153*, 4573-4582.

Thimmappaya, B., Weinberger, C., Schneider, R.J., and Shenk, T. (1982). Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. Cell *31*, 543-551.

Thomas, M.A., Spencer, J.F., La Regina, M.C., Dhar, D., Tollefson, A.E., Toth, K., and Wold, W.S. (2006). Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. Cancer Res *66*, 1270-1276.

Thomas, M.A., Spencer, J.F., Toth, K., Sagartz, J.E., Phillips, N.J., and Wold, W.S. (2008). Immunosuppression enhances oncolytic adenovirus replication and antitumor efficacy in the Syrian hamster model. Mol Ther *16*, 1665-1673.

Thomas, M.A., Spencer, J.F., and Wold, W.S. (2007). Use of the Syrian hamster as an animal model for oncolytic adenovirus vectors. Methods Mol Med *130*, 169-183.

Tollefson, A.E., Ryerse, J.S., Scaria, A., Hermiston, T.W., and Wold, W.S. (1996). The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants. Virology *220*, 152-162.

Toth, K., Spencer, J.F., Dhar, D., Sagartz, J.E., Buller, R.M., Painter, G.R., and Wold, W.S. (2008). Hexadecyloxypropyl-cidofovir, CMX001, prevents adenovirus-induced mortality in a permissive, immunosuppressed animal model. Proc Natl Acad Sci U S A *105*, 7293-7297.

Tuve, S., Liu, Y., Tragoolpua, K., Jacobs, J.D., Yumul, R.C., Li, Z.Y., Strauss, R., Hellstrom, K.E., Disis, M.L., Roffler, S.*, et al.* (2009). In situ adenovirus vaccination engages T effector cells against cancer. Vaccine *27*, 4225-4239.

van Kooten, C., and Banchereau, J. (2000). CD40-CD40 ligand. J Leukoc Biol *67*, 2-17.

Vasey, P.A., Shulman, L.N., Campos, S., Davis, J., Gore, M., Johnston, S., Kirn, D.H., O'Neill, V., Siddiqui, N., Seiden, M.V.*, et al.* (2002). Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted

adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. J Clin Oncol *20*, 1562-1569.

Volk, A.L., Rivera, A.A., Kanerva, A., Bauerschmitz, G., Dmitriev, I., Nettelbeck, D.M., and Curiel, D.T. (2003). Enhanced adenovirus infection of melanoma cells by fiber-modification: incorporation of RGD peptide or Ad5/3 chimerism. Cancer Biol Ther *2*, 511-515.

Waddington, S.N., McVey, J.H., Bhella, D., Parker, A.L., Barker, K., Atoda, H., Pink, R., Buckley, S.M., Greig, J.A., Denby, L*., et al.* (2008). Adenovirus serotype 5 hexon mediates liver gene transfer. Cell *132*, 397-409.

Wadhwa, P.D., Zielske, S.P., Roth, J.C., Ballas, C.B., Bowman, J.E., and Gerson, S.L. (2002). Cancer gene therapy: scientific basis. Annu Rev Med *53*, 437-452.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Wang, L.H., Rothberg, K.G., and Anderson, R.G. (1993). Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J Cell Biol *123*, 1107-1117.

White, E., Sabbatini, P., Debbas, M., Wold, W.S., Kusher, D.I., and Gooding, L.R. (1992). The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol Cell Biol *12*, 2570-2580.

Whiteside, T.L., and Herberman, R.B. (1995). The role of natural killer cells in immune surveillance of cancer. Curr Opin Immunol *7*, 704-710.

Wickham, T.J., Filardo, E.J., Cheresh, D.A., and Nemerow, G.R. (1994). Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. J Cell Biol *127*, 257-264.

Wickham, T.J., Mathias, P., Cheresh, D.A., and Nemerow, G.R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell *73*, 309-319.

Willcox, N., and Mautner, V. (1976). Antigenic determinants of adenovirus capsids. I. Measurement of antibody cross-reactivity. J Immunol *116*, 19-24.

Wille, A., Gessner, A., Lother, H., and Lehmann-Grube, F. (1989). Mechanism of recovery from acute virus infection. VIII. Treatment of lymphocytic choriomeningitis virus-infected mice with anti-interferon-gamma monoclonal antibody blocks generation of virus-specific cytotoxic T lymphocytes and virus elimination. Eur J Immunol *19*, 1283-1288.

Wold, W.S., Doronin, K., Toth, K., Kuppuswamy, M., Lichtenstein, D.L., and Tollefson, A.E. (1999). Immune responses to adenoviruses: viral evasion mechanisms and their implications for the clinic. Curr Opin Immunol *11*, 380-386.

Wong, H.H., Lemoine, N.R., and Wang, Y. (2010). Oncolytic Viruses for Cancer Therapy: Overcoming the Obstacles. Viruses *2*, 78-106.

Worgall, S., Wolff, G., Falck-Pedersen, E., and Crystal, R.G. (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. Hum Gene Ther *8*, 37-44.

Work, L.M., Nicklin, S.A., Brain, N.J., Dishart, K.L., Von Seggern, D.J., Hallek, M., Buning, H., and Baker, A.H. (2004). Development of efficient viral vectors selective for vascular smooth muscle cells. Mol Ther *9*, 198-208.

Xia, H., Anderson, B., Mao, Q., and Davidson, B.L. (2000). Recombinant human adenovirus: targeting to the human transferrin receptor improves gene transfer to brain microcapillary endothelium. J Virol *74*, 11359-11366.

Xia, Z.J., Chang, J.H., Zhang, L., Jiang, W.Q., Guan, Z.Z., Liu, J.W., Zhang, Y., Hu, X.H., Wu, G.H., Wang, H.Q*., et al.* (2004). [Phase III randomized clinical trial of intratumoral injection of E1B gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus.]. Ai Zheng *23*, 1666-1670.

Xie, X., Zhao, X., Liu, Y., Young, C.Y., Tindall, D.J., Slawin, K.M., and Spencer, D.M. (2001). Robust prostate-specific expression for targeted gene therapy based on the human kallikrein 2 promoter. Hum Gene Ther *12*, 549-561.

Yamamoto, M., Alemany, R., Adachi, Y., Grizzle, W.E., and Curiel, D.T. (2001). Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers. Mol Ther *3*, 385-394.

Yamamoto, M., and Curiel, D.T. (2010). Current issues and future directions of oncolytic adenoviruses. Mol Ther *18*, 243-250.

Yang, Y., Jooss, K.U., Su, Q., Ertl, H.C., and Wilson, J.M. (1996). Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes *in vivo*. Gene Ther *3*, 137-144.

Yang, Y., and Wilson, J.M. (1995). Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs *in vivo*. J Immunol *155*, 2564-2570.

Yang, Y., Xiang, Z., Ertl, H.C., and Wilson, J.M. (1995). Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes *in vivo*. Proc Natl Acad Sci U S A *92*, 7257-7261.

Yew, P.R., and Berk, A.J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature *357*, 82-85.

Ying, B., Toth, K., Spencer, J.F., Meyer, J., Tollefson, A.E., Patra, D., Dhar, D., Shashkova, E.V., Kuppuswamy, M., Doronin, K*., et al.* (2009). INGN 007, an oncolytic adenovirus vector, replicates in Syrian hamsters but not mice: comparison of biodistribution studies. Cancer Gene Ther.

Ylosmaki, E., Hakkarainen, T., Hemminki, A., Visakorpi, T., Andino, R., and Saksela, K. (2008). Generation of a conditionally replicating adenovirus based on targeted destruction of E1A mRNA by a cell type-specific MicroRNA. J Virol *82*, 11009-11015.

Yokoyama, T., Iwado, E., Kondo, Y., Aoki, H., Hayashi, Y., Georgescu, M.M., Sawaya, R., Hess, K.R., Mills, G.B., Kawamura, H*., et al.* (2008). Autophagy-inducing agents augment the antitumor effect of telerase-selve oncolytic adenovirus OBP-405 on glioblastoma cells. Gene Ther *15*, 1233-1239.

Yoo, J.Y., Kim, J.H., Kwon, Y.G., Kim, E.C., Kim, N.K., Choi, H.J., and Yun, C.O. (2007). VEGF-specific short hairpin RNA-expressing oncolytic adenovirus elicits potent inhibition of angiogenesis and tumor growth. Mol Ther *15*, 295-302.

Yotnda, P., Zompeta, C., Heslop, H.E., Andreeff, M., Brenner, M.K., and Marini, F. (2004). Comparison of the efficiency of transduction of leukemic cells by fiber-modified adenoviruses. Hum Gene Ther *15*, 1229-1242.

Yu, W., and Fang, H. (2007). Clinical trials with oncolytic adenovirus in China. Curr Cancer Drug Targets *7*, 141-148.

Zhang, K.J., Wang, Y.G., Cao, X., Zhong, S.Y., Wei, R.C., Wu, Y.M., Yue, X.T., Li, G.C., and Liu, X.Y. (2009). Potent antitumor effect of interleukin-24 gene in the survivin promoter and retinoblastoma double-regulated oncolytic adenovirus. Hum Gene Ther *20*, 818-830.

Zhang, W., and Imperiale, M.J. (2003). Requirement of the adenovirus IVa2 protein for virus assembly. J Virol *77*, 3586-3594.

Zhang, Y., Chirmule, N., Gao, G.P., Qian, R., Croyle, M., Joshi, B., Tazelaar, J., and Wilson, J.M. (2001). Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther *3*, 697-707.

Zhang, Z., Zou, W., Wang, J., Gu, J., Dang, Y., Li, B., Zhao, L., Qian, C., Qian, Q., and Liu, X. (2005). Suppression of tumor growth by oncolytic adenovirus-mediated delivery of an antiangiogenic gene, soluble Flt-1. Mol Ther *11*, 553-562.

PART C – ORIGINAL PUBLICATIONS