Adenoviral Gene Therapy for Advanced Head and Neck Cancer

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“No amount of experimentation can ever prove me right;
a single experiment can prove me wrong.”
- Albert Einstein

To my parents and Laura

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Part A

i. List of original publications

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

I- **João D. Dias**, Kilian Guse, Petri Nokisalmi, Minna Eriksson, Dung-Tsa Chen, Iulia Diaconu, Mikko Tenhunen, Ilkka Liikanen, Reidar Grénman, Mikko Savontaus, Sari Pesonen, Vincenzo Cerullo and Akseli Hemminki

Multi-modal approach using oncolytic adenovirus, cetuximab, chemotherapy and radiotherapy in HNSCC low passage tumour cell cultures,

Eur J Cancer. 2010 Feb;46(3):625-635. Epub 2009 Dec 16.

II- Ilkka Liikanen, **João D. Dias**, Petri Nokisalmi, Marta Sloniecka, Lotta Kangasniemi, Maria Rajecki, Thomas Dobner, Mikko Tenhunen, Anna Kanerva, Sari Pesonen, Laura Ahtiainen and Akseli Hemminki

Adenoviral E4orf3 and E4orf6 Proteins, but not E1B55K, Increase Killing of Cancer Cells by Radiotherapy *in vivo*.

Int J Radiat Oncol Biol Phys. 2010 Sep 8. [Epub ahead of print]

III- **João D. Dias**, Ilkka Liikanen, Kilian Guse, Johann Foloppe, Marta Sloniecka, Iulia Diaconu, Ville Rantanen, Minna Eriksson, Tanja Hakkarainen, Monika Lusky, Philippe Erbs, Sophie Escutenaire, Anna Kanerva, Sari Pesonen, Vincenzo Cerullo and Akseli Hemminki,

Targeted Chemotherapy for Head and Neck Cancer with a Chimeric Oncolytic Adenovirus Coding for Bifunctional Suicide Protein FCU1

Clin Cancer Res. 2010 May 1;16(9):2540-9. Epub 2010 Apr 13

IV- **João D. Dias**, Alessandro Bonetti, Kilian Guse, Iulia Diaconu, Sophie Escutenaire, Anna Kanerva, Sari Pesonen, Vincenzo Cerullo and Akseli Hemminki

Targeted Cancer Immunotherapy with a Oncolytic Adenovirus Coding for a Fully Human Monoclonal Antibody Specific for Human Cytotoxic T Lymphocyte-Associated Antigen 4

Submitted

ii. Abbreviations

5-FC	5-fluorocytosine
5-FU	5-fluorouracil
5-FUMP	5-fluorouridine monophosphate
Ad	adenovirus
AAT	antiangiogenic therapies
bp	base pair
CAR	coxsackie-adenovirus receptor
CCL5	chemokine (C-C motif) ligand 5
CD	cytosine deaminase
Cox-2	cyclooxygenase-2
CMV	cytomegalovirus
CR	constant region
CRAd	conditionally replicating adenovirus
CSC	cancer stem cells
CT	computerized Axial Tomography Scan
CTLA-4	cytotoxic T Lymphocyte-Associated Antigen 4
CTL	cytotoxic T-lymphocytes
DLT	dose limited toxicity
DMEM	dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNAPK	DNA-protein kinase
DSB	double strand brakes
EGFR	epidermal growth factor receptor
EpCAM	epithelial cell adhesion molecule
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FCU1	bifunctional fusion enzyme of CD and UPRT
Foxp3	forkhead box p3
GCV	ganciclovir
GM	growth media
GM-CSF	granulocyte macrophage colony-stimulating factor
Gy	gray
HCC	hepatocellular carcinoma
HeLa	Henrietta Lacks tumor cells
HIFU	high-intensity focused ultrasound
HNC	head and neck cancer
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HSV-TK	herpes simplex thymidine kinase
HSPGs	heparin sulfate proteoglycans
hTERT	human telomerase
IFN	interferon
i.ha.	intrahepatic artery
IMRT	intensity-modulated radiation therapy
i.p.	intraperitoneal
irAEs	immune-related adverse events
i.t.	intratumoral

ITR	inverted terminal repeat
i.v.	intravenous
LacZ	β-galactosidase
luc	luciferase
mAb	monoclonal antibody
MAP	mitomycin C + doxorubicin + cisplatin
miRNAs	microRNAs
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
MOI	multiplicity of infection
MRI	magnetic resonance imaging
NF-κB	nuclear factor κB
NK	natural killer cells
NKT	natural killer T cells
OV	oncolytic virotherapy
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC	prostate carcinoma
PCR	polymerase chain reaction
PET	positron emission tomography
PET-CT	positron emission tomography - computed tomography
PFU	plaque forming unit
pK	polylysine
PMA	phorbol myristyl acetate
pRb	retinoblastoma protein family
Rb	retinoblastoma
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RGD	arginine-glycine-aspartic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNAs	small interfering RNAs
TCID50	tissue culture infective dose 50
TCR	T cell receptor
TGF-β	transforming growth factor beta
TH3	T helper type-3 cells
TKI	tyrosine kinase inhibitor
T-regs	regulatory T cells
TSPs	tissue-specific promoters
UPRT	uracil phosphoribosyltransferase
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VP	virus particle

iii. Abstract

Advanced stage head and neck cancers (HNC) with distant metastasis, as well as prostate cancers (PC), are devastating diseases currently lacking efficient treatment options. One promising developmental approach in cancer treatment is the use of oncolytic adenoviruses, especially in combination therapy with conventional cancer therapies. The safety of the approach has been tested in many clinical trials. However, antitumor efficacy needs to be improved in order to establish oncolytic viruses as a viable treatment alternative. To be able to test *in vivo* the effects on anti-tumor efficiency of a multimodal combination therapy of oncolytic adenoviruses with the standard therapeutic combination of radiotherapy, chemotherapy and Cetuximab monoclonal antibody (mAb), a xenograft HNC tumor model was developed. This model mimics the typical clinical situation as it is initially sensitive to cetuximab, but resistance develops eventually. Surprisingly, but in agreement with recent findings for chemotherapy and radiotherapy, a higher proportion of cells positive for HNC cancer stem cell markers were found in the tumors refractory to cetuximab. *In vitro* as well as *in vivo* results found in this study support the multimodal combination therapy of oncolytic adenoviruses with chemotherapy, radiotherapy and monoclonal antibody therapy to achieve increased anti-tumor efficiency and even complete tumor eradication with lower treatment doses required. In this study, it was found that capsid modified oncolytic viruses have increased gene transfer to cancer cells as well as an increased antitumor effect. In order to elucidate the mechanism of how oncolytic viruses promote radiosensitization of tumor cells *in vivo*, replicative deficient viruses expressing several promising radiosensitizing viral proteins were tested. The results of this study indicated that oncolytic adenoviruses promote radiosensitization by delaying the repair of DNA double strand breaks in tumor cells. Based on the promising data of the first study, two tumor double-targeted oncolytic adenoviruses armed with the fusion suicide gene FCU1 or with a fully human mAb specific for human Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4) were produced. FCU1 encodes a bifunctional fusion protein that efficiently catalyzes the direct conversion of 5-FC, a relatively nontoxic antifungal agent, into the toxic metabolites 5-fluorouracil and 5-fluorouridine monophosphate, bypassing the natural resistance of certain human tumor cells to 5-fluorouracil. Anti-CTLA4 mAb promotes direct killing of tumor cells via apoptosis and most importantly immune system activation against the tumors. These armed oncolytic viruses present increased anti-tumor efficacy both *in vitro* and *in vivo*. Furthermore, by taking advantage of the unique tumor targeted gene transfer of oncolytic adenoviruses, functional high tumor titers but low systemic concentrations of the armed proteins were generated. In addition, supernatants of tumor cells infected with Ad5/3-Δ24aCTLA4, which contain anti-CTLA4 mAb, were able to effectively immunomodulate peripheral blood mononuclear cells (PBMC) of cancer patients with advanced tumors.

In conclusion, the results presented in this thesis suggest that genetically engineered oncolytic adenoviruses have great potential in the treatment of advanced and metastatic HNC and PC.

PART B

1 REVIEW OF THE LITERATURE

1.1 Introduction

Cancer is a devastating disease which has been one of the main known causes of death worldwide (Eaton 2003). Despite the improvements made in recent years in conventional cancer treatment modalities, together with more effective diagnostic techniques and earlier access to cancer treatments, the number of cancer cases is still on the rise (Eaton 2003). The high rates of mortality associated with cancer and the complications that arise with its treatments has encouraged the pursuit of alternative therapeutic strategies.

In recent years, developments in the fields of cancer biology, cancer genetics and molecular biology have stimulated a renewed interest in cancer gene therapy with special interest in tumor targeted oncolytic viruses. Several viruses have been used in the past few years (Vähä-Koskela, Heikkilä et al. 2007) but by far adenoviruses are the most studied and to the present day the only approved vector for the treatment of cancer patients (Garber 2006).

Several clinical trials have demonstrated the safety of oncolytic viruses as well as promising results in anti-tumor efficacy (Nemunaitis, Khuri et al. 2001; Xia, Chang et al. 2004; Yu and Fang 2007). The most promising results were obtained with modalities in combination with conventional therapies. However, antitumor efficacy needs to be improved in order to establish oncolytic viruses as a viable treatment alternative.

1.2 Cancer

Cancer is a major public health problem in many parts of the world (Eaton 2003). It has been projected that cancer will become the leading cause of death worldwide this year in World Cancer Report from the International Agency for Research on Cancer. Furthermore, cancer has the most devastating economic impact of any cause of death in the world according to the Global Economic Cost of Cancer Report authored by Dr. Rigo John and Dr.Hana Ross (American Cancer Society and LIVESTRONG®). In 2002, 10.9 million new cases, 6.7 million related deaths and 24.6 million persons alive with cancer (within three years of diagnosis) were reported worldwide (Parkin, Bray et al. 2005). The most recent reports estimate that in Europe there are 1.7 million cancer related deaths every year alone (Ferlay, Parkin et al. 2010). Furthermore, the World Health Organization expects that the worldwide number of newly diagnosed cancers will double to 20 million by 2020, unless preventive measures are taken (Eaton 2003).

1.2.1 Head and Neck Cancer

Each year, more than 635,000 new cases of head and neck cancer (HNC) are diagnosed worldwide with 350 000 related deaths every year (Ferlay, Shin et al. 2010). HNC is the seventh most common cancer type worldwide, with more than 48,000 new cases reported every year in the United States alone (Ferlay, Shin et al. 2010; Jemal, Siegel et al. 2010). The incidence trends are declining in the last decade in the Indian subcontinent, East Asia, Western Europe and the United States for men, but for women, it is generally stable. In the Nordic countries, the incidence of HNC continues to increase for both men and women (Curado and Hashibe 2009). In Finland, only larynx or lip cancers have shown a decrease in incidence (Curado and Hashibe 2009). The major known risk factors are alcohol and tobacco consumption, but recently, the role of human papillomavirus (HPV) 16 in HNC, especially for oropharyngeal cancers, has been reported (Curado and Hashibe 2009). According to the latest cancer registries, in the developed countries 1 in 100 persons will be diagnosed with HNC during their life time and 1 out of 3 diagnosed cases will succumb to the disease (Curado and Hashibe 2009; Jemal, Siegel et al. 2009).

1.2.1.1 Head and Neck Squamous Cell Carcinoma

Approximately 90% of HNC cases are squamous cell carcinomas (HNSCC). HNSCC arises in the mucosal lining of the upper aerodigestive tract and it is an umbrella term that includes cancers at several sites (e.g. oral cavity, pharynx and larynx) having different etiologies and prognoses while sharing common risk factors and treatment options (Baatenburg de Jong, Hermans et al. 2001). The addition of chemotherapy to radiotherapy has been useful in the context of organ preservation. However, despite advances in conventional therapy including surgery, chemotherapy, and radiation, the 5-year mortality rate of patients with HNSCC has not improved (Prince and Ailles 2008). Uncontrolled growth, resulting from dramatic changes in gene expression patterns, combined with the relative accessibility of head and neck tumors to direct inoculation make HNSCC an ideal candidate for gene therapy approaches (Thomas and Grandis 2009).

1.2.1.1.1 Molecular mechanisms of HNSCC

Cascades of several genetic events promoting the inactivation of tumor-suppressor genes and/or activation of proto-oncogenes drive HNSCC progression. Molecular techniques have uncovered several genetic and epigenetic alterations in several stages of disease progression (Califano, van der Riet et al. 1996; Ha and Califano 2006; Perez-Ordonez, Beauchemin et al. 2006). The main risk factors associated with HNSCC are alcohol, tobacco consumption, and/or more recently oncogenic human papillomavirus type 16 (HPV16) exposure; disease occurs as a consequence of their genotoxic activity (Argiris, Karamouzis et al. 2008). Telomerase, which is involved in telomere maintenance and immortalization, thus protecting the acquired genetic changes, has been found to be reactivated in 90% of

HNSCC cases and in premalignant lesions (McCaul, Gordon et al. 2002). The loss of 9p21 is seen in 70–80% of HNSCC (Mao, Lee et al. 1996). Inactivation of p16, which is caused by homozygous deletion, point mutations, or promoter hypermethylation, and loss of 3p, could be early events in HNSCC carcinogenesis (Argiris, Karamouzis et al. 2008). Loss of heterozygosity of 17p and p53 point mutations are seen in over 50% of HNSCC cases (Balz, Scheckenbach et al. 2003). The prognostic significance of p53 mutations is rather controversial; however, disruptive p53 mutations in the DNA of the tumor were shown to be associated with reduced survival after surgical treatment of HNSCC (Argiris, Karamouzis et al. 2008). Amplification of 11q13 and over-expression of cyclin D1 are also detected in HNSCC, and could correlate with more aggressive tumor behavior (Argiris, Karamouzis et al. 2008).

1.2.1.1.2 Treatment options for HNSCC

Currently, the standard of care for HNSCC combines surgery, radiotherapy, chemoradiotherapy and cetuximab. Standard therapy for local disease is surgery, often followed by radiation. Another option is chemoradiation instead of surgery. For locally advanced tumors, the operation can be followed by chemoradiation or radiation with cetuximab. For metastatic disease, chemotherapy + cetuximab is standard treatment modality. Surgery is a standard treatment for HNSCC but is frequently limited by the anatomical extent of the tumor and desire to achieve organ preservation. Advances in microsurgical free tissue transfer for reconstruction of surgical defects have made major reconstructive procedures commonplace at many centers, helping in the resection of locally advanced tumors. By use of modern surgical techniques, substantially improved functional outcomes are often possible for patients who need extensive surgical resections, even in the setting of salvage surgery after failure of organ-preserving treatment (Argiris, Karamouzis et al. 2008).

The mortality rates and the morbid side effects associated with the standard therapies of HNSCC have prompted the pursuit of novel therapies. Therefore, in recent years, molecular targeted agents have been extensively studied and clinically tested in HNCs. These molecular targeted agents mainly centered on epidermal growth factor receptor (EGFR) inhibitors and antiangiogenic therapies (AAT), which include the modulation of vascular endothelial growth factor (VEGF) or its receptor (VEGFR). Examples of EGFR inhibitors include monoclonal antibodies against the extracellular domain of this receptor (e.g., cetuximab and panitumumab) and receptor tyrosine kinase inhibitors (TKIs) that target the intracellular domain (e.g., gefitinib and erlotinib). Other promising agents that produce antitumor effects in conjunction with EGFR receptor inhibitors include trastuzumab and lapatinib. Vandetanib, an antagonist of both VEGFR and the EGFR is in phase II trials. New molecular targets like hypoxia-inducible factor 1 alpha, mesenchymal-epithelial transition factor, insulin-like growth factor or the PI3K/AKT/mTOR pathway are currently under investigation.

1.2.1.1.2.1 Radiation therapy

Radiotherapy is an integral part of primary or adjuvant treatment of HNSCC. Radiotherapy alone results in high tumor control and cure rates for early stage glottic, base of tongue, and tonsillar cancers (Ding, Newman et al. 2005; Voynov, Heron et al. 2006). Advances in imaging and radiation delivery have dramatically changed management approaches. Planning CT scans are now frequently combined with diagnostic CT, MRI, or PET datasets to improve tumor delineation in three dimensions. Additional advances in radiotherapy include tomotherapy (integration of CT or PET-CT technology into a linear accelerator) heavy particle radiation, proton therapy, neutron beam radiation, brachytherapy, and stereotactic radiosurgery; however, in most instances these methods have not been validated in prospective randomized clinical trials (Ding, Newman et al. 2005; Voynov, Heron et al. 2006).

Intensity modulated radiotherapy (IMRT) is an advanced approach to three-dimensional treatment planning and conformal therapy. It optimizes the delivery of irradiation to irregularly-shaped volumes and has the ability to produce concavities in radiation treatment volumes. When treating HNCs, IMRT allows for a greater sparing of normal structures such as salivary glands, upper aero-digestive tract mucosa, optic nerves, cochlea, pharyngeal constrictors, brain stem and spinal cord (Bhide and Nutting 2010). Salivary gland sparing using IMRT in various head and neck sub-sites has been demonstrated in randomized and non-randomised trials (Bhide and Nutting 2010).

Radiation therapy for treatment of HNSCC is typically given in daily fractions of 2·0 Gy, 5 days a week, up to a total dose of 70 Gy over 7 weeks. Long-term interruptions to radiotherapy or delays in starting postoperative radiotherapy are potentially harmful, presumably because of repopulation of cancer cells (Bentzen 2003; Suwinski, Sowa et al. 2003; Bese, Hendry et al. 2007). Phase III trials have showed that despite of the improvements in locoregional control with increased infield toxic effects, the survival rates of hyperfractionation radiotherapy staid the same compared with conventional radiotherapy (Fu, Pajak et al. 2000).

Ionizing radiation targets primarily DNA molecules and produces an array of lesions that include single-strand breaks, base alterations, oxidative damage and double-strand breaks (Li, Story et al. 2001).

1.2.1.1.2.2 Chemotherapy

The role of chemotherapy in HNSCC treatment has evolved from palliative care to a central component of curative programs for locally advanced HNSCC (Cohen, Lingen et al. 2004). Several classes of agents such as platinum compounds, antimetabolites and taxanes have shown single-agent activity against HNSCC (Colevas 2006). The platinum compound cisplatin is regarded as a standard agent in combination with radiation or with other agents.

The main biochemical mechanism of action of cisplatin involves the binding of the drug to DNA in the cell nucleus and subsequent interference with normal transcription, and/or DNA replication mechanisms. If cisplatin-DNA adducts are not efficiently processed by cell machinery, cytotoxic processes may result in cell death (Fuertesa, Castillab et al. 2003). However, there are also other possible mechanisms that may play a role in the activity of cisplatin. Even before cell entry, cisplatin can bind to phospholipids and phosphatidylserine in the cell membrane thereby triggering the Fas death receptor pathway, promoting cell death via apoptosis (Rebillard, Lagadic-Gossmann et al. 2008). Once inside the cell, cisplatin has a number of possible targets: DNA; RNA; sulfur-containing enzymes such as metallothionein and glutathione; and mitochondria (Pil and Lippard 1992).

5-Fluorouracil (5-FU) is a pyrimidine analog that requires cellular uptake and metabolic activation in order to exert cytotoxicity. Routinely in the clinic, 5-FU is used in combined regimens with Cisplatin (Kish, Ensley et al. 1985). As a uracil analog, it serves as a substrate for the same transport processes and enzymes involved in anabolism and catabolism. As such, 5-FU may be utilized by several metabolic routes where it will be converted to its active metabolites for inhibition of DNA and RNA synthesis and interference with DNA repair (Grem 2000; Noordhuis, Holwerda et al. 2004).

1.2.1.1.2.3 Monoclonal antibody therapy

Epidermal growth factor receptor (EGFR) inhibition has emerged as a novel treatment strategy for HNSCC, and the monoclonal antibody cetuximab is the first EGFR targeted agent that has been introduced into standard practice (Karamouzis, Grandis et al. 2007). Other ways of targeting EGFR and other deregulated molecular pathways in HNSCC, using monoclonal antibodies, single-selective or multi-selective tyrosine kinase inhibitors, and nucleic acid-directed approaches, are also being explored (Argiris, Karamouzis et al. 2008). The combination of EGFR inhibitors with other molecularly targeted agents (e.g., angiogenesis inhibitors) has surfaced as a novel strategy, whereas the combination of these novel agents with chemotherapy and radiotherapy is under investigation (Argiris, Karamouzis et al. 2008). In our days, combination of cetuximab with either chemotherapy or radiotherapy is standard care and the triple combination is undergoing investigation.

Monoclonal EGFR inhibiting antibodies have improved the efficacy of conventional chemotherapy in both pre-clinical and clinical studies. Although such therapies may lead to a partial response or disease stabilization in some patients, many patients do not benefit from EGFR inhibitor therapy. Even those who do, eventually develop resistance (Pao, Miller et al. 2005). Great interest therefore exists in elucidating resistance mechanisms for EGFR inhibitor therapy. The molecular mechanisms of resistance can be attributed to several general processes involving emergence of inhibitor insensitive cell populations: (a) resistance due to the activation of alternative tyrosine kinase receptors that bypass the EGFR pathway (e.g. c-Met and IGF-1R), (b) resistance due to increased angiogenesis, (c) resistance based on constitutive activation of downstream mediators (e.g. PTEN, K-ras and others), (d) the existence of specific EGFR mutations (Dempke and Heinemann 2009) and

(e) emergence of EGFR negative clones. Therefore, combination treatments may be useful for avoiding development of EGFR inhibitor resistant disease.

The mechanisms through which cetuximab expresses its antitumor activity are numerous and not completely understood in humans. The main cetuximab activities include the direct inhibition of EGFR tyrosine kinase activity, the inhibition of cell cycle progression, angiogenesis, invasion and metastasization, the increase and activation of pro-apoptotic molecules, and the synergic cytotoxicity with chemotherapy and radiotherapy (Vincenzi, Schiavon et al. 2008). In addition, cetuximab is able to induce antibody-dependent cell-mediated cytotoxicity (Bonner, Harari et al. 2006). Moreover, recent reports suggest that cetuximab is able to also mediate complement system activation (Hsu, Ajona et al. 2010).

1.2.1.1.3 Cancer stem cells

Tumor initiating cells or cancer stem cells (CSC) are defined as cells that have the capacity to self-renew and to cause the heterogeneous lineages of cells that comprise the tumor (Reya, Morrison et al. 2001). Currently, there are two hypothetical explanations for the existence of CSCs. CSCs may arise from normal stem cells by mutation of genes that render the stem cells cancerous. Or, they may come from differentiated tumor cells that experience further genetic alterations and, therefore, become dedifferentiated and acquire CSC-like features (Chen 2009). According to the CSC theory, only a specific subpopulation of cancer cells called CSC have unlimited replicative potential and therefore the ability to sustain cancer growth. All of the other cancer cells or progenitor cells have a limited growth potential or no growth potential at all. Four key characteristics define the CSC subpopulation: (1) only a small portion of the cancer cells within a tumor have tumorigenic potential when transplanted into immunodeficient mice; (2) the CSC subpopulation can be separated from the other cancer cells by distinctive cell surface markers; (3) tumors resulting from the CSCs contain the mixed tumorigenic and non-tumorigenic cells of the original tumor; and (4) the CSC subpopulation can be serially transplanted through multiple generations, indicating that it is a self-renewing population (Prince and Ailles 2008).

CSC have been suggested to represent a distinct subpopulation of cells in many human tumors including HNSCC (Prince, Sivanandan et al. 2007), while more differentiated and less tumorigenic cells constitute the bulk of tumor cells (Reya, Morrison et al. 2001). Several CSC markers have been reported for isolation of CSC, including CD133, CD44, ALDH1A1, and epithelial cell adhesion molecule (EpCAM) (Visvader and Lindeman 2008). However, there is no universal CSC marker for all types of cancer. Tumor initiating HNSCC cells have been proposed to present a distinct phenotype identifiable by surface markers CD44, CD133 (Zhou, Wei et al. 2007; Pries, Witrkopf et al. 2008; Prince and Ailles 2008).

CSC have many properties that separate them from mature, differentiated cells. In addition to their ability to self-renew and differentiate, they are quiescent, dividing infrequently. They also require specific environments comprising other cells, stroma and growth factors for their survival (Blanpain, Lowry et al. 2004). One particularly intriguing property of stem cells is that they express high levels of specific ABC drug transporters (Blanpain, Lowry et al. 2004). An

important implication of this concept is that cancer stem cells by their quiescence, their capacity for DNA repair, and ABC-transporter expression are possibly more resistant to treatment with drugs or radiation that preferentially kill fast replicating cells, which can lead to tumor regrowth and relapse (Dean, Fojo et al. 2005).

1.2.2 Prostate cancer

Prostate carcinoma (PC) is the second most frequently diagnosed cancer of men (914,000 new cases, 13.8% of the total) and the fifth most common cancer overall (Ferlay, Shin et al. 2010). In 2008, it is estimated that there were more than 258,000 PC related deaths. A rising incidence is observed mainly due to early detection programs and increasing of life expectancy (Allen, Howard et al. 2007). In 2008, in Europe alone there were 382,000 new cases diagnosed, placing PC as the fourth most common cancer type in Europe (Ferlay, Parkin et al. 2010).

1.2.2.1 Molecular mechanisms in Prostate Cancer

Data suggest that prostate cancer results from the successive accumulation of gene mutations (Vogelstein and Kinzler 2004). Linkage analyses have indicated several chromosomal loci, such as 1p36 (CABP) (Gibbs, Stanford et al. 1999), 1q24-q25 (HPC1) (Smith, Freije et al. 1996), 1q42.4-q43 (PCAP) (Berthon, Valeri et al. 1998), 8p22-23 (Xu, Zheng et al. 2001), 16q23 (Suarez, Lin et al. 2000), 17p12-p13 (Tavtigian, Simard et al. 2001), 19q13 (Witte, Goddard et al. 2000), 20q13 (HPC20) (Berry, Schroeder et al. 2000), and Xq27-q28 (HPCX) (Xu, Meyers et al. 1998) that may harbor high-penetrance prostate cancer susceptibility genes. However, none of the loci have been verified indisputably by a second independent study confirming the tremendous heterogeneity in the predisposition of prostate cancer (Porkka and Visakorpi 2004). Three candidate susceptibility genes have also been identified. The first positionally cloned prostate cancer susceptibility gene was HPC2/ELAC2, located at 17p12 (Tavtigian, Simard et al. 2001). However, the function of the protein code by HPC2/ELAC2 is still not fully characterized. The second putative susceptibility gene was identified in HPC1-linked (chromosomal region 1q24-q25) families (Carpten, Nupponen et al. 2002). The prostate tumors carrying this mutated gene have reduced RNASEL enzyme activity. RNASEL is an endoribonuclease involved in the mediation of the antiviral and proapoptotic activities of the interferon-regulated 2-5A system (Porkka and Visakorpi 2004). The third identified prostate cancer susceptibility gene is the macrophage scavenger receptor 1 (MSR1) gene, located at 8p22-23 (Xu, Zheng et al. 2002). The expression of MSR1 is induced in macrophages by oxidative stress. It has been suggested that the cancer predisposing effects of MSR1 is mediated by macrophages (Xu, Zheng et al. 2002; DeMarzo, Nelson et al. 2003).

Numerous polymorphisms in many genes have already been suggested to be associated with the risk of prostate cancer (DeMarzo, Nelson et al. 2003; Gronberg 2003). Maybe the most widely studied polymorphic gene is the androgen receptor gene or genes that are involved in androgen metabolism. Other genes, whose sequence variations have been

suggested to be associated with the risk of prostate cancer, include BRCA2 (Edwards, Kote-Jarai et al. 2003), CHECK2 (Dong, Wang et al. 2003), vitamin D receptor (VDR) (Ingles, Ross et al. 1997), 17α-hydroxylase (CYP17) (Lunn, Bell et al. 1999), paraoxonase 1 (PON1) (Marchesani, Hakkarainen et al. 2003) and 5α reductase (SRD5A2) (Makridakis, Ross et al. 1999). However, larger and better controlled studies are needed for more definitive associations.

1.2.2.2 Treatment options for Prostate Cancer

Radiotherapy and surgery are commonly used primary therapies for localized and locally advanced prostate cancer, with or without androgen deprivation therapy, and are the two main curative treatment options for PC (Shelley, Kumar et al. 2009). Radiation therapy may be delivered through external beam irradiation or brachytherapy. The choice of the curative treatment modality remains strongly related to patient features (age, urinary, digestive, sexual status) as well as tumor features such as Gleason score, clinical stage and PSA level (D'Amico, Whittington et al. 1998). Due to technical improvements, effects of radiotherapy in normal tissue are decreasing, and hence, important side effects such as erectile dysfunction or radiation proctitis are lowered, increasing the popularity of this therapeutic approach (Sanda, Dunn et al. 2008; Zelefsky, Levin et al. 2008). Also, great improvements have been achieved in radiotherapy dose planning, which has allowed higher tumor doses which leads to more cures. However, 35% of cases recur or are detected when metastatic (Pound, Partin et al. 1999). Hormonal therapies are usually effective initially, but given enough time, hormone refractory disease eventually emerges (Feldman and Feldman 2001; Shelley, Kumar et al. 2009). Currently, different recognized treatment options are available in case of local failure after radiation therapy such as radical prostatectomy, cryotherapy, high-intensity focused ultrasound (HIFU), chemotherapy (docetaxel), T cell Immunotherapy (Sipuleucel-T), and brachytherapy (Boukaram and Hannoun-Levi 2010; Kantoff, Higano et al. 2010).

1.2.3 Cancer immunity

Immunity has two main distinct effects on cancer. On one side, immunity prevents against the development of nascent tumors, defined as cancer immunosurveillance. In fact, compelling experimental studies in mouse models of cancer together with clinical data from human patients have uncovered cancer immunosurveillance functions as an effective extrinsic tumor suppressor mechanism (Smyth, Dunn et al. 2006). On the other side, immunity sculpts the intrinsic nature of developing tumors through the immunological pressure afforded by cancer immunosurveillance. This combination of host-protective and tumor-sculpting functions of the immune system throughout tumor development is termed cancer immunoediting (Dunn, Old et al. 2004). Cancer immunoediting refers to a dynamic process comprising of three phases: elimination, equilibrium, and escape (Dunn, Old et al. 2004). Elimination consists of the classical concept of cancer immunosurveillance, where pre-malignant and early-stage malignant cells are directly or indirectly removed by immune

cells. Equilibrium is the period of immune-mediated latency after incomplete tumor destruction, and escape refers to the final outgrowth of tumors that have overcome immunological pressure. When the immune system ultimately fails to eliminate all transformed cells, tumors with reduced immunogenicity emerge capable of escaping immune destruction and, in some circumstances, harness or alter ensuing inflammatory reactions to their own benefit (**Figure 1**).

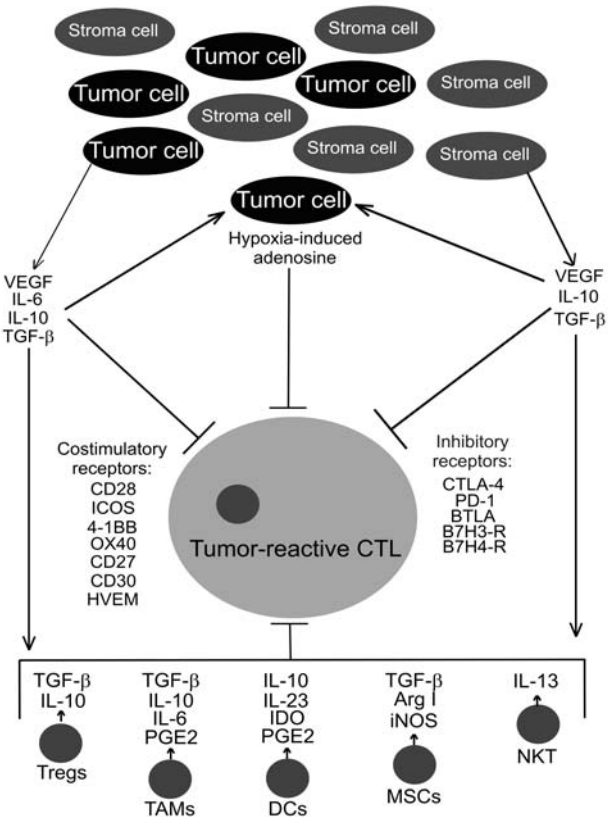

Figure 1. Tumor-associated immune suppression. Several regulatory mechanisms limit the activity of tumor-reactive cytotoxic T lymphocytes (CTLs). Immune regulation can be intrinsic to CTLs through the unbalanced activation of costimulatory and inhibitory receptors. Tumor cells can produce immune-suppressive factors, such as TGF-b, IL-6, IL-10, and VEGF, sometimes as a result of activated oncogenes, such as STAT3. Activation of STAT3 in tumor-infiltrating immune cells further suppresses their activity. Tissue remodeling and hypoxia induce the release of immune-suppressive factors such as adenosine, TGF-b, IL-10, and VEGF. Finally, tumors are often infiltrated with a broad range of regulatory cells such as T-regulatory cells (Tregs), type II NKT cells, myeloid-derived suppressor cells (MSC), tumor-associated macrophages (TAM), and tolerogenic DCs that produce a broad range of immune-suppressive factors, such as TGF-b, IL-6, IL-10, IL-13, IL-23, prostaglandin-E2 (PGE2), IDO, and arginase I (Arg). TGF-b, transforming growth factor-b; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; STAT3, signal transducer and activator of transcription 3; DCs, dendritic cells; IDO, indoleamine 2–3 dioxygenase; NKT, natural killer T cells. (Adapted from (Stagg, Johnstone et al. 2007)).

Given the now well-established importance of the immune system at controlling and shaping developing tumors (Smyth, Dunn et al. 2006; Zitvogel, Tesniere et al. 2006; Swann and Smyth 2007), more effective cancer therapies might be developed by understanding how tumors escape the immune system and more importantly, how to increase the immunogenicity of tumors.

1.2.3.1 T regulatory cells

In recent years, several populations of specialized regulatory cells have emerged as potent regulators of immune responses, including a number of T-cell subsets. One of the main mechanisms that the tumors use to escape anti-tumor immunity is through regulatory T cells (T-regs). T-regs fall into two main categories: those that are continuously produced by the

thymus and dependent on the expression of forkhead box p3 (Foxp3), such as $CD4^+$ $CD25^+$ Tregs, and those that arise as a result of peripheral encounters, such as IL-10-producing, Foxp3-negative Tr1 cells, and TGF-β- producing T helper type-3 (Th3) cells (Stagg, Johnstone et al. 2007). T-regs are activated in an antigen-specific manner but are generally believed to suppress T cells in an antigen-non-specific manner. Although the precise mechanisms of action of T-regs are not entirely clear, it has been suggested that T-reg-mediated suppression is regulated by, among other molecules, CTLA-4 (Miyara and Sakaguchi 2007). Therefore, CTLA-4 is of important interest as a molecular target to breakdown the tumor immune tolerance.

In addition to T-regs, T cells expressing both TCR and NK-cell receptors have also been shown to possess regulatory properties (Kronenberg 2005). CD1d-restricted NKT cells include two subsets: invariant type I NKT cells and non-invariant type II NKT cells. Whereas activation of type I NKT cells has potent immune stimulatory effects, type II NKT cells were shown to be sufficient to suppress tumor immunosurveillance (Terabe, Swann et al. 2005). Regulatory NKT cells can produce IL-13, which in turn can activate myeloid-derived $Gr1^{high}$ $Mac1^+$ suppressor cells to produce TGF-β (Terabe, Matsui et al. 2003).

1.2.3.2 A specific monoclonal antibody for the negative costimulatory receptor cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152)

Regulatory pathways that determine the immune response to cancer are becoming increasingly well characterized. One of these pathways involves the negative costimulatory receptor cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152). CTLA-4 is an activation-induced Type I transmembrane protein of the Ig superfamily which is expressed by T lymphocytes as a covalent homodimer andfunctions as an inhibitory receptor for the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) (Ribas, Hanson et al. 2007) **(Figure 2)**. CTLA-4 blockade with mAbs results in increased interleukin-2 (IL-2) and interferon-gamma (IFN-γ) production by lymphocytes, and increased expression of major histocompatibility complex (MHC) class I molecules (Lee, Chuang et al. 1998; Paradis, Floyd et al. 2001). The preclinical antitumor efficacy of antagonistic antibodies to CTLA-4 has been previously shown in several tumor models, including decreased relapses when given as adjuvant immunotherapy in a model of metastatic PC (Leach, Krummel et al. 1996; Kwon, Foster et al. 1999). Currently, two fully human monoclonal antibodies (mAbs) with CTLA-4 antagonistic activity are in clinical testing; ipilimumab (IgG1 isotype) (formerly MDX-010; developed by Medarex Inc.,Bloomsburg, NJ, and codeveloped with Bristol-Myers Squibb, Princeton, NJ) and tremelimumab (IgG2) (formerly CP-675,206; developed by Pfizer Pharmaceuticals Inc., NY). Several previous studies have accessed the biologic and clinical activity of ipilimumab and tremelimumab in patients with melanoma and other cancer types (Ribas, Hanson et al. 2007; Hodi, O'Day et al. 2010; Kirkwood, Lorigan et al. 2010). However, these therapeutic agents also have the potential to create long-lasting severe immune-related adverse events (irAEs) and even death as a result of the disruption of T cell homeostasis or the breaking of tolerance to self antigens (Maker, Phan et al. 2005; Hodi, O'Day et al. 2010). A recent phase III clinical trial with ipilimumab in combination with

glycoprotein 100 (gp100) revealed that anti-CTLA4 alone had a median overall survival of 10.1 months, similar to the combination group (Hodi, O'Day et al. 2010). However, grade 3 or 4 immune-related adverse events occurred in 10 to 15% of patients treated with ipilimumab and in 3% treated with gp100 alone. There were 14 deaths related to the study drugs (2.1%) and 7 were associated with immune-related adverse events (Hodi, O'Day et al. 2010).

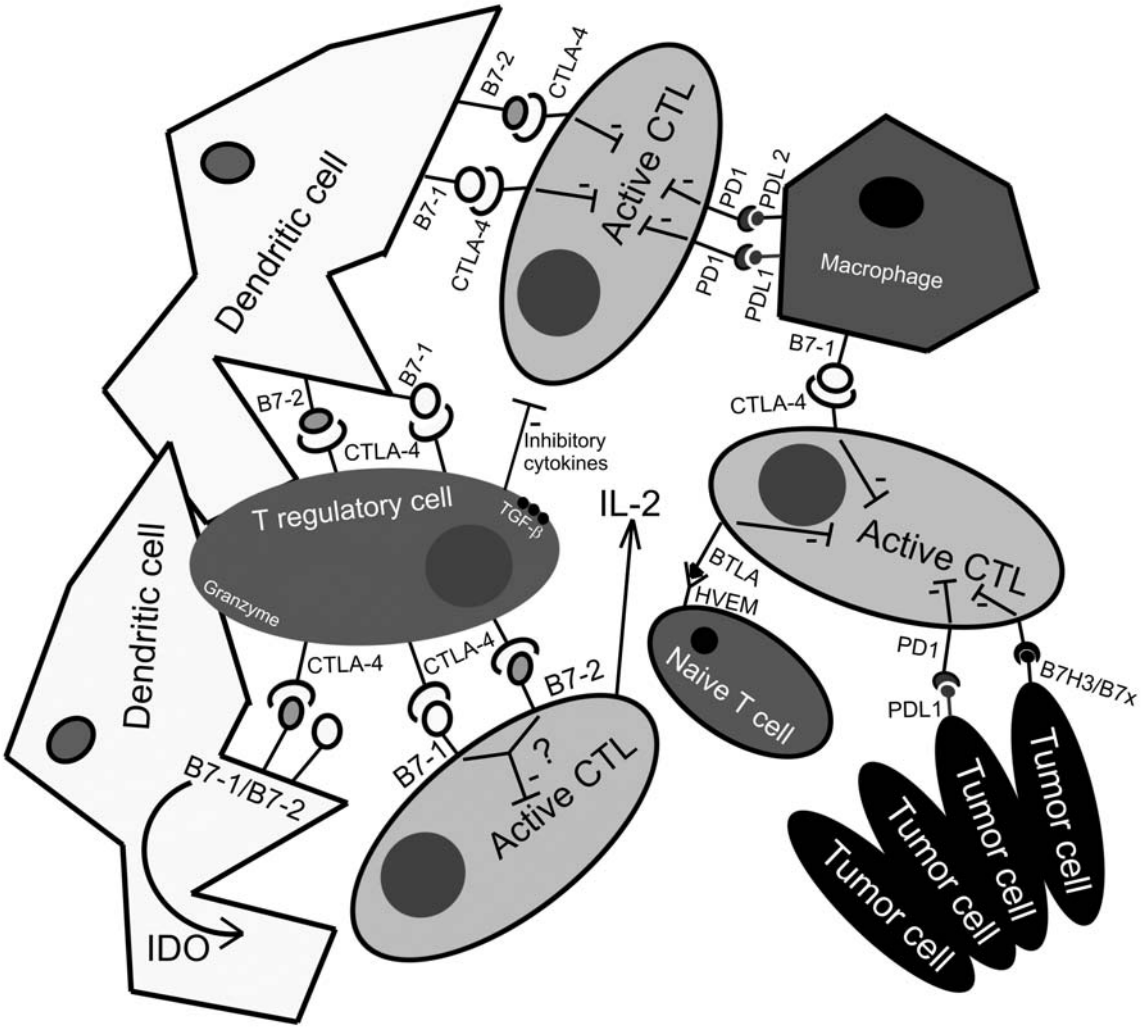

Figure 2. CTLA-4 expressing cells and their respective interactions. All members of the immunoglobulin superfamily that act as inhibitory checkpoints are potential targets for manipulation in immunotherapies. CD28, CTLA-4, B7-1, and B7-2 are centrally important for the initial activation of naïve T cells of the clonal composition of the responding repertoire following migration of activated dendritic cells to lymphoid activation organs. As activated effectors traffic back into peripheral tissues, they come under the influence of PD-1–PDL-1–and PD-1–PDL-2–mediated signaling, as a result of interactions with both tissue macrophages and ligands expressed on malignant cells. B7-H3 and B7x might act as the final arbiters of the fate of T-cell effector interactions with nonlymphoid target tissues, and might protect tumor cells that express them from cytotoxic T-cell–mediated killing. The potential for crosstalk between T-cell populations via many of these pathways is complex, particularly because activated cells can upregulate receptors and/or ligands that can potentially signal bidirectionally.

Blockade of BTLA might remove inhibitory restraints imposed by HVEM-expressing cells, but effects on T-cell–T-cell interactions mediated by blockade of CTLA-4, PD-1 or PDL-1, or B7-H3 are also possible. Regulatory T cells provide an additional therapeutic target. Their mode of function *in vivo* is not entirely clear. Experimental evidence points to important roles for inhibitory cytokines, membrane bound TGF-β, and granzyme. The role of CTLA-4 remains controversial, but could be mediated via outside-in signaling through the B7 ligands.
Abbreviations: BTLA, B- and T-lymphocyte attenuator; CTLA-4, cytotoxic T-lymphocyte antigen 4; HVEM, herpes virus entry mediator; IDO, indoleamine 2,3-digoxygenase; IL-2, interleukin-2; LIGHT, lymphtoxins, inducible, competes with herpes simplex virus glycoproteins D for HVEM, expressed by T cells; PD, programmed death; PDL, programmed death ligand; TGF-β, transforming growth factor β. Adapted from (Peggs, Quezada et al. 2006)

Based on several *in vitro* studies, Ribas and colleagues (Ribas, Hanson et al. 2007) have proposed several potential mechanisms of antitumor responses mediated by anti-CTLA-4 blocking antibodies (Abs). (A): Anti–CTLA-4 Abs can block the negative signaling derived from the activation-induced CTLA-4 molecule on the surface of activated T cells triggered by B7 costimulatory molecules on dendritic cells (DCs). (B): A subset of T regulatory cells that constitutively express CTLA-4 may provide reverse signaling by binding to B7 molecules on DCs, which can upregulate indoleamine 2,3-dioxygenase and thereby tolerize T cells in the microenvironment. (C): CTLA-4–expressing T cells may also bind directly to activated T cells, because B7 costimulatory molecules are expressed on the surface of activated human T cells. CTLA-4–blocking Abs would interfere with this negative signaling and result in the local expansion of tumor antigen-specific T cells. (D): CTLA-4 can be expressed on the surface of tumor cells. CTLA-4–blocking Abs may induce direct killing of tumor cells by triggering apoptosis or Ab-dependent cellular cytotoxicity. (E): Tumor-expressed CTLA-4 may trigger increased indoleamine 2,3-dioxygenase in tumor-infiltrating DCs, and CTLA-4–specific monoclonal Abs would also block this effect (Ribas, Hanson et al. 2007). However, the mechanism or mechanisms of action that mediate the increased anti-tumor effect in cancer patients is still not fully understood.

1.3 Cancer Gene Therapy

The ability of viruses to kill cancer cells has been known for more than a century (Kelly and Russell 2007). Their antitumor potency is obtained by several mechanisms, including direct lysis, apoptosis, expression of toxic proteins, autophagy and shut-down of protein synthesis, as well as the induction of anti-tumoral immunity. Even though clinical trials of several naturally-occurring oncolytic viruses date back to the 1950s, it was only in 1991 that a herpes simplex virus-1 (HSV-1) with deletion of its thymidine kinase UL23 gene became the first genetically-engineered, replication-selective oncolytic virus to be tested in the laboratory (Martuza, Malick et al. 1991). In 2005, an adenovirus (Ad) with E1B 55K and E3B genes deletion (H101(Oncorine); Shanghai Sunway Biotech, Shanghai, China) was approved in China as the world's first oncolytic virus for HNC in combination with chemotherapy (Garber 2006). Besides oncolytic virotherapy (OV) other cancer gene therapy strategies have been explored, mainly gene replacement therapies or expression of toxic proteins. However, promising laboratory results have not always been translated to improved clinical outcomes, and this appears to be determined by the complex interactions between the tumor and its

microenvironment, the virus, and the host immunity (Wong, Lemoine et al. 2010). Therefore, there is the need to develop more potent antitumor vectors as well as more effective therapeutic strategies.

1.3.1 Oncolytic viruses

Oncolytic viruses are viruses that are able to replicate specifically in and destroy tumor cells, and this property is either inherent or genetically-engineered. After replication, cancer cells are lysed and virus progeny is released to infect neighboring cancer cells. In principle, infection and replication can continue until all tumor cells are eliminated including distant metastasis (Qiao, Kottke et al. 2008). Several DNA and RNA viruses have been studied for their ability to replicate and lyse tumor cells such as adenoviruses, herpes viruses and pox viruses, (Vähä-Koskela, Heikkilä et al. 2007), however, not all were further developed for OV. Tumor-selective viruses can specifically target cancer by exploiting the very same cellular aberrations that occur in these cells, such as surface attachment receptors, activated *Ras* and *Akt*; and defective Rb/p16 and interferon (IFN) pathways (Sherr 1996; Wong, Lemoine et al. 2010).

1.3.1.1 Adenoviruses

Adenoviruses (Ad) were isolated in 1953 from human adenoid tissue samples in culture undergoing “spontaneous” regression, and were dubbed adenoidal–pharyngeal–conjunctival viruses based on their capacity to induce disease symptoms in experimentally infected humans (Rowe, Huebner et al. 1953). Since then adenoviruses have become the most widely used and most extensively studied viruses for gene delivery/therapy purposes. Oncolytic adenoviruses were the first and so far the only approved oncolytic viruses in combination with chemotherapy for the treatment of refractory HNC (Garber 2006).

Several characteristics made possible that Ad was the first OV approved virus for treatment of cancer patients (Garber 2006). Ads have a natural lytic replication cycle and they are able to infect cells regardless of cell cycling status (Hemmi, Geertsen et al. 1998). Also, Ad production is efficient and stable particles are produced in high titers. The Ad genome is easily manipulated, being able to accommodate up to 105% of the wild type’s 36 kb genome, and there are several genome manipulating tools available to facilitate this process. In consequence, therapeutic transgenes can be easily incorporated into the viral genome in order to further improve the viral anti-tumor efficacy. In addition, the adenoviral genome stays episomal and thus mutational risk of infected cells is low. Finally, Ads have been extensively used in vaccination programs and the possible side effects are well known and easily resolved in normal conditions.

1.3.1.1.1 Adenoviral general virology

Adenoviruses are nonenveloped, icosahedral particles of approximately 90 nm in diameter containing linear, double-stranded DNA, and projecting fibers from the vertices of the

icosahedrons **(Figure 3)**. As in many other nonenveloped viruses, Ad virions are mainly constituted of proteins and DNA, and some carbohydrates can also be found but not lipids (Russell 2000). The protein fraction is the main Ad constituent and it is formed by three major proteins (hexon (II), penton base (III), knobbed fiber (IV)) and five minor proteins (VI, VIII, XI, IIIa and IVa2) (Fig. 3). The virus's genomic DNA is covalently bounded to a terminal protein (TP) in the 5' termini containing inverted terminal repeats (ITRs) (Rekosh, Russell et al. 1977). In addition, the viral DNA is also associated with protein VII and the small peptide mu (Anderson, Young et al. 1989). Protein V is packaged with this DNA-protein complex and seems to provide a structural link to the capsid together with protein VI (Matthews and Russell 1995). Lastly, the protein fraction also contains a protease necessary for processing some of the structural proteins to produce mature infections particles.

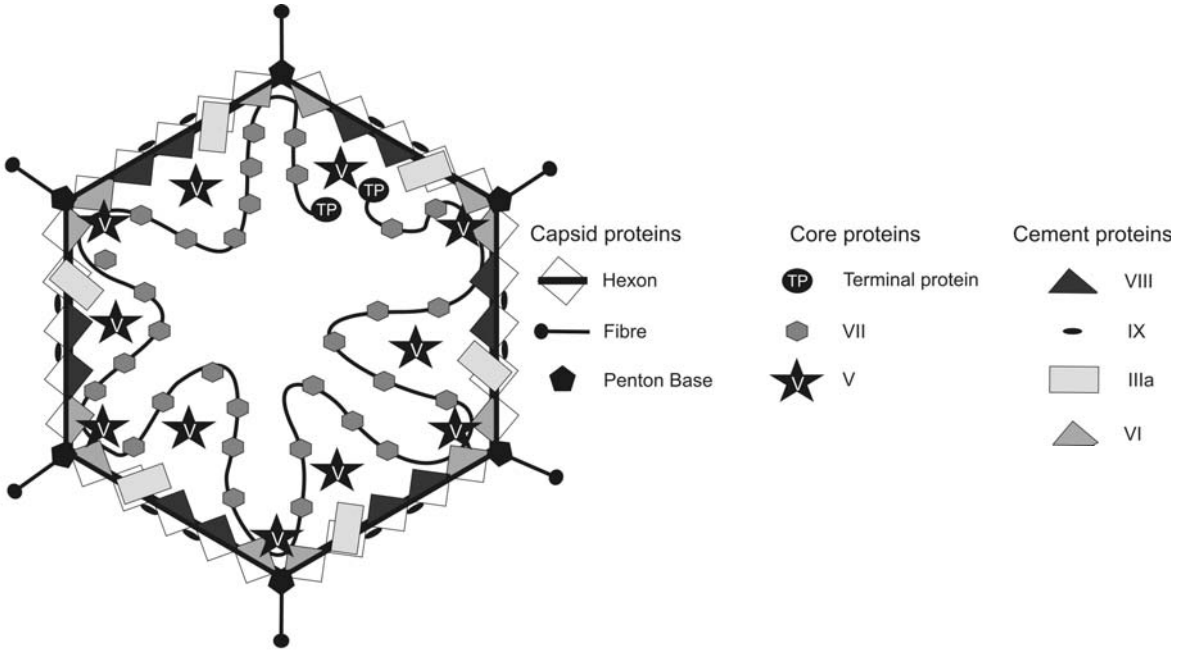

Figure 3. Adenovirus structure; adapted from (Russell 2000).

Besides respiratory diseases (Rowe, Huebner et al. 1953), Ads cause epidemic conjunctivitis (Jawetz 1959) and have been associated with a variety of additional clinical syndromes, especially infantile gastroenteritis (Mautner, Steinthorsdottir et al. 1995). In immune-competent patients, wild type Ads usually cause a mild, self-limiting acute infection. While in neonates and immune-suppressed patients, wild type Ads can cause severe infections (Krilov 2005).

Currently, more than 100 members of the Ad group that infect a broad range of vertebrate hosts have been isolated. The *Adenoviridae* family is divided mainly in 4 clades: Mastadenovirus, Aviadenovirus, Atadenovirus and Siadenovirus; nomenclature is based on the vertebrate host origin (Davison, Benko et al. 2003). Bioinformatics analysis has proposed a fifth new clade (Davison, Benko et al. 2003). Fifty-one human Ad serotypes have been distinguished on the basis of their resistance to neutralization by antisera to other known human Ad (De Jong, Wermenbol et al. 1999). The various serotypes are classified into six

(A-F) subgroups (species) based on their ability to agglutinate red blood cells (Rosen 1960). For example, Ad serotype 3 belongs to the subgroup B and the Ad serotype 5 belongs to the subgroup C.

The human Ad replication cycle has been mainly studied in Ad2 and Ad5 serotypes as models and so far it has been found to be equivalent to the other serotypes. The viral replication cycle is divided by convention into two phases that are separated by the onset of viral DNA replication. The early phase starts with the viral interaction with the host cell and it further includes adsorption, penetration, movement of partially uncoated virus particles to a nuclear pore complex (NPC), transport of viral DNA through the NPC into the nucleus and finally expression of an early set of genes. Early viral gene products mediate further viral gene expression and DNA replication, induced cell cycle progression, block apoptosis, and antagonize a variety of host antiviral measures. The late phase of the cycle begins with expression of late viral genes and assembly of progeny virions. Early transcription cassettes are termed E1-E4 and late transcription cassettes are divided into L1-L5 (Berk 2006).

The initial viral interaction with the host cell is mediated by the knob fiber and the respective receptor on the cell surface. *In vitro*, the main receptor for the Ad subgroups A and C-F is the coxsackie-adenovirus-receptor (CAR) (Roelvink, Lizonova et al. 1998). However, for the other subgroups or *in vivo* more complex interactions might occur. On the consequence of the primary interaction a secondary interaction involving the cellular $\alpha_v\beta$ integrins and the viral penton base arginine-glycine-aspartic acid (RGD) is established. Once the secondary binding is established endocytosis mediated by clathrin coated pits occurs (Berk 2006). The newly formed endosome then migrates towards nucleus. During this migration the endosomal pH is acidified, resulting in the partial degradation of the viral capsid. When the endosome reaches the nuclear membrane the now partially degraded virion binds to the nuclear pore and injects the viral DNA into the nucleus (Berk 2006).

The early E1 gene products are divided into E1A and E1B (E1B55K) proteins and they are expressed promptly upon adenovirus entry into a cell. Normally, the products of these genes act together to force the host cell to enter S phase, a prerequisite for the rest of the viral replication process. Deletion of E1A will render the virus susceptible to the anti-viral mechanisms of the retinoblastoma (Rb) protein, specifically by blocking the G1 to S transition. Deletion of E1B, on the other hand, allows p53 to induce apoptosis in infected cells, aborting replication and spread of the virus. In addition, the protein encoded in E4 (E4orf6) alone or in complex with E1B-55k inhibits p53 mediated apoptosis (Berk 2006). The E2 gene products provide the machinery for virus DNA replication (Hay, Freeman et al. 1995). E3 genes encode for several proteins to overcome the host defense mechanisms (Russell 2000). The E3 gp19K is localized in the ER membrane and binds the MHC class I heavy chain preventing transport to the cell surface, where it would activate cytotoxic T-lymphocytes (Bennett, Bennink et al. 1999). Another important viral protein for this study is the E4orf3 encoded in E4, this protein as well as the E1B-55K/E4orf6 complex interact with the MRN complex inhibiting the cellular DNA damage response (Berk 2006). In HeLa cells the early phase lasts for 5 to 6 hours. The late genes L1-L5 result in the production of the viral structural components and the encapsidation and maturation of the particles in the

nucleus (Russell 2000). The complete viral cycle is complete after 24 to 36 hours in HeLa cells.

1.3.1.1.2 Adenoviruses as gene transfer vehicles

The presently available recombinant tools together with an easily modified and well studied genome made Ads the most used tools for gene transfer in scientific research as well as in gene therapy (Russell 2000). Conventionally, the term gene therapy is used to indicate gene delivery by insertion of nucleic acids into cells of an individual to treat a disease. The therapeutic transgene can supplement a defective gene (e.g. a tumor suppressor gene for the treatment of cancer or delivery of a functional gene into the target tissue in monogenic diseases), or encode RNA or protein with a therapeutic function.

In the past few years several modifications have been performed in order to transform adenoviruses to be more effective, to be able to include more foreign genetic material in their genomes and to be less immunogenic. The initial viruses, also called first generation, were engineered by replacing the E1 region by the gene of interest. This first generation is the most widely used tool in basic research to achieve transient gene expression and several trials have also been performed based on this approach (Russell 2000). In order to create less immunogenic vectors and with a bigger capacity harbour genes of interest, a second generation was created by deleting the E1 and E3 regions in addition to the E2 or E4 regions (Shen 2006). Finally, a third generation called gutless or helper-dependent virus was created by deleting basically all the viral genes with exceptions of the ITRs and the packaging signal. Of the engineered Ads for gene transfer, helper dependent viruses are the least immunogenic and with the biggest capacity for foreign genetic material (Shen 2006).

1.3.1.1.3 Transductional targeting

Ad5 is the most used serotype in adenoviral gene therapy and similarly to other serotypes, CAR is, *in vitro,* the primary receptor (Roelvink, Lizonova et al. 1998). Consequently, efficiency of gene transfer is conditioned by the CAR expression levels. CAR is highly expressed in epithelial cells as well as in heart, pancreas, the central and peripheral nervous system, prostate, testis, lung, liver and intestine; but little or no CAR is expressed on lymphocytes or adult muscle (Meier and Greber 2004). This makes Ad5 a broad tool for gene transfer. However, cancer progression is correlated with a decrease in CAR expression levels on tumor cells (Anders, Christian et al. 2003). Thus, it would be advantageous to transductionally retarget adenovirus to non-CAR receptors for increased tumor transduction and/or reduced infection of non-target tissues (Kanerva, Zinn et al. 2003; Bauerschmitz, Guse et al. 2006). Several strategies can be used to achieve a none-CAR dependent transduction, mainly divided in adapter-molecule based retargeting, and genetic manipulation of the viral capsid.

The adapter-molecule based retargeting consists of a bi-specific ligand that bridges a connection between a receptor in the cell surface and the Ad. Several strategies have been tested as, for example, bi-specific antibodies (Korn, Muller et al. 2004), cell-selective ligands such as folate (Douglas, Rogers et al. 1996) and chemical conjugates (Reynolds, Zinn et al.

2000). However, by using a two component system the risk of unexpected side effects is increased and the production of such bi-specific ligands is often complex, ending up with impure mixes of ligands that increases even further the risk of side effects.
To the present day, transductional retargeting by genetic manipulation of the viral capsid has been obtained by ligand incorporation into the fiber, replacing fiber regions with a ligand, or by serotype fiber knob switching (Bauerschmitz, Barker et al. 2002; Glasgow, Everts et al. 2006). Several ligands have been studied as well as different localization modifications in the fiber. The C-terminus and the HI-loop within the fiber revealed promising regions for ligand insertion. A polylysine tail constituted of 7 lysine residuesthat retargets the virus to cell surface heparin sulfate proteoglycans (HSPGs), has been successfully inserted into the fiber C-terminus with increased transduction of cancer cells (Kangasniemi, Kiviluoto et al. 2006; Ranki, Kanerva et al. 2007). Enhanced infectivity of tumor cells was also obtained by inserting the RGD motif targeting $\alpha_v\beta$ integrins into the HI-loop (Kanerva, Wang et al. 2002; Kangasniemi, Kiviluoto et al. 2006) as well as into the hexon monomer protein (Vigne, Mahfouz et al. 1999). Furthermore, combination of pK7 in the C-terminus and RGD motif in the HI-loop revealed increased transduction of CAR deficient cells (Wu, Seki et al. 2002). Transductional retargeting can also be obtained by replacing the knob or other fiber regions with a ligand. For example, by replacing the penton base RGD motif responsible for the secondary viral interaction with receptor specific motifs, Ad can be targeted to different cancer tissues (Wickham, Carrion et al. 1995). Finally, serotype fiber knob switching has revealed promising results by replacing the knob of the Ad5 by another serotype knob e.g. serotype 3 knob retargeting to the Ad3 receptor (Kanerva, Mikheeva et al. 2002).

1.3.1.1.4 Transcriptional targeting

Since the first generation Ad, the anti-tumor efficiency of adenoviral cancer gene therapy has been improved by taking advantage of viral replication and by arming the vectors with therapeutic transgenes. However, despite these improvements there was also the need to decrease possible off-target side effects. For this purpose, transcriptional targeting to tumor cells has been explored where viral genes or other transgenes in the viral genome can only be transcribed in malignant cells and not in normal cells. Transcriptional targeting can be obtained by placing viral genes fundamental for viral replication under the control of tissue-specific promoters (TSPs) that are activated in tumor cells but not usually in normal cells. Consequently, the early viral genes, especially the E1A gene has been placed under the control of several promoters, such as E2F (Tsukuda, Wiewrodt et al. 2002), cyclooxygenase-2 (Cox-2) (Bauerschmitz, Ranki et al. 2008) and human telomerase (hTERT) (Hashimoto, Watanabe et al. 2008) with increased tumor retargeting.
More recently, gene silencing by RNA interference technology has been utilized to confer tumor selectivity. MicroRNAs (miRNAs) or small interfering RNAs (siRNAs) regulate gene expression post-transcriptionally by translation block or cleavage of specific, complementary mRNA via the RNA-induced silencing complex (RISC). By inserting a complementary sequence next to a critical viral gene, it is possible to restrict virus replication to tumor but not normal cells that express high levels of the corresponding miRNA. This has been

demonstrated by several groups (Kelly, Hadac et al. 2008; Ylosmaki, Hakkarainen et al. 2008).

1.3.1.1.5 Transcomplementation targeting

Several clinical trials have revealed Ads as a safe and promising therapy for cancer (Harvey, Maroni et al. 2002; Peng 2005; Pan, Zhang et al. 2008; Ylä-Herttuala 2008). However, the main conclusion from most cancer trials is that tumor transduction and tumor penetration have been too low for a significant therapeutic antitumor effect (Harvey, Maroni et al. 2002; Pan, Zhang et al. 2008; Ylä-Herttuala 2008). Therefore, oncolytic viruses have been explored for enhanced tumor transduction and amplification of effect (Kanerva and Hemminki 2004). As a result, transductional and transcriptional targeting have been explored to target the viral replication and in this way abrogate the viral replication in normal healthy tissue. In addition, viral replication can be restricted to tumor cells by deleting adenoviral genes that are necessary for viral replication in normal cells but not in tumor cells. In fact, adenoviral infection induces several signaling pathways that are also abnormally induced in tumor cells (such as cell cycle deregulation and inhibition of apoptosis) (Yew and Berk 1992; Lukas, Muller et al. 1994; Han, Modha et al. 1998).

The first engineered, oncolytic Ad to enter clinical trials for cancers including those of the HNC was dl1520 (ONYX-015; Onyx Pharmaceuticals, California, USA) (Heise, Sampson-Johannes et al. 1997; Khuri, Nemunaitis et al. 2000; You, Yang et al. 2000; Kirn 2001; Reid, Galanis et al. 2002; Hecht, Bedford et al. 2003). This virus is an oncolytic Ad2/Ad5 hybrid with deletion of its E1B 55K and it has shown safety (Nemunaitis, Cunningham et al. 2001).Furthermore, the virus H101 was the first oncolytic Ad to be approved for cancer treatment. H101 is similar to ONYX-015 but with an additional deletion of the E3B genes deletion. However, the specificity of E1B-deletion mutants to p53-negative cells is not absolute, but instead it is the capacity of cells to compensate for late mRNA transport (another function of 55k) which determines selectivity (O'Shea, Johnson et al. 2004). Also, the lack of E1B can be compensated by drugs or hyperthermia, enhancing the replication of E1B-deleted adenoviruses (Vähä-Koskela, Heikkilä et al. 2007). In addition, durable objective responses with this virus as a single agent have been limited and this could be partly due to the loss of other essential functions of the E1B 55K and E3B genes that resulted in significantly lower efficiency than the wild type virus in lysing cells in G1 status (Harada and Berk 1999). Thus, there is a need to improve these viruses by identifying mutations that result in tumor selectivity but not those that result in attenuated virus replication and oncolysis.

The adenoviral E1A is the first gene to be transcribed after virus entry into the host cell (Frisch and Mymryk 2002). E1A normally interacts with the retinoblastoma protein (pRb) thereby releasing E2F and thus pushing quiescent cells into S phase to allow virus replication **(Figure 4)**. E1A-deletion mutants, such as Δ24 (dl922–947), have shown superior oncolytic efficacy compared to E1B mutants both *in vitro* and *in vivo* (Fueyo, Gomez-Manzano et al. 2000; Heise, Hermiston et al. 2000). The approach is based on the fact that most advanced human tumors are deficient in the pRb/p16 pathway (Sherr 1996; Hernando,

Nahle et al. 2004). Therefore, by deleting the Δ24 bp (dl922–947) of E1A, the interaction with pRb is lost and the virus can not replicate in normal cells. However, the abnormally activated pRb/p16 pathway in tumors cells which have already free E2F that transcomplements with the viruses allowing viral replication.

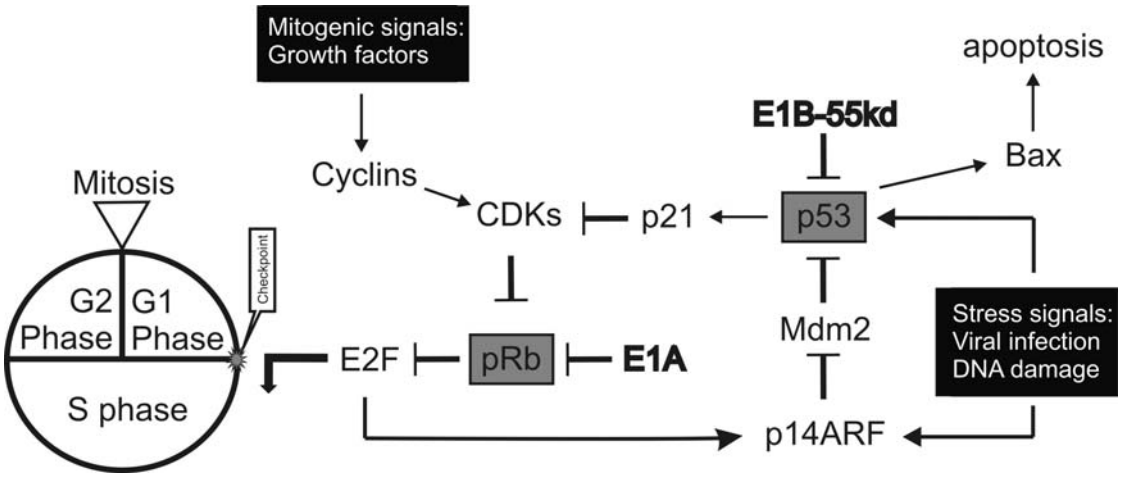

Figure 4. Early adenoviral proteins lead to loss of cell cycle control and cell arrest. The retinoblastoma protein family (pRb) regulates the G1 to S-phase cell cycle checkpoint. pRb prevents progression of the cell cycle from G1 to S phase by binding to and repressing transcription factor E2F, which normally induces expression of genes needed for DNA synthesis. When pRb is phosphorylated by cyclin-dependent kinase 4 (CDK4), it is unable to bind to and repress E2F. Another protein relevant for the cell cycle control is the tumor suppressor transcription factor p53, which is upregulated and activated upon stress signals like DNA damage or viral infection. p53 can activate transcription of genes coding for proteins that either induce apoptosis (for example Bax) or cell cycle arrest (for example p21). p21 is able to inactivate CDKs, thereby inhibiting progression through the cell cycle. The activity of p53 is blocked by Mdm2, which inactivates p53 through binding and mediating degradation. The effect of Mdm2 can be inhibited by $p14^{ARF}$, a tumor suppressor protein that is upregulated by stress signals and mitogenic signals, like E2F. As E2F activity is blocked by pRb, inactivation of pRb leads to activation of p53 and functions as a safety mechanism during progression through the cell cycle. However, targeted deletions in E1B-55 kD or E1A result in mutant proteins that are unable to bind p53 or pRb, respectively. Therefore, modified viruses replicate only in cells deficient in these pathways such as most cancer cells. Adapted from (Everts and van der Poel 2005)

The strategies of transductional, transcriptional and conditionally replicating targeting are not independent of each other. Consequently, increased tumor targeting and decreased possible side effects can be accomplished by combining the three approaches. Bauerschmitz and colleagues showed that a triple targeted virus exhibits increased tumor cell selectivity while retaining oncolytic potency (Bauerschmitz, Guse et al. 2006).

1.3.2 Arming approaches for enhanced antitumor efficacy

Advanced tumor masses are very complex and heterogeneous having several barriers that impair an efficient oncolytic viral spread (Hay 2005; Cheng, Sauthoff et al. 2007). Heretofore,

oncolytic viruses' anti-tumor efficacy can be further improved by taking advantage of the unique viral efficiency of gene delivery and arm the viruses with anti-tumor transgenes. In comparison to first generation none replicative viruses, to couple the expression of anti-tumor genes to virus replication promotes higher transgene expression as well as during longer periods (Hawkins, Johnson et al. 2001). The reason has been that transgene expression is not limited to a single cycle of replication as in the first generation viruses (Peng 2005). Furthermore, the oncolytic virus tumor targeting features permit high transgene expression levels locally to the tumor and therefore decreasing possible systemic side effects of the therapeutic proteins. Recently, arming oncolytic viruses with anti-cancer genes has been a major focus in cancer virotherapy, and transgenes exploited include tumor suppressor, pro-apoptotic, anti-angiogenic, "suicide", and immunomodulatory genes.

With the increased knowledge of tumor genetics a logic strategy would be to correct the deficient genes in the tumor cells. Therefore, tumor suppressor and pro-apoptotic transgenes such as p16INK4A (Ma, He et al. 2009) or p53 (Wang, Su et al. 2008) armed viruses were among the first to be tested. However, targeting a single gene is not enough to achieve a significant result with regard to the extreme complexity of mutations in the tumor cells and the difficulty in transducing sufficient amounts of cells (Stratton, Campbell et al. 2009). For this reason it would be more advantageous to target signaling pathways instead of individual genes such as arming with transforming growth factor beta (TGF-β) (Hu, Robbins et al. 2009).

Tumor increased vasculature is directly correlated with disease progression. Therefore, several anti-angiogenic molecules have been developed in order to reduce tumor vasculature and tumor progression. Recently, oncolytic viruses have been armed with a broad spectrum of anti-angiogenic factors such as endostatin/angiostatin (Su, Na et al. 2008; Fang, Pu et al. 2009; Tysome, Briat et al. 2009), interleukin-18 (IL-18) (Zheng, Pei et al. 2009; Zheng, Pei et al. 2009), canstatin (He, Su et al. 2009), and trichostatin A (Liu, Castelo-Branco et al. 2008), as well as arming viruses with genes that inhibit pro-angiogenic molecules such as IL-8 (Yoo, Kim et al. 2008) and vascular endothelial growth factor (VEGF) (Guse, Diaconu et al. 2009). In addition, oncolytic viruses coding for matrix metalloproteinases (MMPs) inhibitors have shown promising results in delaying tumor growth and angiogenesis (McNally, Rosenthal et al. 2009).

Another strategy used for arming oncolytic viruses, gene-directed prodrug activation therapy (or suicide gene therapy), involves the delivery of a gene that would lead to the expression of an enzyme, followed by the administration of a prodrug that is activated selectively by this enzyme. The best known example is herpes simplex thymidine kinase (HSV-TK), where the non-toxic drug ganciclovir (GCV) is converted into triphosphorylated forms, blocking DNA synthesis and inducing cell death (Wong, Lemoine et al. 2010). In addition, the phosphorylated toxic metabolites can spread to non-infected cells creating a cytotoxic bystander effect. The efficiency of the approach has been tested in oncolytic Ad vectors (Raki, Hakkarainen et al. 2007). Alternative combinations include nitroreductase with the prodrug CB1954 (converted into an alkylating agent) (Braidwood, Dunn et al. 2009), and cytosine deaminase (CD) with 5-fluorocytosine, which is converted into the cytotoxic and radiosensitizing 5-fluorouracil (Chalikonda, Kivlen et al. 2008; Foloppe, Kintz et al. 2008).

With the discovery of immunosurveillance and the importance of the immune system in the development of cancer, a plethora of immunomodulatory genes have been inserted into the genome of oncolytic viruses with the aim of stimulating effective anti-tumoral immune responses. Furthermore, viral mediated cell oncolysis has the potential to work as a tumor vaccine increasing the anti-tumor efficiency of immunodulatory molecules. Recent examples include the heat shock proteins (Li, Liu et al. 2009), chemokine (C-C motif) ligand 5 (CCL5) (Lapteva, Aldrich et al. 2009), IFN (Willmon, Saloura et al. 2009), granulocyte macrophage colony-stimulating factor (GM-CSF) (Chang, Zhao et al. 2009; Cerullo, Pesonen et al. 2010), IL-12 (Bortolanza, Bunuales et al. 2009), IL-18 (Zheng, Pei et al. 2009), and IL-24 (Luo, Xia et al. 2008).

A possible problem of arming oncolytic viruses with anti-tumor transgenes is that the viral replication and spread can in theory be hindered by the transgenes. Nevertheless, the several studies so far have revealed that it is feasible and efficacious to arm oncolytic viruses with transgenes resulting in more potent anti-tumor vectors. Furthermore, side effects of the treatments are usually non-overlapping, which might facilitate increased efficacy without increasing toxicity.

1.3.3 HNC clinical trials with oncolytic adenoviruses

Over 1000 cancer gene therapy clinical trials have been done so far representing 64.5% of all clinical trials in gene therapy (source The Journal of Gene Medicine Clinical Trial site). The currently employed oncolytic adenoviruses in clinical trials are all based on human adenovirus serotype 5, although they employ different modes of tumor selectivity. The first oncolytic adenoviruses tested in clinical trials was ONYX-015 in 1996 (described in chapter 1.3.1.1.5) and successfully established the clinical proof-of-concept of adenoviral gene therapy for HNC (Ganly, Kirn et al. 2000). Single agent efficacy has been relatively limited to about 14% of local tumor regression rates. Nevertheless, in a phase II clinical trial the anti-tumor efficacy of the vector was improved when in combination with chemotherapy (Khuri, Nemunaitis et al. 2000). A promising Phase III clinical trial of ONYX-015 in combination with chemotherapy for HNSCC was started in 2003 but unfortunately due to funding problems was stopped.

Another oncolytic Ad to undergo clinical testing and so far the only one to be approved in 2005 for the treatment of HNC was H101 (described in chapter 1.3.1.1.5). H1O1 clinical testing started in the year 2000 in a phase I clinical trial for HNC in a dose-escalation manner similar to ONYX-015 (Yuan, Zhang et al. 2003). The highest given dose was intratumorally at $1.5x10^{12}$ VP and no dose limited toxicity (DLT) nor serious adverse events were seen. The phase II multicenter open-label clinical trial was performed from 2001 to 2002 showing the safety of the virus (Xu, Yuan et al. 2003). Also, in this phase II clinical trial promising anti-tumor results were observed when in combination with chemotherapy (Xu, Yuan et al. 2003). In addition similar results as single agent in comparison to Onyx-015 were observed (Xu, Yuan et al. 2003). These findings prompted a multi-center, randomized and controlled phase III clinical trial from 2002 to 2004 (Xia, Chang et al. 2004). The clinical trial of H101 in combination with cisplatin and 5-FU showed an impressive response rate of

78.8% (41/52) compared to just 39.6% (21/53) of chemotherapy alone (Xia, Chang et al. 2004). Other selected clinical trials with oncolytic Ads are listed in **Table 1**.

In summary, clinical trials with oncolytic adenoviruses have been proven safe. However, to access antitumor efficacy further phase III randomized clinical trials are needed.

Table 1. Selected clinical trials with oncolytic adenoviruses.

Virus/ treatment agents	Genetic modification	Phase	Route of administration	Cancer type	Efficacy / number of patients	Ref
ONYX-015	E1B-55kD deletion	I	i.t.	HNSCC	2/22	(Ganly, Kirn et al. 2000)
ONYX-015	E1B-55kD deletion	I	i.t.	Pancreatic cancer	0/23	(Mulvihill, Warren et al. 2001)
ONYX-015	E1B-55kD deletion	I	i.v.	Cancer metastatic to the lung	0/10	(Nemunaitis, Cunningham et al. 2001)
ONYX-015	E1B-55kD deletion	I	i.p.	Ovarian cancer	0/16	(Vasey, Shulman et al. 2002)
ONYX-015	E1B-55kD deletion	I	i.v. + i.t.	HCC	1/5	(Habib, Salama et al. 2002)
ONYX-015	E1B-55kD deletion	I	i.t.	Glioma	3/24	(Chiocca, Abbed et al. 2004)
ONYX-015 + etarnercept	E1B-55kD deletion	I	i.v.	Advanced cancers	0/9	(Nemunaitis, Senzer et al. 2007)
CV706	PSA promoter controlling E1A	I	i.t.	Prostate cancer	5/20	(DeWeese, van der Poel et al. 2001)
ONYX-015 + 5-FU	E1B-55kD deletion	I-II	i.t. + i.ha + i.v.	HCC / colorectal cancer metastatic to liver	3/16	(Habib, Sarraf et al. 2001)
ONYX-015 + 5-FU + leukovorin	E1B-55kD deletion	I-II	i. ha	Metastatic colorectal cancer	2/24	(Reid, Freeman et al. 2005)
ONYX-015	E1B-55kD deletion	II	i.t.	SCCHN	5/40	(Nemunaitis, Khuri et al. 2001)
ONYX-015 + cisplatin + 5-FU	E1B-55kD deletion	II	i.t.	SCCHN	19/37	(Khuri, Nemunaitis et al. 2000)
ONYX-015 + gemcitabine	E1B-55kD deletion	I-II	i.t.	Pancreatic cancer	2/21	(Hecht, Bedford et al. 2003)
ONYX-015	E1B-55kD deletion	II	i.v.	Metastatic colorectal cancer	0/18	(Hamid, Varterasian et al. 2003)
H101 +cisplatin/adriamycin +5-FU	E1B-55kD deletion	III	i.t.	HNSCC	71/160	(Xia, Chang et al. 2004)
ONYX-015 + MAP chemotherapy	E1B-55kD deletion	I-II	i.t.	Sarcoma	1/6	(Galanis, Okuno et al. 2005)

Ad5-yCD/*mut*TK_{SR39}*rep*-ADP + GCV/5-FC + radiation	yeast CD-HSV TK fusion gene in E1, ADP in E3	I	i.t.	Prostate cancer	Significant decline in PSA level in 9/9	(Freytag, Movsas et al. 2007)
Ad5-yCD/mutTK_{SR39}rep-ADP+ GCV/5-FC + radiation	yeast CD-HSV TK fusion gene in E1, ADP in E3	II-III	i.t.	Prostate cancer	not available yet	*
CG7870 (CV787)	Tumor specific promoters driving E1A and E1B	I	i.v.	Hormone refractory prostate cancer	5/23 decline in PSA, 3/8 at highest dose levels	(Small, Carducci et al. 2006)
KH901	hTERT driving E1A, GM-CSF in E3	I	i.t.	HNSCC	not accessed	(Chang, Zhao et al. 2009)

Abbreviations: 5-FU, 5-fluorouracil; ADP, adenoviral death protein; CD, cytosine deaminase; GCV, ganciclovir; HCC, hepatocellular carcinoma; MAP, mitomycin-.C-doxorubicin-cisplatin; i.ha, intrahepatic artery; i.t., intratumoral; i.v., intravenous; mitomycin-C-doxorubicin-cisplatin; PSA, prostate specific antigen; TK, tyrosine kinase; GMCSF, granulocyte-macrophage colony stimulating factor;

*, clinicaltrials.gov NCT00583492

2 AIMS OF THE STUDY

This study was performed in order to develop and evaluate new adenovirus based treatment modalities for HNSCC and PC. Adenoviral gene therapy was utilized and new vectors were developed. The aims of this study were as follows:

1. To investigate the anti-tumor efficiency of the combination of oncolytic adenoviruses with cetuximab, radiotherapy and/or chemotherapy in HNSCC. (I)

2. To study the mechanism contributing to viral radiosensitization of tumors *in vivo*. (II)

3. To generate and evaluate a transductionally and transcomplementary targeted oncolytic adenovirus armed with a prodrug converting suicide transgene to enhance specificity and increase antitumor effect due to the bystander effect prompted by the pro-drug enzymatic conversion. (III)

4. To generate and evaluate a transductionally and transcomplementary targeted oncolytic adenovirus armed with a fully human monoclonal antibody specific for CTLA-4 to enhance specificity and increase antitumor effect due to immune response prompted by the immunomodulating molecule. (IV)

3 MATERIALS AND METHODS

Detailed description of the used methodology can be found in the original publications.

3.1 Cell lines; low passage tumor cell cultures (I-IV)

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

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

Cell line name	Description	Used in
293	Transformed embryonic kidney cells	I-IV
911	Transformed embryonic retinoblasts	III
A549	Lung adenocarcinoma	I-IV
DU-145	Prostate carcinoma	II
Jurkat (E6-1)	Leukemic T cell lymphoblast	IV
M4A4-LM3	Breast carcinoma	II
PC-3MM2	Prostate carcinoma	II, IV
SKOV3-ip1	Ovarian adenocarcinoma	IV
UT-SCC 8	Supraglottic larynx HNSCC low passage tumor cell cultures	I-IV
UT-SCC 9	Glottic larynx HNSCC low passage tumor cell cultures	I
UT-SCC 10	Mobile tongue HNSCC low passage tumor cell cultures	I
UT-SCC 29	Glottic larynx HNSCC low passage tumor cell cultures	I, III

Cell lines were subcultured according to the recommended conditions and not used with passage numbers above thirty.

HNSCC low passage tumor cell cultures were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (PromoCell GmbH, Heidelberg, Germany), 1% nonessential amino acids (Gibco, Invitrogen, Carlsbad, CA, USA) 2 mmol/L glutamine, 100 units/mL penicillin, and 100 Ag/mL streptomycin (all from Sigma, St. Louis, MO) (Erjala, Sundvall et al. 2006).

3.2 Human specimens

Peripheral blood mononuclear cells (PBMC) of healthy individuals and patients with advanced metastatic tumors refractory to conventional therapies were obtained with written informed consent. The study was completed according to Good Clinical Practice and the Declaration of Helsinki. This Advanced Therapy Access Program is approved by the Medicolegal Department of the Finnish Ministry of Social Affairs and Health and the Gene Technology Board, and is regulated by Finnish Medicines Agency Fimea.

3.3 Adenoviruses

Large scale production of replication deficient viruses and replication competent viruses were performed respectively in 293 and A549 cells and purified on double cesium chloride gradients. 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 (PFU) titers were assessed with standard TCID50 assay on 293 cells.

3.3.1 Replication deficient adenoviruses

The main features of the replication deficient adenoviruses used in the studies are described in Table 3.

Table 3. List of replication deficient adenoviruses used in this study

Virus name	E1 *	Fiber	Used in	Reference
Ad5(GL)	GFP + luciferase	Wild type serotype 5	I, II	(Wu, Seki et al. 2002)
Ad5luc1	Luciferase	Wild type serotype 5	I	(Kanerva, Mikheeva et al. 2002)
Ad5lucRGD	Luciferase	RGD motif in HI loop	I	(Dmitriev, Krasnykh et al. 1998)
Ad5.pK7(GL)	GFP + Luciferase	7 lysine residues at COOH terminus	I	(Wu, Seki et al. 2002)
Ad5.RGD.pK7(GL)	GFP + Luciferase	7 lysine residues at COOH terminus and RGD motif in HI loop	I	(Wu, Seki et al. 2002)
Ad5/3-aCTLA4	anti-CTLA mAb	5/3 serotype chimerism	IV	Study IV
Ad5/3-FCU1	FCU1	5/3 serotype chimerism	III	Study III
Ad5/3luc1	Luciferase	5/3 serotype chimerism	I, III, IV	(Kanerva, Mikheeva et al. 2002)
AdTG14800	FCU1	Wild type serotype 5	III	(Erbs, Regulier et al. 2000)
rAdE4orf3	E4orf3 + GFP	Wild type serotype 5	II	(Araujo, Stracker et al. 2005)
rAdE1B55K	E1B55K	Wild type serotype 5	II	(Marcellus, Teodoro et al. 1996)
rAdE4orf6	E4orf6	Wild type serotype 5	II	(Querido, Marcellus et al. 1997)

* The marker genes and transgenes in E1 are under control of the CMV promoter

3.3.2 Replication competent adenoviruses

The main features of the replication competent adenoviruses used in this study are described in Table 4.

Table 4. List of replication competent adenoviruses used in this study

Virus name	E1	E3	Fiber	Used in	Reference
Ad300wt	Wild type	Wild type	Wild type serotype 5	I, II	ATCC[1]
Ad5-Δ24E3	24 bp deletion[2]	Δgp19	Wild type serotype 5		(Kanerva, Zinn et al. 2003)
Ad5-Δ24RGD	24 bp deletion[2]	Wild type	RGD motif in HI loop	I	(Suzuki, Fueyo et al. 2001)
Ad5/3-Δ24	24 bp deletion[2]	Wild type	5/3 serotype chimerism	I, III, IV	(Kanerva, Zinn et al. 2003)
Ad5/3-Δ24aCTLA4	24 bp deletion[2]	anti-CTLA mAb	5/3 serotype chimerism	IV	Study IV
Ad5/3-Δ24FCU1	24 bp deletion[2]	FCU1	5/3 serotype chimerism	III	Study III
Ad5.pK7-Δ24	24 bp deletion[2]	Wild type	7 lysine residues at COOH terminus	I	(Ranki, Kanerva et al. 2007)

[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-FCU1, Ad5/3-Δ24FCU1, Ad5/3-aCTLA4, Ad5/3-Δ24aCTLA4

For construction of Ad5/3-FCU1 and Ad5/3-aCTLA4, expression cassettes with either FCU1 fusion gene (Erbs, Regulier et al. 2000) or the heavy and light chains of IgG2 type anti-CTLA4 mAb were inserted into the multiple cloning site of pShuttle (Stratagene, La Jolla, CA, USA). Shuttle plasmids were recombined with pAdeasy-1 plasmid (Stratagene), which carries the whole adenovirus genome, and resulting rescue plasmids were transfected to 293 cells to generate Ad5/3-FCU1 and Ad5/3-aCTLA4. For construction of oncolytic Ad5/3-Δ24FCU1 and Ad5/3-Δ24aCTLA4, the gene for FCU1 fusion gene (Erbs, Regulier et al. 2000) or the heavy and light chains of IgG2 type anti-CTLA4 mAb was cloned into pTHSN plasmid that contains the E3 region of the adenoviral genome replacing the 6.7K/gp19K genes (Kanerva et al., 2005). The resulting plasmid was recombined with pAdeasy-1.5/3-Δ24, an adenovirus rescue plasmid containing the serotype 3 knob and a 24 bp deletion in E1A (Kanerva, Zinn et al. 2005), resulting in pAdeasy-1.5/3-Δ24-FCU1 or pAdeasy-1.5/3-Δ24-aCTLA4, which were transfected to 911 or A549 cells for generation of Ad5/3-9HIF-Δ24-FCU1 and Ad5/3-9HIF-Δ24aCTLA4.

3.4 *In vitro* studies

3.4.1 Marker gene transfer assays (I)

Cell lines were seeded on day 1 at 25,000 cells per well on 24-well plates in 1 ml growth media (GM). On day 2, cells were infected with 40, 200, 1000 or 5000 VP/cell for 30 minutes in 2% GM on a shaker. Afterwards, cells were washed once with 1 ml PBS and 1 ml GM was added per well. After 24 hours the GM was removed, cells were lysed with 200 µl lysis buffer (Reporter Lysis Buffer, Promega, Madison, WI) and freeze-thawed once. 20 µl of these samples was mixed with 100 µl of luciferase assay reagent (Reporter Lysis Buffer, Promega, Madison, WI) and measured with Berthold Lumat LB9501. Standardization was accomplished by setting the values obtained with isogenic control virus with an unmodified serotype 5 capsid, which was given the value of 100%.

3.4.2 Cytotoxicity assay (I-IV)

Cells were seeded at a density of 1.5 x 10^4 cells/well for HNSCC cells or 1 x 10^4 cells/well for the other cell lines on 96-well plates and cultured overnight. Briefly, cells were either infected with variable concentrations of viruses or treated with agents, or treated with a combination of both. Detailed description of doses and schedules utilized in different studies can be found in the original publications. Cells were incubated at 37°C until almost complete cell killing was visually evident. Thereafter, cell viability was measured with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS assay) (Promega, Madison, WI, USA).

3.4.3 Radiotherapy (I, II)

In vitro, cells were irradiated on cell culture plates through a 1 cm thick plastic phantom bottom and 1 cm thick layer of water in the phantom. Mice remained free in standard plastic cages that were placed in the middle of the radiation field. Irradiation was performed with a linear accelerator (model: Clinac 600 C/D, Varian Medical Systems, Palo Alto, USA) using a 6 MV photon beam and dose rate 400 MU/min (≈4 GY/min).

3.4.4 Western blot (II, III, IV)

Cancer cells were seeded on 6-well plates and infected with viruses. Detailed description of doses and schedules utilized in different studies can be found in the original publications. Briefly, cell lysates, proteins or supernatants were run in SDS–polyacrylamide gel electrophoresis (PAGE) gel under reducing or native conditions and transferred to a nitrocellulose membranes. The membrane was incubated with primary antibody against the protein of interest, washed and incubated with secondary antibody coupled to horseradish peroxidase. Signal detection was done by enhanced chemiluminescence (GE Healthcare, Amersham, UK).

3.4.5 Immunofluorescence microscopy (I, II)

Tumor cryosections or PC-3MM2 cells were fixed in 4% paraformaldehyde (room temperature), permeabilized and blocked at room temperature. Briefly, indirect immunofluorescent labeling was used (I, II) by incubating the slides with primary antibody, washed and incubated in the dark with secondary antibody. Also, direct immunofluorescent labeling technique was used (I) by incubating the slides with primary antibody conjugated with the APC, washed and tumor sections were post-fixed with 4% paraformaldehyde. In either indirect or direct immunofluorescent the slides were washed in PBS and mounted with Vectashield. Intensity ratios were calculated using Image J 1.39a (Wayne Rasband, National institutes of Health, USA)

3.4.6 Clonogenic assay (II)

On day 1, PC-3MM2 cells were seeded at $5x10^4$cells/well into 24-well plates and infected on day 2 with the respective indicated viruses at 100 VP/cell. On day 3, plates were irradiated with 0 or 8 Gy and the cells were transferred to six-well plates at densities of 1000 cells/well. On day 13, the cells were fixed with 10% paraformaldehyde and stained with 1% crystal violet in 70% ethanol. Colony formation was counted under a microscope.

3.4.7 Enzymatic assays by HPLC (III)

On day 1, PANC-1 cells were seeded at 2×10^6 cells/well and on the following day infected with the respective viruses at an MOI of 25 VP/cell. Twenty four hours later, enzymatic activities were determined using HPLC separation as described in Erbs et al. (Erbs, Findeli et al. 2008).

The CDase activity in tumors and plasma was determined using HPLC separation. Nude mice bearing s.c. HNSCC tumors infected with the respective viruses at an MOI of $3x10^8$ VP/day (days 0, 2 and 4) and treated intraperitoneally (i.p.) with 5-FC (250 mg/kg/day) or phosphate buffered saline (PBS). Plasma was separated by centrifugation from blood collected via tail vein in heparinized tubes, 1 hour post-5-FC i.p. injection. Tumors were homogenized using a Polytron homogenator. Tumor or plasma samples were quenched with 1 ml of ethyl acetate/2-propanol/0.5 m acetic acid solution (84:15:1). The organic supernatant was reconstituted in 50 µl of water and analyzed by HPLC as described above.

3.4.8 Immunostaining for apoptosis or human IgG (III-IV)

Cryosections of 4–5µm thickness of frozen tumors embedded in Tissue Tek OCT. (Sakura, Torrance, CA, USA) were fixed in acetone for 10 min at -20◦C. Detailed description of doses and schedules utilized in different studies can be found in the original publications. Briefly, tissue sections were incubated with primary antibody against active caspase 3 or human IgG. Further, sections were incubated according to manufacturer's instructions with LSAB2 System-HRP kit (K0673, DakoCytomation, Carpinteria, CA, USA). Bound antibodies were

visualized using 3,3'-diaminobenzidine (DAB,Sigma, St Louis, MO, USA). Lastly, sections were counterstained with hematoxylin and dehydrated in ethanol, clarified in xylene and sealed with Canada balsam. Representative pictures were captured at 20X or 40X magnification using an Leica DM LB microscope equipped with Olympus DP50 color camera.

The immunohistochemistry images of tumor cryosections stained for apoptosis were analysed using Matlab from MathWorks (III). The color space of each image was simplified to the 10 most representative different colors by using K-means algorithm (Ilea and Whelan 2006). The simplified colors were labeled as being red, blue or background. The resulting areas were filtered based on shape features to increase accuracy (Stanescu, Burdescu et al. 2007).

3.4.9 Quantitative real-time PCR (III)

Total DNA was extracted from UT-SCC8 HNSCC low passage tumor cell cultures or tumors with QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) and DNA concentration was measured by spectrophotometry. PCR amplification was based on primers and probe targeting of the E4 gene (Kanerva, Wang et al. 2002). Human beta-actin primers and probe were used as internal control and to normalize the number of viral DNA copies for the amount of genomic DNA (Alvarez-Lafuente, Garcia-Montojo et al. 2007).

The real-time PCR conditions were as follows: 2X LightCycler480 Probes Master Mix (Roche, Mannheim, Germany), 500nM each forward and reverse primer, 150nM each probe and 5 µl extracted DNA. PCR reactions were carried out in a LightCycler (Roche, Mannheim, Germany) under the following cycling conditions: 10 min at 95°C, 50 cycles of 15 s at 95°C and 1 min at 60°C, and 10 min at 40°C. A regression standard curve was established using DNA extracted from serial dilutions of pAd5easy plasmid. Known amounts of human genomic DNA were used to generate a standard curve for the beta-actin gene.

3.4.10 Biological activity of anti-CTLA4 measured by flow cytometry array of IL-2 or INF-γ (IV)

Cancer patients with advanced solid tumors or healthy donor PBMCs as well as Jurkat cells were incubated with 0.03µg/ml of phorbol myristyl acetate (PMA) (Sigma-Aldrich Co.), 0.3µg/ml of ionomycin (Sigma-Aldrich Co.) and 1µg/ml of Recombinant Human B7 Fc Chimera (R&D systems); and treated with 0.02 µm filtrated (Anotop, Whatman, England) supernatants of virus infected PC3-MM2 cells. In the loss of function assay 0.1µg/ml of recombinant human CTLA-4/Fc Chimera (R&D Systems) was added to the previous stimulating and treated media. The next day after PBMC stimulation or two days after Jurkat cells stimulation, interleukin-2 (IL-2) or interferon-γ (IFN-γ) levels in the growth media were analyzed by BD Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson) according to the instructions of the manufacturer. PC3-MM2 cells were infected with 10 VP/cell and 48h later the supernatants collected. Mouse anti-human CTLA-4 (=CD152) mAb

(BD Pharmingen™, Europe) was used as a positive control. FCAP Array v.1.0.2 (Soft Flow) software was used for analysis.

3.4.11 Immunofluorescence flow cytometry (IV)

Indirect immunofluorescent labeling was used by incubating cancer cells with primary antibody against human CTLA-4 (BD Pharmingen™, Europe) at 4ºC for 30 minutes, washed and incubated in the dark with secondary antibody at 4ºC for 30 minutes. Subsequently, fluorescence intensity was measured on a LSR flow cytometer (BD Pharmingen™, Europe). Clontech Discovery Labware Immunocytometry systems (BD Pharmingen™, Europe) and the FlowJo 7.6.1 software were used for analysis.

3.4.12 Measurement of human IgG concentrations by Elisa

Tissues were minced with a scalpel and incubated with 5 µl of protease inhibitor (P8340; Sigma-Aldrich, St Louis, MO) and 600 µl of digestion mixture consisting of RPMI 1640 medium with 10 mmol/l HEPES buffer and 1.6 mmol/l phenylmethylsulfonyl fluoride (Sigma-Aldrich), 40 µg/ml gentamycin (Amresco, Solon, OH), 100 µg/ml bovine serum albumin (Sigma-Aldrich) and 100 µg/ ml Zwittergent 3-12 (Merck4Biosciences, Darmstadt, Germany). After incubation of 90 minutes at 37 °C under continuous agitation the tumor lysates were subjected to 30 seconds of sonication and centrifuged at 2,000*g* for 10 minutes at 4 °C. Supernatants were collected and stored (Koski, Kangasniemi et al. 2010). Plasma was separated by centrifugation from blood collected by heart puncture.

Human IgG concentrations in tissue lysate supernatants and plasma samples were measured using Elisa for Human IgG (Immunology Consultants Laboratory, USA) according to the manufacturer's protocol. Total protein concentration was measured by BCA Protein Assay (Thermo Scientific Pierce) according to the manufacturer's instructions.

3.5 *In vivo* studies

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

In all the present studies the tumor growth rate was assessed. For tumor volume determination, the largest diameter of the tumor and the diameter perpendicular to it were measured with calipers. Volumes were calculated using the formula: (larger diameter) x (smaller diameter)^2 x 0.5.

3.5.1 Animals models in study I

For the HNSCC cancer xenograft model, female NMRI nude mice were subcutaneously injected with $5x10^6$ UT-SCC 8 HNSCC low passage tumor cell culture together with matrigel (BD Pharmingen, Franklin Lakes, NJ) in both flanks.

3.5.1.1 Comparison of different transductionally targeted oncolytic adenoviruses

When the HNSCC cancer xenograft tumors (n=10/group) reached a volume of ca. 80 mm^3 , they were randomized into 3 groups and injected for 3 consecutive days (days 0, 1 and 2) with the indicated vectors at a dose of $3x10^8$ VP and control tumors were injected with growth medium only.

3.5.1.2 Combination of oncolytic adenoviruses with chemotherapy, radiotherapy and monoclonal antibody against EGFR

When the HNSCC cancer xenograft tumors (n=10/group) reached a volume of ca. 30 mm^3, they were injected intratumorally with Ad.pK7-Δ24 (1×10^8 VP) on days 1, 4, 8 and 11. Cetuximab (750 µg), and/or chemotherapy (25 µg cisplatin+ 250 µg 5-FU) was given intraperitoneally on days 0, 3, 7 and 10. Whole body radiation (1 Gy) was given on days 0, 3, 7 and 10. On day 97, the remaining tumors were collected and stored at -80°C.

3.5.2 Animals models in study II

For the prostate cancer xenograft model, NMRI nude mice were subcutaneously injected with $5x10^6$ PC3-MM2 tumor cells in both flanks. Mice were randomized into 12 groups (n=6/group; tumor volume ca. 0.55 cm^3): mock (injected with growth media only), rAdE1B55K, rAdE4orf3, rAdE4orf6, Ad5(GL), Ad300wt, with and without radiotherapy (RT). Intratumoral virus injections of $1x10^9$ VP were administered every other day (total of $4x10^9$ VP). Fractionated whole- body irradiation was given every other day (4 x 2 Gy) during 8 treatment days.

3.5.3 Animals models in study III

For the HNSCC cancer xenograft model, female NMRI nude mice were subcutaneously injected with $3x10^6$ UT-SCC 8 HNSCC low passage tumor cell culture together with matrigel (BD Pharmingen, Franklin Lakes, NJ) in both flanks. After 7 days, the tumor bearing mice were randomized into 6 groups (n=8/group, ca. 25 mm^3) tumors were injected 3 times every other day with the indicated vectors at a dose of $3x10^8$ VP (days 0, 2 and 4) and control tumors were injected with growth medium only. 5-FC was given intraperitoneally at 250 mg/kg/day for 3 times (from day 1 to day 11, from day 28 to day 38 and from day 48 to day 56) and phosphate buffered saline (PBS) injections were used as negative controls.

3.5.4 Animals models in study IV

For the prostate cancer xenograft model, NMRI nude mice were subcutaneously injected with $5x10^6$ PC3-MM2 tumor cells in both flanks. After 7 days, the mice were randomized into 5 groups (n=8/group, tumor volume ca.130 mm^3),tumors were injected for 3 times every other day with $3x10^8$ VP of the indicated vectors (days 0, 2 and 4) and control tumors were injected with growth medium only.

3.6 Statistical analysis (I-IV)

In all studies, statistical analyses were performed using a two-tailed Student's t-test and/or the nonparametric Mann–Whitney U-test (SPSS 15.0, Chicago, IL). For all analyses a P value of <0.05 was considered statistically significant.

In study I, combination index (CI) values were calculated using the Chou Talalay's median-effect method (Chou T-C. 1983) under assumption of mutually nonexclusive drug interactions with S-PLUS 6.0 (Insightful Corporation, Seattle, WA). In CI analysis <1 indicates synergism, 1= additivity, and CI >1 indicates antagonism. One-sample t-test was performed to determine whether the mean CI from separate experiments at multiple effects levels were significantly different from a value of 1.0.

4 RESULTS AND DISCUSSION

4.1 High frequency of CD133+/CD44+ cancer initiating cells in HNSCC tumors recurr after anti-EGFR monoclonal antibody treatment (I)

Currently, there is an increased clinical use of epidermal growth factor receptor (EGFR) inhibitors (e.g. cetuximab), which have shown utility in combination with chemotherapy, radiotherapy and chemoradiotherapy (Bonner, Harari et al. 2006; Nyati, Morgan et al. 2006). However, increasing evidence suggests that patients who initially respond to EGFR inhibitors may subsequently become refractory (Pao, Miller et al. 2005). Therefore, it is important to study the mechanisms of treatment resistance in order to further developments and improvements.

Tumor initiating cells or cancer stem cells have been suggested to be a possible reason for tumor relapse to the current treatment modalities (Baumann, Krause et al. 2008; Prince and Ailles 2008). It has been proposed that current treatment modalities used for HNSCC and other cancer types may selectively kill differentiated cancer cells, producing tumor regression, while sparing tumor initiating cells, which can lead, in time, to tumor relapse (Baumann, Krause et al. 2008; Prince and Ailles 2008). In agreement with such reports, relapsed tumors to cetuximab were found to have higher ratio of CD44/CD133 positive cells in relation to EGFR positive cells (Figure 1, **Study I**), suggesting that cancer stem cells might play a role in the mechanism of resistance to cetuximab. In addition, EGFR inhibition is usually not effective enough to be of clinical benefit as a single therapy and the best responses are achieved in combination regimens (Bonner, Harari et al. 2006). Also, it has been reported that capsid modified viruses may be able to kill tumor initiating cells (Eriksson, Guse et al. 2007; Bauerschmitz, Ranki et al. 2008). This prompted us to study if a combination regimen with oncolytic Ad could be utilized to increase the therapeutic efficacy of standard cancer therapies such as monoclonal antibody therapy, chemotherapy and radiotherapy.

4.2 Capsid modified adenoviruses exhibit increased gene transfer to HNSCC low passage tumor cell cultures (I)

Clinical trials have revealed Ads as a promising tool against cancer as well as showing increased tumor targeting. Previous reports have demonstrated that transductionally targeted oncolytic viruses enter tumor cells through CAR-independent mechanisms (Ranki, Kanerva et al. 2007). This might be useful to overcome the inefficient transduction of many tumor types, especially when they are advanced due to variable or low CAR expression (Okegawa, Li et al. 2000). A panel of capsid modifications was tested in several HNSCC low passage tumor cell cultures in order to select which capsid modifications allowed increased gene transfer of human HNSCC and it was found that 5/3, pK7, RGD and pK7.RGD capsid modifications promote increased gene transfer when compared with the wild Ad5 capsid

(Figure 1, **Study I**). Furthermore, 5/3 and pK7 capsid modification promoted the highest gene transfer (Figure 1, **Study I**). Low passage tumor cell cultures might resemble patient tumors more closely than conventional cell lines (Erjala, Sundvall et al. 2006).

4.3 Capsid modified oncolytic adenoviruses are effective in killing tumor cells both *in vitro* and *in vivo* (I, III, IV)

Previous reports indicate that by deleting the Rb binding site of the E1A (Δ24), increased tumor targeting can be obtained (Kanerva, Zinn et al. 2003). Also, it has been reported that transcomplementally and transductionally double targeted oncolytic viruses have an enhanced anti-tumor effect both *in vitro* and *in vivo* in several different tumor types (Kanerva, Zinn et al. 2003; Raki, Kanerva et al. 2005; Ranki, Kanerva et al. 2007). In line with such reports, Δ24 transcriptional targeting in combination with 5/3 chimera or pK7 capsid modifications promoted increased cell killing of HNSCC low passage tumor cell cultures *in vitro* but only pK7 presented a significant anti-tumor effect *in vivo* (Figure 2, **Study I**). The high levels of heparan sulfate proteoglycan (HSPG) reported in HNSCC might explain the high efficacy of pK7 (Hussein and Cullen 2001). Furthermore, *in vitro,* Ad5/3-Δ24 was found to kill prostate cancer cells (Supplementary Figure 1, **Study III** and Figure 3, **Study IV**) as well as lung cancer and ovarian cancer (Figure 3, **Study IV**) confirming previous reports (Särkioja, Kanerva et al. 2006; Rajecki, Kanerva et al. 2007; Raki, Särkioja et al. 2008).

4.4 Combination of oncolytic adenoviruses with chemotherapy, radiotherapy and/or monoclonal antibody treatment resulted in significantly increased killing of tumor cells *in vitro* and complete tumor eradication *in vivo* (I)

In order to test our initial hypothesis that the combination of oncolytic viruses with conventional HNSCC therapies has an increased antitumor effect, the most promising oncolytic viruses (Ad5.pK7-Δ24 or Ad5/3-Δ24) were combined with cetuximab, chemotherapy or radiotherapy, and the increased cell killing was assessed by MTS assay. *In vitro* data suggested that there was a positive synergistic effect between the virus and chemotherapy or radiotherapy when pair-wise analysis was performed (Figure 4, **Study I**), in accordance with previous reports (Khuri, Nemunaitis et al. 2000; Lamfers, Idema et al. 2007). It is also worth to report that cetuximab, despite the poor results as a single agent *in vitro* (Figure 3, **Study I**), was observed to have a greater response *in vivo.* A possible explanation for these findings is that the Fc tail may activate the complement and antibody-dependent cell-mediated cytotoxicity against the tumor (Bonner, Harari et al. 2006). Further, indirect effects on angiogenesis, invasion and metastasis may play a role in the *in vivo* effects of cetuximab (Vincenzi, Schiavon et al. 2008). The same reasons might also explain the modest results of the quadruple combination *in vitro*, although *in vivo* a complete tumor reduction was observed (Figure 5, **Study I**).

According to this study, tumors relapsing after cetuximab are enriched in CD133 and CD44+ cells (Figure 1, **Study I**). Therefore, it is tempting to hypothesize that the quadruple

combination was able to kill these cells. This seems to be confirmed by the finding that tumors were completely eradicated by the quadruple combination and also, *in vitro,* all cells could be killed (Figure 5, **Study I**). Tumor initiating cells may be resistant to radiotherapy, chemotherapy and kinase inhibitors (Reya, Morrison et al. 2001; Dean, Fojo et al. 2005). Therefore, the oncolytic virus present in the quadruple combination may have been important with regard to complete tumor eradication. In particular, previous reports suggest that capsid modified viruses may be able to kill tumor initiating cells (Eriksson, Guse et al. 2007; Bauerschmitz, Ranki et al. 2008).

4.5 Infection with recombinant adenoviruses expressing the adenoviral radiosensitizing proteins E4orf6, E4orf3 and E1B55K prior to radiotherapy significantly increases tumor cell killing *in vitro* but only E4orf6 and E4orf3 were able to radiosensitize *in vivo*. (II)

The radiosensitizing effect of oncolytic serotype 5 adenoviruses has been recently reported *in vivo* (Kim, Kim et al. 2009; Rajecki, af Hallstrom et al. 2009). Also, a synergistic effect was observed in *in vitro* experiments with HNSCC previously in this study (Study I). The Ad proteins E1B55K, E4orf3 and E4orf6 have evolved to inhibit cell cycle arrest and DNA double-strand break (DSB) repair signaling to maintain effective replication (Boyer, Rohleder et al. 1999; Stracker, Carson et al. 2002; Hart, Yannone et al. 2005; Carson, Orazio et al. 2009) (section 1.3.1.1.1). In order to assess if the radiosensitization of tumor cells by Ad is mediated through the impairment of a DSB repair mechanism, replicative deficient viruses expressing the proteins E4orf6, E4orf3 or E1B55K were used. These viruses expressed high levels of the respective radiosensitizing proteins and are able to transduce prostate cancer cells with rates of more than 90% at 24h after infection (Figure 1, **Study II**).

In vitro, prostate cancer, breast cancer and HNSCC cells infected with the E4orf6, E4orf3 or E1B55K expressing viruses and irradiated the next day showed increased cell killing in comparison with only irradiated cells or to cells irradiated and infected with replicative deficient control virus A5(GL) expressing GFP and luciferase (Figure 2, **Study II**). The best radio sensitizing effect was observed with the E4orf6 (Figure 4, **Study II**). Furthermore, double or triple combinations of the recombinant viruses did not further improve the cell killing of the most effective viral component alone (Figure 3, **Study III**). Despite this, there have been molecular level indications that E1B55K and E4orf6 as well as E1B55K and E4orf3 might work in complexes to inhibit the DSB repair (Leppard and Everett 1999; Mohammadi, Ketner et al. 2004; Schwartz, Lakdawala et al. 2008).

In vivo, rAdE4orf3 was the most effective of the transgene expressing viruses when used with radiation, and resulted in significant reduction of tumor growth compared to Ad5(GL) with RT (Figure 5, **Study II**). Also, rAdE4orf6 with radiation resulted in a statistically significant reduction of tumor growth. In contrast, rAdE1B55K with radiation did not inhibit tumor growth. Replicative control wild type virus Ad300wt caused a significant reduction of tumor growth combined with RT but not alone. Interestingly, in line with this study and others' *in vitro* results (Brand, Klocke et al. 1999; Kim, Kim et al. 2009; Wang, Sima et al. 2009), recombinant viruses had anti-tumor efficacy even in the absence of radiation (Figure 5, **Study II**).

4.6 Infection with rAdE4orf6 and rAdE4orf3 results in persistence of double-strand breaks at 24h post-irradiation. (II)

Inhibition of DSB repair has been suggested to be the main mechanism contributing to the radiosensitizing effect of replication-competent adenoviruses (Stracker, Carson et al. 2002; Rajecki, af Hallstrom et al. 2009). In line with such reports, Ad300wt wild type virus showed 50% persistence of DSBs 24h after irradiation as well as rAdE4orf3 or rAdE4orf6 (40-50%) (Figure 6, **Study III**), indicated by γH2AX foci. In contrast, the control virus Ad5(GL) or RT-only casued fewer DSBs (10%, <5%, respectively). rAdE1B55K showed some persistence in DSBs (25%) (Figure 6, **Study III**).

Despite rAdE4orf3 or rAdE4orf6 having revealed similar inhibition of DSBs repair compared to replicative competent wild type virus (Figure 6, **Study III**), *in vivo* the highest tumor growth inhibition was achieved by the replicative competent wild type virus in combination with radiotherapy (Figure 5, **Study II**). These findings support that gene transfer alone is not enough to achieve sustained an anti-tumor effect *in vivo,* but when combined with viral replication increased anti-tumor efficacy can be obtained.

The MRN complex (Mre11, Rad50 and NBS1) is important in DSB sensing, stabilization, signaling, and repair (Rogakou, Pilch et al. 1998; Williams, Williams et al. 2007). It upregulates ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) signal transduction pathways (Rogakou, Pilch et al. 1998; Kuo and Yang 2008), which are involved in both homologous recombination and non-homologous end-joining (Lilley, Schwartz et al. 2007). Earlier data suggested DNA-dependent protein kinase (DNAPK) inhibition as the main mechanism for E4orf6 and E4orf3 mediated DSB repair inhibition (Boyer, Rohleder et al. 1999). However, ATM/ATR pathway inhibition by E4 proteins is evident, although the specific targets remain unknown, and plays a more central role in DSB signaling (Rogakou, Pilch et al. 1998).

4.7 FCU-1 fusion enzyme or anti-CTLA monoclonal antibody armed oncolytic and replication-deficient adenoviruses retain their efficacy of infecting tumor cells and express high levels of functional proteins *in vitro* and *in vivo* (III, IV)

Clinical trials have demonstrated the increased anti-tumor effect when oncolytic Ads are combined with conventional cancer therapies (You, Yang et al. 2000; Xia, Chang et al. 2004). In addition, **Study I** showed that transductionally and transcomplementally double targeted oncolytic Ad can also be combined with conventional cancer therapies such as radiotherapy, chemotherapy and monoclonal antibody treatment to achieve increased and even synergistic anti-tumor effects. These findings support that lower doses of conventional cancer therapies can be used in a multimodal approach to achieve an increased anti-tumor effect. The side effects of the treatments are nonoverlapping, which might facilitate increased efficacy without increasing toxicity. Furthermore, **Study II** supported the radiosensitization effect of Ad but as well the need to use viral replication to obtain a sustained anti-tumor efficacy. Therefore, in order to take advantage of the oncolytic Ad

synergy with conventional cancer therapies and further reduce possible side effects, double targeted oncolytic Ad was armed with FCU-1 fusion enzyme or anti-CTLA monoclonal antibody (Figure 1, **Studies III** and **IV**). Also, in order to further demonstrate the need of viral replication to achieve sustained anti-tumor efficiency, the isogenic replicative deficient Ad was produced (Figure 1, **Studies III** and **IV**). The newly produced viruses feature a 5/3 chimera capsid modification and the oncolytic viruses additionally have a 24 bp deletion in the E1A (Δ24 transcomplementary targeting). All the viruses produced high levels of the respective proteins measured by Western blot (Figure 2, **Studies III** and **IV**).

Viral expressed FCU1 enzymatic CDase and UPRTase activities were confirmed by the analysis of the enzymatic conversions of 5-FC to 5-FU and 5-FU to 5-FUMP in HPLC separation *in vitro* (Figure 2, **Study III**) and 5-FC to 5-FU *in vivo* (Figure 4 and 5, **Study III**). The highest FCU1 enzymatic activities were observed in cells infected with the oncolytic virus Ad5/3-Δ24FCU1 both *in vitro* and *in vivo*. Furthermore, chemotherapeutic 5-FU product of the 5-FC conversion was found only in the tumors treated with Ad5/3-Δ24FCU1 and 5-FC up to day 57. This finding supports the hypothesis that combined anti-tumor transgene expression to viral replication results in a sustained long term targeted anti-tumor effect. In fact, on day 57, increased apoptosis was observed in the tumors treated with Ad5/3-Δ24FCU1 and 5-FC, resulting from the bystander effect of the FCU1 enzymatic conversion of 5-FC to the chemotherapeutic metabolites 5-FU and 5-FUMP (Figure 6, **Study III**).

Virally expressed anti-CTLA4 mAb biological activity was confirmed by measuring the increased IL-2 production of stimulated Jurkat cells (clone 6.1) as described previously (Lee, Chuang et al. 1998). This analysis showed that mAb anti-CTLA4 activity was found in the supernatants of cells infected with Ad5/3-Δ24aCTLA4 and Ad5/3-aCTLA4 compared to the respective isogenic controls Ad5/3-Δ24 or Ad5/3Luc1-infected tumor cells (Supplementary Figure 1 and Figure 2, **Study IV**). Furthermore, when rCTLA-4 was added to the medium, the anti-CTLA4 mAb T cell stimulating activity was lost, confirming the specificity of the anti-CTLA4 mAb produced (Supplementary Figure. 1 and Figure. 2, **Study IV**). In addition, tumors treated with Ad5/3-Δ24aCTLA4 showed increased IgG production correlated with increased apoptosis, confirming further that the production *in vivo* of functional fully human monoclonal antibody specific for CTLA-4 in cells infected with Ad5/3-Δ24aCTLA4 (Figure 4, **Study IV**). Finally, tumors infected with Ad5/3-Δ24aCTLA4 showed 43.3-fold ($p<0.05$) more anti-CTLA4 mAb in the tumors than in the plasma and 81-fold ($p<0.05$) more than tumors treated with the replicative deficient Ad5/3-aCTLA4. These findings are in line with **Studies II and III** in that increased transgene production can be obtained by combining transgene expression to viral replication

Taken together, our data indicates that infection with Ad5/3-Δ24FCU1 or Ad5/3-Δ24aCTLA4 leads to high expression of functional transgenes in targeted tumors.

4.8 Oncolytic adenovirus armed with a suicide gene system or immunomodulatory agent showed increased cell killing *in vitro* and tumor growth inhibition *in vivo*. (III, IV)

Arming oncolytic viruses with powerful transgenes such as FCU1 + 5-FC suicide system or with anti-CTLA4 mAb has been previously a concern because of their size and biological properties. 5-FC enzymatic conversion by the FCU1 fusion enzyme results in the chemotherapeutic agent 5-FU and 5-FUMP, which interfere with DNA and RNA synthesis and consequently could inhibit viral replication. In fact, a certain decrease on viral copy number was observed when 5-FC was added to Ad5/3-Δ24FCU1 infected cells or tumors (Figure 3 and 6, **Study III**). Nevertheless, a significantly increased anti-tumor effect was observed when Ad5/3-Δ24FCU1 was combined with 5-FC than with component alone both *in vitro* and *in vivo* (Figure 3 and 4, **Study III**). The increased efficacy suggests that the additional cell killing and bystander effect provided by 5-FU and 5-FUMP, together with the putative synergy with oncolytic adenovirus (**Study I**), are more important than the relative reduction in virus replication.

CTLA-4 blockade with mAbs results in increased production of IFN-γ and major histocompatibility complex (MHC) class I molecules that can potentially inhibit virus replication (McCart, Puhlmann et al. 2000; Nakamura, Mullen et al. 2001). However, this may not be a problem in tumor cells which are often deficient in interferon signaling and MHC-I presentation (Sherr 1996). Furthermore, the anti-CTLA mAb gene cassette brings the Ad5/3-Δ24aCTLA4 genome size close to the maximum 105%. Nevertheless, increased cytotoxicity was observed *in vitro* and increased anti-tumor efficacy *in vivo* (Figure 3 and 4, **Study IV**). The replicative competent control Ad5/3-Δ24 was not able to significantly increase the anti-tumor efficacy in this very aggressive prostate PC3-MM2 xenograft model despite similar cytotoxicity efficiency *in vitro* (Figure 3, **Study IV**). Also, the replicative deficient Ad5/3-aCTLA4 despite showing some cytotoxicity *in vitro* (Figure 3, **Study IV**), *in vivo* was not able to significantly reduce the tumor growth (Figure 4, **Study IV**). Ad5/3-aCTLA4 cytotoxicity *in vitro* can be explained by increased tumor cell apoptosis mediated by the anti-CTLA4 mAb (Figure 4, **Study IV**). These findings, together with the increased apoptosis, indicate that oncolytic adenoviruses can effectively target the expression of functional anti-CTLA4 mAb in high amounts, resulting in increased anti-tumor efficacy. Furthermore, the main anti-tumor effect of the anti-CTLA4 mAb is mediated by the activation of the immune system against the tumor. However, this promising mechanism of action could not be tested *in vivo* because the human anti-CTLA4 mAb does not work in mice (Hanson 2004).

4.9 Effective immunomodulation of cancer patient T-cells by anti-CTLA4 monoclonal antibody expressing viruses and the effect of anti-CTLA4 monoclonal antibody on PBMCs from healthy individuals (IV)

With the inability to fully assess the anti-tumor efficacy of the anti-CTLA4 mAb in mice and to extend **Study IV** preclinical findings into humans, PBMCs of patients with advanced solid tumors refractory to chemotherapy were simulated as previously indicated (Lee, Chuang et

al. 1998) (Figure 5, and rationale in Supplementary Figure 1, **Study IV**). In all four patients, supernatant from anti-CTLA4 mAb expressing viruses was able to increase T-cell activity, as measured by IL-2 and interferon gamma (Figure 5, **Study IV**). Similar data were obtained in a loss function assay (Suppl. Fig 2, rationale in Suppl. Fig 1, **Study IV**). However, in healthy donors, no significant immunomodulation was observed with supernatant from Ad5/3-Δ24aCTLA4 infected cells but some immunomodulation was achieved with a high concentration of the positive control mAb. These findings support the suggestion that cancer patients have a higher degree of immunosuppressive processes ongoing due to the advanced tumor present (**Figure 1** and (Stagg, Johnstone et al. 2007)), thus requiring lower concentrations of anti-CTLA4 mAb to activate T cytotoxic cells.

5 SUMMARY AND CONCLUSIONS

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

In **study I** it was found that cetuximab relapsed tumors have a higher proportion of cancer stem cells. *In vitro* studies revealed that Ad5.pK7-Δ24 and Ad5/3-Δ24 are the most promising capsid modifications for the treatment of HNSCC. Furthermore, the quadruple treatment modality using the double targeted Ad5.pK7-Δ24 with radiotherapy, chemotherapy and cetuximab mAb therapy was able to completely eradicate HNSCC xenograft tumors. Further, the low LC50 values obtained with the quadruple combination *in vitro* support that lower doses of therapeutic agents can be used in this modality and consequently with fewer possible side effects.

Study II brought some light onto the mechanism of how Ads radiosensitize tumors. Data shows that endogenous adenoviral proteins E4orf3 and E4orf6 can sensitize prostate cancer cells to radiotherapy *in vivo*. Further, multiple administrations of adenovirus together with fractionated radiotherapy can restrict aggressive prostate tumor growth.

The mechanisms of augmented therapeutic effects obtained by combining oncolytic adenoviruses, EGFR inhibitors, chemotherapy and radiotherapy require further studies. Nevertheless, our data together with others allow several hypotheses to be put forth. Cultured cells treated with ionizing radiation show increased levels of phosphorylated EGFR (Schmidt-Ullrich, Valerie et al. 1996) with subsequent EGFR import to the nucleus, where it activates the DNA-protein kinase (DNAPK) leading to DNA repair such as DSBs (Figure 5, **Study II**) and cell survival (Schmidt-Ullrich, Mikkelsen et al. 1997). Tumor cells treated with various cytotoxic drugs (e.g. cisplatin and 5-FU (Van Schaeybroeck, Karaiskou-McCaul et al. 2005)) also promote EGFR phosphorylation in order to enable cell survival. Cetuximab inhibits radiation-induced activation of DNAPK, as well as EGFR nuclear import, DNA repair and survival from radiation induced damage (Dittmann, Mayer et al. 2005). The adenoviral protein E4orf6 enables prolonged auto-phosphorylation of DNAPK after ionizing radiation. This inhibits damage repair and reduces cancer cell survival (Hart, Yannone et al. 2005). In **Study II,** E4orf3, and to a lesser extent E1B55K, promoted delayed DSBs repair possibly via ATM/ATR or DNAPK (Rogakou, Pilch et al. 1998; Boyer, Rohleder et al. 1999). It has also been shown that adenoviral E1A gene products can inhibit HER-2/c-erbB-2 expression (Yan, Chang et al. 1991) for further inhibition of the erbB signaling pathway already partially blocked by cetuximab. Cetuximab and adenovirus use different ways to inhibit the activation of the erbB survival pathway. This might make it more difficult for tumor cells to overcome pathway inhibition for gaining resistance to chemo and radiation regimens.

To take advantage of the synergistic effects between double targeted oncolytic Ad with chemotherapy and mAb observed in **Study I**, Ad5/3-Δ24FCU1 and Ad5/3-Δ24aCTLA4 were produced. Ad5/3-Δ24FCU1 with 5-FC seemed to result in improvements in anti-tumor activity (**Study III**). Ad5/3-Δ24aCTLA4 to our knowledge, is the first study showing that a full

length mAb can be produced from an oncolytic adenovirus. Also, it is the first fully human anti-CTLA4 mAb expressed by a tumor targeted replicative competent platform that resulted in improved anti-tumor activity (**Study IV**). No side effects were observed in these experiments, but this requires further studies. Also, these viruses confirm the hypothesis that arming tumor targeted oncolytic viruses with potent anti-tumor transgenes results in high tumor concentrations and low systemic concentrations of the transgenes. The anti-tumor potency of both these viruses' might be further improved by combining them with radiation (**Studies I and II**)

The data in these studies provide a rationale for the clinical translation of Ad5/3-Δ24FCU1 and Ad5/3-Δ24aCTLA4 as two new oncolytic viral vectors for treating HNSCC and PC. Since the p16-Rb pathway is defective in many if not all solid tumors (Sherr 1996), Ad5/3-Δ24FCU1 and Ad5/3-Δ24aCTLA4 are attractive candidate agents for treatment of many other types of cancer that are refractory to available treatments.

However, clinical trials are needed to confirm the data in humans. Recent breakthroughs in clinical gene therapy have demonstrated that small incremental improvements have the potential for large differences to patients. Often, these advances have been achieved through combination therapies (Ylä-Herttuala 2008; Aiuti, Cattaneo et al. 2009).

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Part C- Original Publications