ONCOLYTIC ADENOVIRUSES WITH RADIATION THERAPY FOR TREATMENT OF PROSTATE CANCER

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Sometimes the journey is more important than the destination.

*To mice and men.*
ABSTRACT

Prostate cancer is the most common cancer in males. Although many patients with localized disease can be cured with surgery and radiotherapy, advanced disease and especially castration resistant metastatic disease remains incurable, with a median life expectancy of less than 18 months. Oncolytic adenoviruses (Ads) are a new promising treatment against cancer due to their innate capacity to kill cancer cells. Viral replication in tumor cells leads to oncolysis and production of a multiplicity of new virions that are capable of further destroying cancerous tissue. Oncolytic Ads can be modified for tumor targeted infection and replication and be armed with therapeutic transgenes to maximize the oncolytic effect.

Worldwide, clinical trials with oncolytic Ads have demonstrated good safety while the antitumor efficacy remains to be improved. Importantly, the best responses have been reported when oncolytic adenoviruses have been combined with standard cancer treatments, such as chemotherapy and radiation. Further, a challenge in many virotherapy approaches has been the monitoring of virus replication in vivo. Reporter genes have been extensively used as transgenes to evaluate the biodistribution of the virus and activity of specific promoters. However, these techniques are often limited to preclinical evaluation and not amenable to human use.

The aim of the thesis was to find and develop new oncolytic Ads with maximum efficacy against metastatic, castration resistant prostate cancer and study them in vitro and in vivo combined to different forms of radiation therapy. Using combination therapy, we were aiming for better antitumor efficacy with reduced side effects. Capsid modified Ads for enhanced transduction were studied. Serotype 3 targeted chimera, Ad5/3, was found to have enhanced infectivity for prostate cancer and was used for developing new viruses for the study. Correlation between Ad-encoded marker peptide secretion and simultaneous viral replication was evaluated and the effects of radiotherapy on viral replication were studied in detail. We found that the repair of double strand breaks caused by ionizing radiation was inhibited by adenoviral proteins and led to autophagic cell death. Both subcutaneous models and intrapulmonary tumor models mimicking metastatic, aggressive disease were used in vivo. Virus efficacy was evaluated by intratumoral injections. Also,
intravenous administration was evaluated to study the effectiveness in metastatic disease. Oncolytic adenovirus treatment led to significant tumor growth control and increased the survival rate of the mice. These results were further improved when oncolytic Ads were combined with radiation therapy.

Oncolytic Ads expressing human sodium/iodide transporter (hNIS) as a transgene were evaluated for their oncolytic potency and for the functionality of hNIS in vitro and in vivo. Monitoring of viral replication was also assessed using different imaging modalities relative to clinical use. SPECT imaging of tumor-bearing mice was evaluated and combined with simultaneous CT-scanning to obtain important anatomical information on biodistribution, also in a three-dimensional form. It was shown that hNIS-expressing adenoviruses could harbour a bi-functional transgene allowing for localization and imaging of viral replication. Targeted radiotherapy was applied by systemic radioiodide administration and resulted in iodide accumulation into Ad-infected tumor. The combination treatment showed significantly enhanced antitumor efficacy in mice bearing prostate cancer tumors.

In summary, the results presented above aim to provide new treatment modalities for castration resistant prostate cancer. Molecular insights were provided for better understanding of the benefits of combined radiation therapy and oncolytic adenoviruses, which will hopefully facilitate the translation of the approach into clinical use for humans.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


SPECT/CT Imaging of Human Sodium-Iodide Symporter (hNIS) Expression after Intravenous Delivery of an Oncolytic Adenovirus in Combination with 131I Radiotherapy. Submitted.

* The authors have equal contribution

The publications are referred in the text by their Roman numerals.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>Ad</td>
<td>adenovirus</td>
</tr>
<tr>
<td>ADP</td>
<td>adenoviral death protein</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia-teleangiectasia mutated</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackie-adenovirus receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cytosine deaminase</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclo-oxygenase 2</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>CR</td>
<td>constant region</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DHA</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DKC1</td>
<td>dyskerin</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GCV</td>
<td>ganciclovir</td>
</tr>
<tr>
<td>GM</td>
<td>growth media</td>
</tr>
<tr>
<td>GMCSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>hNIS</td>
<td>human sodium/iodide symporter</td>
</tr>
<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IU</td>
<td>infectious units</td>
</tr>
<tr>
<td>KC</td>
<td>Kupffer cell</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone-releasing hormone</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule associated protein 1 light chain</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLP</td>
<td>major late promoter</td>
</tr>
<tr>
<td>MRN</td>
<td>mre11, rad50 and NSB1-complex</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>sCAR</td>
<td>soluble coxsackie-adenovirus receptor</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TSP</td>
<td>tumor specific promoter</td>
</tr>
<tr>
<td>Vp</td>
<td>viral particle</td>
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</table>
REVIEW OF THE LITERATURE

1. Introduction

Prostate cancer is the most common male cancer in the Western world attributing to significant morbidity and mortality. Initially, the response rate of localized prostate cancer to therapies with curative intent (hormonal ablation therapies, surgery and radiation) is usually good and even watchful waiting is an option for well differentiated, low-volume tumors. However, about 30% of prostate cancers relapse after first-line treatment (Pound et al., 1999). In advanced prostate cancer, despite the initial good response rate to hormonal therapies, many patients develop castration resistant prostate cancer that has only one very limited standardized therapy available (Tannock et al., 2004). Thus, new treatments are urgently needed.

Gene therapy has traditionally aimed to correct defective genes by replacing affected parts of the genome with functional genes. Apart from a few exceptions, cancer is the result of several accumulated genetic mutations, making the correction of single genes arduous. However, the differences in genetic imprint between normal and cancer cells and the similarities between cancer cells and requirements for viral DNA replication set the background for using and targeting oncolytic adenoviruses for cancer treatment. The natural infection of adenoviruses leads to lysis and death of the infected cell and releases viral progeny for further dissemination. Besides being oncolytic by nature, these vectors can be further equipped with therapeutic substances for an enhanced cell killing effect. Importantly, adenoviral genome can be engineered to render the virus tumor selective and to de-target them from normal tissues.

The clinical history of oncolytic adenoviruses dates back to 1956 when cervical cancer patients were treated with wild type adenoviruses of several serotypes (Huebner et al., 1956). The treatment was well tolerated and although the delivered dose could not be quantified and most likely involved low concentrations, local responses were observed in the majority of patients. Thereafter, the developments in molecular biology and the improvements in modifying adenoviral genome have provided tools to optimize
adenoviral therapy. Hundreds of approaches have been used to improve the oncolytic capacity and tumor selectivity of the vectors while retaining safety. Some of the innovative techniques have even been implemented as treatment regimens in clinical practice. However, none of the strategies has been above all the others reflecting the complexity of both the tumor and viral kinetics in a tumor microenvironment. Thus the challenge remains to develop replicative viruses with higher specificity, low toxicity and strong oncolytic capacity to treat various cancers in a heterogeneous patient population. Ultimately, if these vectors are to be constructed, the biggest challenge remains to get them into randomized trials where their efficacy can be truly demonstrated.

2. Prostate cancer

Prostate cancer is the most common male cancer. It is often considered slowly progressing and even a clinically unimportant disease of the elderly; yet it killed 814 Finnish men in 2008 (www.cancerregistry.fi). Moreover, it is estimated that in the United States alone, annually roughly 20,000 out of the 217,730 total new cases are diagnosed in men under 55 (http://seer.cancer.gov/statfacts/html/prost.html). While surgery and radiation therapy can cure many patients, more than 30% will relapse after initial treatment or are detected when the disease is locally advanced or metastatic (Pound et al., 1999). For disseminated or recurrent disease, androgen ablation therapies are often initially effective, but emergence of castration resistant, locally recurrent or distant disease is usually fatal and the median survival time is about 18 months (Tannock et al., 2004).

2.1. Risk factors

Age is the most important risk factor. The mean age of diagnosis is around 70 years (http://seer.cancer.gov/csr/1975_2006/results_single/sect_01_table.11_2pgs.pdf and http://www.cancerregistry.fi). Inherited risk factors are estimated to contribute up to 43
% of the prostate cancer cases (Lichtenstein et al., 2000). However, hereditary prostate
cancer, which accounts only for 3% of the cases, is a multigenetic disease with a large
genetic heterogeneity. Even though several chromosomal regions have been linked to
prostate cancer, so far only three genes (MSR1, ELAC2 and RNASEL) have been shown to
be strongly associated with hereditary prostate cancer (Langeberg et al., 2007; Foulkes,
2008). These single genetic mutations seem to account for only a fraction of the overall
genetic variance of prostate cancer (Tavtigan et al., 2001; Carpten et al., 2002; Verhage
and Kiemenev, 2003; Pakkanen et al., 2007). Prostate cancer is more prevalent in black
men, who also have higher mortality rates (Merrill and Brawley, 1997). The lowest rates
of prostate cancer are found in Asian countries, namely in China and Japan (Hsing et al.,
2000). The causes for racial differences are unclear but probably multiple factors are
involved including socioeconomic, environmental, dietary, and genetic factors (Hoffman
et al., 2001).

A lot of discrepancy exists between nutritional factors and prostate cancer. The
strongest data exists for a positive relationship between animal products, red meat
intake and prostate cancer and suggests a role for fat (Chan et al., 2005). A number of
studies support a protective role for lycopene (Etminan et al., 2004), however a recent
study has not been able to corroborate the effectiveness (Peters et al., 2007).

Non-microbial inflammation and infections of the prostate may represent one
mechanism through which prostate cancer develops (Dennis et al., 2002; Wagenlehner et
al., 2007). Also, a previously unknown prostate-directed retrovirus, xenotropic murine
leukemia virus-related virus, has been isolated from prostate specimens and associated
to cancer development (Schlaberg et al., 2009) but the relation remains unconfirmed
(Hohn et al., 2009).
2.2. Symptoms and diagnosis

Prostate cancer does not have a specific clinical manifestation and it is most often asymptomatic in localized stage. The symptoms are usually similar to those caused by benign prostatic hyperplasia (BPH) i.e. difficulties in initiating and maintaining urination, weak urine stream, nocturia and dysuria. However, most tumors arise from the peripheral zone of the prostate and hence are asymptomatic when in an organ confined stage.

Several approaches to detect and screen prostate cancer are in use. The most relevant include digital examination, prostate specific antigen (PSA) testing from blood samples and transrectal ultrasound. Much dispute exists for PSA screening since PSA values are organ, not tumor, specific (Barry, 2009; Schroder et al., 2009). Increases in PSA values are observed in both BPH and prostate cancer. The rate of PSA increase (Potter and Carter, 2000) or the ratio of free PSA to total PSA in plasma (Stenman et al., 1994) are more indicative for cancer and should be determined if cancer is suspected. However, further evaluation of the suitability of PSA for prostate cancer screening is also needed. Since the introduction of PSA-measurements in the late 1980’s, a clear shift for diagnosing localized disease has been observed (van Leeuwen et al., 2010). A very recent multicenter study showed that PSA screening decreases prostate cancer mortality but with the cost of high over-diagnosis and over-treatment (Schroder et al., 2009).

Consequently, to confirm cancer of the prostate, a biopsy is always needed. Twelve different sites of the prostate are typically biopsied, the microscopic findings are analyzed and histological differentiation is graded according to the Gleason score and/or WHO classification. Gleason scoring is currently the most widely used method for prostate cancer classification. The score defines the two most common cellular patterns in the tumor and determines an overall Gleason score from 2 to 10. A Gleason score under 6 determines the prostate cancer as low-risk, a score of 7 is intermediate-risk and a score above 8 is classified as high-risk. Thus, the most aggressive cancers have the highest score (Gleason, 1966).

In most cases, prostate cancer is diagnosed as organ confined and non-metastatic. For local, well differentiated tumors, even active surveillance in younger patients or
watchful waiting in older patients are a treatment option (Käypähoito, 2007; Klotz, 2010). However, there are no readily available diagnostic tests that could reliably segregate these cases from the aggressive and rapidly progressing prostate cancers. Nomograms combining biochemical, histological and clinical parameters are used in clinical decision making for selecting the best treatment option, but more accurate prognostic factors are urgently needed (Stephenson et al., 2006). Consequently, many low-risk cancers are aggressively treated for certainty. Thus, a major challenge in the field of prostate cancer diagnosis is to find reliable tests to distinguish between slowly progressing, non-aggressive prostate cancer and the rapidly progressing cancer that will spread and metastasise early.

When prostate cancer diagnosis is set, further evaluation is needed in intermediate and high-risk patients (Gleason >7 or PSA >20 μg/l) for disease staging. Typically, a γ-camera imaging is used for detection of the typical bone metastasis and if needed, an MRI or CT-scan can be run for detection of soft tissue metastasis.

2.3 Treatment options for prostate cancer

As for many common cancers, surgery and radiation therapy are treatment options for prostate cancer. Already in the early 1940’s Huggins and Hodges demonstrated that prostate cancer is under the trophic influence of male hormones (see chapter 2.4 below) and that ablation of androgens could cause cancer regression. However, later randomized investigations have shown that hormonal treatments are palliative rather than curative. Table 1 provides information on the currently approved treatment options for non-castration resistant prostate cancer.
### Table 1. Current approved treatment options for prostate cancer

<table>
<thead>
<tr>
<th>Localized disease</th>
<th>Advantages</th>
<th>Disadvantages/complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>Acquisition of samples for pathological examination, precise clinical staging, (complete) removal of tumor mass</td>
<td>Impotence (10-70 %)</td>
</tr>
<tr>
<td>Radiation therapy</td>
<td>Less strenuous than surgery, lower amounts of acute complications than with surgery</td>
<td>Impotence (30-70 %), bowel and bladder irritation, bladder outlet obstruction</td>
</tr>
<tr>
<td>- Brachytherapy</td>
<td>Less strenuous than surgery, lower amounts of acute complications than with surgery</td>
<td>Impotence (10-70 %), bowel and bladder irritation, secondary malignancies</td>
</tr>
<tr>
<td>- Intensity modified radiation therapy (IMRT)</td>
<td>Less strenuous than surgery, lower amounts of acute complications than with surgery</td>
<td>Impotence (10-70 %), bowel and bladder irritation, secondary malignancies</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Advanced disease</th>
<th>Advantages</th>
<th>Disadvantages/complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation therapy with hormonal treatment</td>
<td>See above and below</td>
<td>See above and below</td>
</tr>
<tr>
<td>Hormonal treatment</td>
<td>Prevention of androgen effect on the growth stimulation of the cancer</td>
<td>Breast and mamilla pain/enlargement, side effects related to the liver</td>
</tr>
<tr>
<td>- Antiandrogens</td>
<td>Prevention of androgen secretion and thus androgen-mediated growth stimulation</td>
<td>Changes in lipid profile, weight gain, decrease of muscular mass, hot flushes, osteoporosis, libido loss and impotence</td>
</tr>
<tr>
<td>- Luteinizing hormone-releasing hormone (LHRH) analogues</td>
<td>Efficient treatment option in advanced disease, prevention of the androgen-mediated growth stimulation</td>
<td>As above</td>
</tr>
<tr>
<td>- Surgical castration</td>
<td>Combination of the advantages of above-mentioned hormonal treatments</td>
<td>As above</td>
</tr>
<tr>
<td>- Maximal androgen blockage</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4. Castration resistant prostate cancer

Like normal prostate tissue, early stage prostate cancer requires androgen hormones for growth and survival. Specifically, 5α-reductase converts testosterone to dihydrotestosterone (DHA) that interacts with cytoplasmic androgen receptors (AR) for transcriptional regulation of AR target genes. Initially, prostate tumors respond to hormonal therapies aiming to impede with the proliferative signals. Unfortunately the emergence of castration resistant prostate cancer is seen in 30% of men with biochemical failure (Pound et al., 1999). This type of cancer might not be completely insensitive to hormonal therapies, but current hormonal treatments fail to block efficiently the AR-signalling cascade in these cases. In metastatic disease, the androgen ablation therapy is of palliative nature before the progression to a deathly disease. Thus, the castration resistance reflects the aggressive nature of the disease. Despite the unresponsiveness of hormone depletion, the tumors continue to express the AR. The development of castration resistant prostate cancer can be the consequence of 1) amplification or over expression of the AR (Linja et al., 2001), 2) mutations in the AR (Marcelli et al., 2000), 3) activation of the AR by other factors than DHA, such as growth factors or cytokines (Culig et al., 1994; Hobisch et al., 1998), 4) androgen independent activation of a truncated form of AR (Libertini et al., 2007), 5) increased local production of androgens (Titus et al., 2005), 6) AR negative tumor cells, and finally 7) mutations and changes in other proto-onco- and tumor suppressor genes. Importantly, it has been shown that aberrations in p53 and p16 pathways lead to highly aggressive and rapidly progressing disease (Zhou et al., 2006). The multifactorial causes leading to refractory disease reflects the difficulties in treating advanced prostate cancer.

Table 2 presents the current treatments options and therapies under investigation for castration resistant prostate cancer.
Table 2. Treatment options for castration resistant prostate cancer

<table>
<thead>
<tr>
<th></th>
<th>Mechanism of action</th>
<th>Outcome</th>
</tr>
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<tbody>
<tr>
<td>Docetaxel</td>
<td>Chemotherapeutic of the taxane-group, inhibits mitosis by interfering with microtubulus assembly</td>
<td>Limited advantage for survival benefit (2-3 months) when combined to prednisone. Approved drug for castration resistant prostate cancer treatment.</td>
</tr>
<tr>
<td>Abiraterone</td>
<td>Inhibits CYP17A1 enzyme that forms DHEA and androstenedione, which can be converted to testosterone</td>
<td>Improvements in progression-free survival in phase II-trials in to such an extent that the control-group started also receiving the drug. Phase III trial is ongoing.</td>
</tr>
<tr>
<td>MDV3100</td>
<td>Androgen receptor antagonist</td>
<td>Phase II trial demonstrated &gt;50 % of PSA reduction in 50 - 60 % of the patients</td>
</tr>
</tbody>
</table>

3. Adenoviruses for cancer gene therapy

3.1. Oncolytic viruses

The definition of oncolytic viruses is used for viruses that are able to specifically replicate in and destroy tumor cells. This property can be either inherent (measles, vesicular stomatitis, parvo and Newcastle viruses) or genetically engineered (adeno-, herpes simplex and vaccinia viruses). The oncolytic activity can result from a multiple of mechanisms, including induction of apoptosis, autophagy or direct cell lysis, the expression of toxic proteins, or from the activation of lethal pathways. Also, in recent years, much attention has been put on the immunological responses evoked by oncolytic viruses that are considered important in breaking the immuno-tolerance.
Vaccinia virus has a long history as a vaccine against small pox. Later, deletions of two genes encoding the vaccinia growth factor and thymidine kinase have rendered it suitable for evaluation as an oncolytic agent (Thorne et al., 2005). Since vaccinia viruses have large genome size, they allow for greater transgenic insertions as well.

Herpes simplex virus -1 (HSV-1) is a DNA virus, that posses intrinsic neuronal tropism and is therefore suitable for treatment of neuronal malignancies (Todo, 2008). However, the use of HSV as an oncolytic virus has not been restricted to only neuronal tissue. Vesicular stomatitis viruses are relatively apathogenic and therefore have natural tropism for tumor cells which lack defensive anti-viral mechanisms (Barber, 2004). The same applies for oncolytic paroviruses, among which a rat parovirus H-1PV has been the most studied, even in humans. H-1PV is smaller than most other oncolytic viruses, which might favor its better tumor penetrance. Besides being oncolytic, the antitumor efficacy of H-1PV is partly mediated by immunotolerance break and tumor rejection by immune system (Rommelaere et al., 2010). Other examples include Newcastle disease virus and measles, which was recently evaluated even in a clinical trial (Galanis et al., 2010).

3.2. Adenoviruses

Adenoviruses are non-enveloped, double-stranded DNA viruses that belong to the family of *Adenoviridae*. The family contains genera of closely related viruses that are able to infect many vertebrates from fish to humans although replication remains host-specific.

Based on their ability to agglutinate human erythrocytes, human adenoviruses are divided into seven species (A–G) which together comprise 52 serotypes (Bailey and Mautner, 1994; Jones et al., 2007). As pathogens, adenoviruses cause mainly conjunctivitis (B, D and E), respiratory disease (B1, C and E) and gastrointestinal infections (F). Generally, the infections are mild and based on seroprevalence studies, it is estimated that 85% of humans are infected with at least one serotype during their lifetime (Mast et al., 2010).

In the context of gene therapy, the most used serotypes are adenoviruses 5 and 2 from subgroup C since their structure and biology are well known.
3.2.1. Adenoviral structure

Adenoviruses are composed of double-stranded DNA and associated proteins surrounded by an icosahedral nucleocapsid of about 80–100 nm with 12 protruding fiber-knobs (Figure 1). The 20 triangular facets of the capsid are principally built up of hexon homotrtrimers surrounding the penton base units at each apex. The penton bases are docks for protruding, trimeric fiber-knob domains that are responsible of the primary attachment of the virus to the cell surface. The capsid also contains minor proteins (IIIa, VI, VIII and IX) that are proposed to have a structural function in capsid stabilization.

Inside the core lies an approximately 36,000 bp long DNA molecule. The double stranded, linear DNA is associated with core proteins (IVa2, V, VII, Mu, Proteinase and Terminal protein) that have a variety of functions from the initiation and priming of viral DNA replication to the viral particle assembly (Russell, 2009).

![Figure 1. Adenoviral structure. A coronal section is seen on the left and an outer view is presented on the right (adapted from Rux JJ et al., 2004 and Vellinga J et al., 2005 with the permission of publishers).]

3.2.2. Adenoviral transduction pathway

For Ad serotype 5, the primary attachment to cell surface occurs through knob mediated binding of the coxsackie and adenovirus receptor (CAR) that is the primary receptor for Ad entry (Bergelson et al., 1997). CAR is a glycoprotein located at the vicinity of tight
junctions of epithelial cells (Coyne and Bergelson, 2005). Apart from acting as a viral attachment receptor, the function of CAR is not entirely clear. Several serotypes share the highly conserved amino acid residues binding to CAR (Roelvink et al., 1998). However, subgroup B and D viruses do not anchor to CAR (Stevenson et al., 1995; Arnberg et al., 2000) and their cell entry pathway is not completely elucidated. For subgroup B1, cluster of differentiation 46 (CD46) has been proposed as a receptor (Segerman et al., 2003) and a receptor X has been identified for subgroup B2 (Tuve et al., 2006). Despite CAR being the major receptor, alternative binding of Ad5 and Ad2 viruses to heparan sulphate glycosaminoglycans and MHC class I has been reported (Hong et al., 1997; Dechecchi et al., 2001).

The primary binding to CAR bends the viral fiber and facilitates the subsequent interaction with cellular αvβ3 and αvβ5 integrins with an arginine-glycine-aspartate (RGD) motif on the Ad penton base (Mathias et al., 1994). This step is crucial for efficient internalization. Integrin binding leads to a signalling cascade and the virus is rapidly internalized through clathrin-coated pits. Subsequently, an early endosome is formed around the viral capsid. The endosomal acidic pH disrupts the viral structure and results in the release of the fibers and peri-pentonal hexons leaving a partially uncoated viral particle. Adenoviral protein VI functions to rupture the membrane of early endosomes (Wiethoff et al., 2005), thus enabling the semi-stable hexon shells to enter the cytoplasm where they are transported via microtubuli to the nuclear pores (Leopold et al., 2000). The DNA and attached terminal protein are then released from the viral core to the nucleus where the attachment to the chromatin scaffold initiates the replication. A schematic presentation of the adenoviral infection pathway is provided in Figure 2.

### 3.2.3. Adenoviral life cycle

The adenoviral genome can be divided into two regions based on the timing of gene transcription before or after the viral DNA replication. The early genes (E) are transcribed before commencement of DNA replication and encode for proteins regulating DNA synthesis and start of the replication. Late genes (L) are expressed after DNA replication initiation and code for viral structural proteins (Russell, 2000). All late genes are
Figure 2. Adenovirus infection pathway and life cycle. Initial binding through CAR is followed by integrin attachment and endocytosis. After disruption of viral shell and endosomal escape, the DNA is transported to nucleus where replication and transcription occur. Viral proteins are produced in the cytoplasm and brought back to nucleus for viral assembly. New virions are released by cell lysis. Adapted from Hakkarainen et al., Duodecim 2005.

Indispensable for the replication initiation, the E1A protein is produced immediately after the viral DNA enters host cell nucleus. E1A is responsible for interacting with cellular proteins needed for active DNA synthesis. Importantly, it interacts with the Retinoblastoma (Rb) growth control protein in the cytoplasm and releases the host transcription factor E2F, normally bound to Rb (Berk, 1986). E2F, in turn, transactivates cellular transcriptional and translational machinery for entry into a state similar to the S-phase.

Important direct downstream targets of E1A are other early genes. E1B gene product 19K prevents induction of apoptotic pathways to ensure sustained production of viral particles (Rao et al., 1992). The 55kD product of E1B binds to the tumor suppressor
Figure 3. Adenovirus 5 transcription map. The ~36 000 bp long genome can be divided into 100 map units (1 map unit = 360 bp). The early genes (E) are transcribed before the commencement of the DNA replication and regulate the process. Late transcripts (L) are produced after DNA replication and specify structural proteins.

protein p53 and blocks its activation (Yew and Berk, 1992). E2 transcripts provide the machinery for DNA replication (DNA polymerase, terminal protein and single-strand binding protein) and are also required for late gene transcription.

E3 gene products are not necessary for viral replication in vitro, but have an important role in in vivo replication. Namely, the E3gp19K product prevents the host immune system from recognizing infected cells by blocking the transport of MHC class I mediated antigen presentation to the cell surface. E3 also provides the adenoviral death protein (ADP) that late in the infection cycle facilitates the disruption of cell membrane for enhanced release of viral progeny (Tollefson et al., 1996).

E4 transcripts are essential for viral DNA replication in aiding the adenoviral RNA transport to the cytoplasm (Weigel and Dobbelstein, 2000). E4orf3 and E4orf6 also have protective function of the productive infection by preventing the host responses to linear DNA ends that can be misinterpreted as DNA double strand breaks (DSB) and result in cell cycle arrest (Tauber and Dobner, 2001).

The DNA replication begins from both termini where the terminal proteins act as primers for DNA synthesis. After initiation of DNA replication, late regions are transcribed through the activation of the major late promoter (MLP) (Russell, 2000). The five late transcription cassettes encode for structural proteins (L2 penton, L3 hexon and L5 fiber) and for proteins needed in encapsidation and maturation of viral particles in the nucleus.
DNA packaging signal at the left termini directs the orthodox packaging of DNA into virions.

The final step in the adenoviral life cycle consists of exporting the viral particles into the cytoplasm and subsequent release into the extracellular space by cell lysis (Figure 2). The mechanism by which adenoviruses induce the lytic pathway and cell membrane disintegration remains poorly understood.

3.3. Adenoviral vectors

The feasibility to modify adenoviral DNA and to produce high viral titers of genetic constructs make adenoviruses useful tools for cancer gene therapy. Adenoviruses are able to infect both dividing and non-dividing cells with efficient gene transfer. Additionally, adenoviral DNA remains episomal prohibiting the possibility of host DNA mutations and also retain genetic stability in vivo. Four basic approaches and their combinations are used to modify adenoviruses for cancer gene therapy: I) adenoviral expression of tumor suppressor genes or oncogene inactivators, II) expression of immunostimulatory genes and inhibitors of angiogenesis, III) adenovirus mediated delivery of enzymes for local prodrug activation and IIII) oncolytic adenoviruses.

3.3.1. Replication deficient vectors

Since the discovery that Ads were highly efficient gene transfer vectors, their potential for expressing foreign DNA has been exploited. Ad genomes containing more than 105 % of the normal DNA length package poorly (Bett et al., 1993), so the E1 and E2 transcription units were replaced by therapeutic genes of considerable size, making the first modified Ad vectors replication deficient. Use of replication deficient constructs soon made evident that Ads are most useful for gene therapy applications requiring
short-term transgene expression as immunoresponses quickly extinguish the transgenic expression from Ad vectors.

3.3.1.1. First-generation adenovirus vectors
In first-generation Ad vectors, both the E1 and E3 regions are deleted. The E1 region is often replaced by a transgene. Since an adenovirus vector can package approximately 38 kb to retain optimal packaging and cloning capacity, an $E1$-deleted Ad can accept insertions of up to 5.1 kb. Frequently, an additional deletion is also inserted into the E3 region to allow the cloning of about 8.2 kb of foreign DNA. However, E1 transcripts are fundamental for viral growth and the production of $E1^-$ vectors requires the transcomplementation of E1 genes. This can be achieved by using specific cell lines expressing E1, such as 293 (Graham et al., 1977), 911 (Fallaux et al., 1996) or PER.C6 (Fallaux et al., 1998).

The first-generation vectors are in principle replication deficient when transfected to cells lacking transcomplementing E1 genes. However, in many studies it has been noted that despite the absence of E1A, activation of other early genes may occur leading to viral replication at some extent. It has been proposed that E1 can be substituted by E1A-like functions that are particularly active in cancer cells (Imperiale et al., 1984).

Another important problem encountered with first-generation Ad vectors is the strong immune response that allows only for a transient expression of the desired transgene. One of the first gene therapy trials made with recombinant Ads was to deliver the cystic fibrosis transmembrane regulator (CFTR) gene into the lungs of cystic fibrosis (CF) patients. The gene transfer resulted in CFTR production, but it was soon noticed that the transgene was expressed only for a limited time and repeated administration did not improve the outcome (Harvey et al., 1999). Several innate and adaptive immunological factors contribute to this, including acute inflammatory response (Worgall et al., 1997), generation of anti-Ad antibodies and T-cells and induction of acute and chronic toxicity (Yang et al., 1994; Lieber et al., 1996).
In the context of cancer gene therapy, replication deficient Ads have been used to deliver a “healing” gene into the cancer tissue. A widely used technique is the so-called suicide gene therapy, where E1 is replaced by herpes simplex virus (HSV) thymidine kinase (TK) or E. coli derived cytosine deaminase (CD) for local expression in tumor cells (Hirschowitz et al., 1995; Sandmair et al., 2000). The function of these enzymes is to convert a harmless prodrug into its active, cytotoxic form. When the expression of these enzymes is restrained to tumor tissue by Ad delivery, local prodrug conversion leads to selective elimination of the cells. Benefits are reduced systemic side effects and the bystander effect: the active compounds are transmitted from infected cells to neighbouring tumor cells. In clinical setting, the prodrug activation method has been implemented in the treatment of various tumors, including brain tumors, gliomas (Eck et al., 1996), mesothelioma (Sterman et al., 1998), recurrent prostate (Herman et al., 1999; Nasu et al., 2007) and ovarian cancer (Hasenburg et al., 2002). In general, the prodrug-converting enzyme therapies have been safe and well tolerated, even when repeated cycles have been used (Shalev et al., 2000; Ben-Gary et al., 2002).

First-generation Ad vectors encoding for immunostimulatory molecules have also been evaluated in a number of preclinical studies and clinical trials. The tumor microenvironment is immunosuppressive mainly due to the presence of regulatory T-cells, giving the tumor an uncontrolled growth advantage (Curiel et al., 2004; Ghiringhelli et al., 2005). In this regard, Ads expressing universal immunostimulatory molecules such as granulocyte macrophage colony stimulating factor (GMCSF) (Soiffer et al., 2003), interferons (Khorana et al., 2003; Sangro et al., 2004; Sterman et al., 2007), CD40 ligand (Wierda et al., 2000) or tumor-specific antigens (Butterfield et al., 2008) would potentially evoke an immune response towards the cancer cells. Ad-derived inactivation of mutated proto-oncogenes has also shown promise in a study by Zhang et al., who created an adenoviral vector expressing small hairpin RNA. The virus encodes a sequence specifically targeting mutated K-Ras (AdH1/siK-Rasv12) to knock down the mutated RNA to treat non-small cell lung cancer (Zhang et al., 2006).

Since cancer cells are also dependent on the vascular supply of the tumor for sustained growth, antiangiogenic therapies have been successfully used in cancer
treatment (Van Meter and Kim; Escudier et al., 2007; Hudes et al., 2007; Motzer et al., 2007). Accordingly, several studies have demonstrated the utility of antiangiogenic transgenes targeting vascular endothelial growth factor receptor-1 encoded by oncolytic Ads (Zhang et al., 2005; Yoo et al., 2007; Guse et al., 2009). However, a potential problem with the approach is the proposed dependency of replicative adenoviruses on tumor vasculature for optimal intratumoral dissemination.

Data obtained from the studies has generally demonstrated that first-generation Ad vectors are safe, but efficacy remains suboptimal due to limited time of transgene expression and difficulties in gene transfer to every target cell.

3.3.1.2. Second- and third-generation adenovirus vectors

The above-mentioned immunological dilemmas encountered with E1/E3-deleted vectors led to the hypothesis by which reducing adenoviral gene expression would lead to subsequent decrease in the host immune response and to increased transgene persistence. Ad vectors with additional mutations in E2 and/or E4 have been constructed (Dedieu et al., 1997; Amalfitano et al., 1998) and are called second-generation adenoviruses. These vectors allow for greater transgene insertions and have decreased potential to generate replication-competent Ads, since multiple recombination events would be required. However, it has remained unclear whether the safety and/or transgene expression potential of the multiple-deleted Ads is superior to first-generation Ads since controversial data exist on these issues (Engelhardt et al., 1994; Engelhardt et al., 1994; Dedieu et al., 1997; O’Neal et al., 1998). Consequently, third-generation (“gutless”) adenoviruses have been developed. In these vectors, inverted terminal repeats are retained and all genes except for packaging signal have been deleted (Fisher et al., 1996). These vectors are also known as helper-dependent Ads, as the production and replication require co-infection with a wild type helper Ad (Mitani et al., 1995).
3.3.2. Oncolytic Ad vectors

The effective but only transient gene expression from replication-deficient Ad vectors provides a platform for cancer therapy approaches that require short-term gene expression rather than correction of genes for sustained expression. To achieve more efficient therapeutic effect, cancer cells can be targeted using oncolytic adenoviruses. Unlike in traditional viral gene therapy, the antitumor effect is not delivered (only) by a therapeutic gene but by the replication of the virus per se leading to oncolysis. The killing of tumor cells by selective viral infection, replication, cell lysis, and spread of progeny viruses in the tumor would in theory carry on until all cancer cells are destroyed. Moreover, the transgene expression is highly enhanced as compared to replication-deficient vectors due to replication of the viral genome.

In the case of oncolytic Ads, viral replication takes advantage of tumor-specific cellular changes to allow the replication and propagation only in target tissue. This demands stringent control to retain safety while restraining replication only into tissues that need to be destroyed. Many different strategies have been developed for achieving this (see section 3.4).

Due to their anticancer potency, replication-selective oncolytic Ads have been rapidly translated into clinical trials in patients with refractory disease (Yamamoto and Curiel, 2010). One of the most studied oncolytic adenovirus in the clinic has been ONYX-015, also known as dl1520 or H101 (Bischoff et al., 1996). The construct lacks a part of the E1B region and is designed to achieve selective replication in cancer cells with mutated p53 (Heise et al., 1997). Significantly, the first commercial product (H101) has been approved in China as a drug for head and neck squamous cell carcinoma (Yu and Fang, 2007).

Further steps in vector design have led to the concept of “armed oncolytic” viruses. In this approach, the oncolytic activity is combined with gene transfer for increased antitumor efficacy. The aim is to amplify gene transfer by replication and spread of the transgene-containing oncolytic virus (Cody and Douglas, 2009). Numerous approaches of arming have been used preclinically (Cody and Douglas, 2009). Approaches using secreted, immunostimulatory factors (Cerullo et al., 2010), and genes encoding prodrug-activating enzymes (Freytag et al., 2003), have also been investigated in cancer
treatment of patients. While preclinical studies have shown promise and clinical trials have already been initiated with oncolytic adenoviruses, the approaches have not been entirely effective, and important issues still remain unsolved. Namely, efficacy improvements still remain a challenge.

3.4. Targeting of adenoviruses

Targeting adenoviruses to malignant cells is a key consideration for the development of effective cancer therapeutics while reducing side effects. Adenoviruses do not pose obvious inherent tumor selectivity and thus regulations in either viral entry (entry targeting) or in gene expression (post-entry targeting), or in both, are required to achieve tumor-selective replication.

Traditionally, two categories have been used to classify vector targeting, transductional and transcriptional targeting. Transductionally targeted adenoviruses contain genetic or physical changes in their cell binding properties to enable their entry into a specific cell type. Transcriptional targeting restricts viral gene expression at a post-cell entry level and is further divided into two categories (Figure 4). Type one adenoviral vectors contain genomic deletions that are transcomplemented in cancer cells. The deletions are based on the similarities between Ad-infected cells entering the S-phase and the rapidly dividing cancer cells. The aim of the deletions is to hinder the viral replication in normal cells. In type two Ads, transcriptional promoters that are found active in certain cancer or tissue types are placed in front of key adenoviral genes to control the transcription of vector DNA.

3.4.1. Transcriptional targeting

3.4.1.1. Targeting via genetic deletions (Type I adenoviruses)

Most approaches with type I adenoviruses are based on deletions in the immediate early genes (E1A and E1B) required for viral replication. The mutant E1-encoded proteins can be functional only in tumor cells where these mutations are compensated by differential
protein expression that is a result from carcinogenesis. The first deletion-based adenovirus was ONYX-015, initially named dl1520. It has two deletions in the gene coding for E1B-55kDa (Bischoff et al., 1996). The deletions are located in a p53-interacting sequence aiming the virus to replicate in cells with a defective p53 status. p53 aberrancy is a common feature in malignomas (Ries et al., 2000) and ONYX-015 showed increased replication selectivity in p53 cancer cells as compared to wild type Ad (Heise et al., 2000). However, the deletion of E1B-55kDa was shown to cripple adenovirus cancer cell killing potency (Dix et al., 2001). Also, it was later demonstrated that cells with normal p53 supported ONYX-015 replication (Goodrum and Ornelles, 1998). The plausible explanation is that Ad proteins other than E1B-55kDa, including E1A, E1B-19kDa and E4orf6, have p53-suppressing functions (Dobner et al., 1996; Hale and Braithwaite, 1999). Also, the limited capacity of ONYX-015 to infect and express early proteins in cancer cells has been proposed to underlie the observations (Steegenga et al., 1999).
Nevertheless, ONYX-015 was the first modified oncolytic adenovirus that was used in humans. Heretofore, more than 20 phase I–III trials have been conducted with several cancer types, also combined with standard treatments. The responses have been variable, but one key finding has been the good tolerability of the therapy (Raki et al., 2009).

Another set of oncolytic Ads targeted by genomic deletions ($\Delta 24$) are dysfunctional in their retinoblastoma protein (pRb) binding properties. pRb is a tumor suppressor protein that normally binds to the cellular transcription factor E2F. Adenoviral E1A constant region 2 (CR2) encodes a binding site to pRb, that functions to release E2F upon E1A binding. The release of E2F leads to cellular S-phase entry and induces the activation of host cell machinery for efficient replication of the viral genome. This step is essential in the viral replication pathway. However, most human malignancies harbour a dysfunctional pRb pathway (Sherr, 1996). This observation was first taken advantage of by Heise and Fueyo and co-workers. Heise et al. constructed $dl922-947$ that produced an E1A unable to bind pRb (Heise et al., 2000). The virus was tumor selective in replication and produced anti-tumor effects in vivo. A very similar construct, Ad5-$\Delta 24$ carries a 24-bp deletion in the CR2 leading to attenuated replication in nonproliferating normal cells (Fueyo et al., 2000). Also, mutants harbouring deletions in both CR1 and CR2 regions of E1A have been constructed, but they have not unequivocally demonstrated increased specificity over single-deletion mutants (Doronin et al., 2000).

### 3.4.1.2. Tumor specific promoters (Type II adenoviruses)

In type II Ads, the expression of therapeutic or essential viral genes is placed under the control of promoters that are specifically expressed in tissue that the replication is aimed at. Many tissue and tumor specific promoters (TSP) that are active in cancer cells, but inactive in the somatic cells that are the origin of cancer, have been identified. The use of a TSP not only directs the gene expression into the cancer cells but also hampers the replication in liver, where Ad particles are rapidly sequestered after systemic administration (Su et al., 2004; Rots et al., 2006).
Tissue specific approaches attempt to target Ad vectors into a specified tissue type that gives rise to the cancer. The first TSP-driven adenovirus was described by Rodriguez et al. who used the PSA promoter for prostate specific expression (Rodriguez et al., 1997). Other examples include α-fetoprotein promoter in hepatic cancer (Ohashi et al., 2001), carcinoembryonic antigen (CEA) promoter in colorectal cancer (Li et al., 2003) and chromogranin A promoter in neuroendocrine cancers (Leja et al., 2007). Whereas tissue-specific promoters are useful for untargeting virus replication from most healthy tissues, they are not able to spare the parental tissue from which the malignancy originates.

Tumor specific promoters, the pan-cancer promoters, take advantage of regulatory elements common in tumor tissue versus normal cells. It is noteworthy that these promoters are not specific to only a certain tumor type, since they can be applied universally for the treatment of many cancers. The E2F promoter has been used in cancer cells where the Rb/p16 pathway is abnormal (Tsukuda et al., 2002; Nokisalmi et al., 2010). Cyclo-oxygenase 2 (Cox-2) over-expression has been associated with many tumor types, and modifications of this promoter for Ad gene expression have been applied (Yamamoto et al., 2003; Pesonen et al., 2010). Also, the survivin promoter has shown promise in treatment of different cancer types (Kamizono et al., 2005).

Though holding a great promise for tumor specific targeting, drawbacks exist. First, deregulation of a promoter can occur by unspecific read-through from enhancers in the Ad terminal regions or from cis-acting viral proteins (Hearing and Shenk, 1983). As an improvement, poly-adenylation signals and insulators have been used (Vassaux et al., 1999). Other improvements include the use of virus-derived promoters instead of cellular ones. Oncogenic viruses have inherent tissue tropism and e.g. the Epstein-Barr virus promoter has been used to target nasopharyngeal carcinoma (Li et al., 2002) while a human papilloma virus-derived promoter was introduced to treat carcinoma of the cervix (Balague et al., 2001). Second, transcriptional targeting cannot prevent the viral entry to healthy tissues, which may lead to toxicity through native immunoresponses that are independent of gene expression.
3.4.1.2.1. Human Telomerase Reverse Transcriptase (hTERT) Promoter

Because human DNA polymerase cannot synthesise the linear chromosomal ends, a nucleic acid repeat is lost at each replication cycle. Chromosomes contain hundreds to thousands of repetitive non-coding TTAGGG sequences at their termini. These structures, called telomeres, function to protect dividing cells from losing genetic material during cell divisions (Dahse et al., 1997). The progressive shortening of telomeres results in genetic instability leading to cellular senescence or apoptosis. Consequently, in most human cells the divisions are limited through telomeric shortening. In cells that are required to maintain their self-renewal capacity, such as stem-cells or germ-line cells, the chromosomal shortening would be fatal. To maintain the telomeres, these cells possess an inherent activity of telomerase, an enzyme that elongates telomeric repeats de novo (Greider and Blackburn, 1985). Telomerase consists of three subunits, dyskerin (DKC1), an RNA template and hTERT, the catalytic subunit that uses the RNA template for DNA synthesis (Cohen et al., 2007). hTERT expression strongly correlates to telomerase activity, while expressions of the other subunits have minor effect on telomerase function (Nakayama et al., 1998).

During the rapid cell divisions, cancer cells face the problem of telomere shortening. Consequently, the majority of human malignancies express telomerase to maintain the rapid DNA replication for growth benefit (Kim et al., 1994; Meyerson et al., 1997). Many studies have postulated a possibility to intervene with telomerase function as a cancer therapeutic and interest in using hTERT promoter to drive viral or therapeutic genes has gained attention. hTERT promoter fragments have been successfully used in the context of genetically modified adenoviruses. Specific E1A expression and replication from the hTERT promoter has been demonstrated in studies comparing either tumor cells to normal cells or telomerase-negative tumor cells to telomerase-positive tumor cells (Huang et al., 2003; Kawashima et al., 2004). Many preclinical models have shown promising responses in vivo (Huang et al., 2003; Wirth et al., 2003; Kawashima et al., 2004; Huang et al., 2008). Importantly, the first construct aimed for commercialisation, Telomelysin, has been evaluated in a clinical trial (Nemunaitis et al., 2010). Telomelysin is an oncolytic Ad where the E1A is controlled by hTERT promoter and E1B by an internal
ribosomal entry site sequence to hinder the transcriptional leakiness from E1A. One partial response and seven stable diseases were observed in the 16 patients treated with Telomelysin suggesting preliminary disease control and the treatment was well-tolerated.

3.4.2. Transductional targeting

The gene expression from oncolytic Ads is mostly determined by the capability of the virotherapeutic agents to enter their target cells (Douglas et al., 2001). CAR expression is often limited on cancer cells (Rauen et al., 2002; Shayakhmetov et al., 2002) resulting in low infectivity with natural viral tropism. Further, as Ads are pathogens to humans, they are effectively cleared from circulation when administered systemically. The systemic delivery of adenoviral vectors results in rapid CAR-independent hepatic uptake by Kuppfer cells (KC) (Wolff et al., 1997; Alemany et al., 2000). The active hepatic clearance is mediated by KC scavenger receptors, the adenoviral fiber shaft and circulatory components such as blood factors, antibodies and parts of the complement (Stone et al., 2007; Smith et al., 2008; Xu et al., 2008).

However, the KC uptake of adenoviral vectors is dose dependent and saturation occurs after high intravenous doses (Tao et al., 2001) leading to efficient transduction of hepatocytes. The adenoviral entry into hepatocytes together with KC activation results in a rapid immune response that has been suggested to be the major determinant of adenovirus-induced toxicity (Lieber et al., 1997; Liu et al., 2000; Raper et al., 2003).

In the development of alternative targeting strategies, researchers have tried to overcome these issues by providing more targeted/liver de-targeted constructs. Many promising genetic modifications of the adenoviral capsid and knob have shown increased tumor transduction while diminishing the viral accumulation into the liver (Figure 5).
3.4.2.1. Adapter-based targeting

Approaches in adapter-based targeting provide a molecular bridge between Ad and the target receptor on cell surface. Bispecific molecules work as cross-linkers to provide novel tropism and ablate CAR binding. Typically, the Fab fragment from a neutralizing antibody against the Ad knob is conjugated with a molecule targeted to a component on a cancer cell, resulting in a so-called bispecific antibody. This approach was pioneered by Douglas et al. who used an anti-knob Fab linker to folate for targeting cancers overexpressing the folate receptor (Douglas et al., 1996). A similar fibroblast growth factor- (FGF) linked bispecific antibody was used to target Ads to FGF receptor-expressing ovarian cancer and Kaposi’s sarcoma (Goldman et al., 1997; Rogers et al., 1997). This resulted in prolonged survival in vivo when applied with HSV-TK encoding Ad (Gu et al., 1999; Printz et al., 2000). More recent studies have targeted Ads to oral surface mucosa by Ly-6D conjugated antibody (van Zeeburg et al., 2010).

Another method implies the use of soluble CAR (sCAR), linked covalently or by recombinant fusion molecules, to ligands of cellular receptors. Cancer cell entry through CD40-sCAR- or epidermal growth factor-sCAR-mediated binding has been reported (Dmitriev et al., 2000). By addition of a fibrin polypeptide to allow trimerisation of the adapter, the stability and infectivity of an sCAR-anti-erbB2 construct was further increased (Kashentseva et al., 2002).

The obvious drawback of using adapter-based targeting is the discontinuous targeting of viral progeny in replication-competent Ads. As linkers are physically conjugated, they are not present during further rounds of replication resulting in loss of targeting specificity. Thus, adapter based strategies are best suited for replication deficient Ads.

3.4.2.2. Genetic modification of Ad fiber

The genetic modification of the Ad fiber is achieved through ligand sequence incorporation to viral genes encoding for the coat proteins. The advantage is the production of stable, homogenous progeny population where all the viral particles possess the same targeting moiety. Fiber modified Ads have expanded tropism since the
CAR-binding ability is retained. Knowledge of the three-dimensional (3D) conformation of the fiber together with mutagenesis studies has provided information of two important domains for knob modification: the carboxyl-terminus (C-terminus) and the HI-loop. These sites allow amino acid modifications without interfering with fiber structure or hampering its trimerisation (Roelvink et al., 1999).

The first genetic modifications were achieved by inserting polypeptides into the C-terminus. Insertion of polyanionic oligolysine residues (pK) to this site enhanced the Ad transduction of multiple cell types (Wickham et al., 1996). The rationale in pK insertion is to bind positively charged heparin sulphate moieties present on most vertebrate cells and highly expressed on most tumor cells. Addition of integrin-binding RGD sites into the C-terminus has also yielded promising results (Wickham et al., 1997).

Another widely used location for transductional targeting has been the knob HI-loop. Initially, Krasnykh et al. demonstrated that the exposed location of the HI-loop and the natural variation of its length between Ad species made the HI-loop an ideal target for binding modifications (Krasnykh et al., 1998). RGD and pK motifs have been inserted to the HI-loop and resulted in increased tropism over wild type Ad (Dmitriev et al., 1998; Cripe et al., 2001) and expanded tissue transduction via systemic administration (Reynolds et al., 1999). Strategies of double or triple targeting have also been evaluated by combining C-terminus-, HI-loop-modifications and CAR ablation (Mizuguchi et al., 2002; Wu et al., 2002).

Alternatively, the whole knob domain can be replaced by a knob from another Ad species. The approach has yielded excellent results in cancer cell transduction. Substitution of the Ad5 fiber or knob protein with Ad7 (Gall et al., 1996), Ad35 (Shayakhmetov et al., 2000), Ad17 (Chillon et al., 1999), Ad11 (Stecher et al., 2001) and Ad19 or Ad37 (Denby et al., 2004) has demonstrated altered vector tropism. Of note, Ad5/3 pseudotype has shown very promising results by entering through the yet unidentified serotype 3 receptor, which seems to be over expressed in relation to CAR on cancer cells (Tuve et al., 2006). Ad5/3 constructs have shown tumor specific transduction and enhanced gene expression of many cancer types, including primary tissue specimens and in vivo xenografts (Kanerva et al., 2002; Kangasniemi et al., 2006; Guse et al., 2007).
3.4.2.3. Liver de-targeted Ads

Ad5 exhibit natural tropism to the liver as shown by rapid clearance from the circulation and liver sequestration after systemic administration in murine models. The uptake occurs mainly by KCs after Ad binding to platelets and blood factors. The vitamin K dependent coagulation factors IX and X and complement protein C4BP have been shown to play a major role in the liver tropism of Ads (Parker et al., 2006). It has also been suggested that binding of Ads to heparan sulphate proteoglycans, by interaction with the KKTK motif in the shaft, may contribute to the liver uptake. Thus, ablation of mediators for liver uptake has been widely studied to overcome Ad liver tropism for improved target tissue transduction and reduced toxicity.

Depletion of KCs has shown to affect the bioavailability of adenovirus (Ranki et al., 2007). However, if the Ad5 tropism is not further modified, KC ablation leads to efficient hepatocyte transduction (Wolff et al., 1997). Moreover, agents used for KC ablation may cause toxicity limiting the implementation of this approach (Ranki et al., 2007). The depletion of coagulation factors, or modification of the fiber regions binding to blood factors, have also been evaluated (Shayakhmetov et al., 2005; Parker et al., 2006). The studies have shown a reduced liver uptake but left unsolved the fate of adenoviruses remaining in circulation. On the contrast, Stone et al. showed that treatment with platelet antibody prior to Ad delivery can reduce virus sequestration into the reticulo-endothelial system of the liver without affecting the delivery to other organs (Stone et
Serotype 5 adenoviruses mutated in the KKTK motif have been ineffective in transducing the liver tissue but also resulted in poor transduction of other tissues including tumors, thus rendering their usability low (Bayo-Puxan et al., 2006; Kritz et al., 2007). Taken together, these data together with studies combining the aforementioned strategies, implicate that the biodistribution of adenovirus is determined by several mechanisms, and liver de-targeting seldom results in enhanced target tissue tropism (Koski et al., 2009).

### 3.4.3. Double-targeted Ad vectors

To optimize tumor specificity, methods combining targeting strategies have arisen. Typically, transcriptional targeting has been combined with transductional targeting. More specific vector targeting may also allow the use of more efficient virotherapeutics by limiting side effects in non-target tissues.

Suzuki et al. were one of the first to demonstrate the improved oncolytic potency of double-targeted adenoviruses. They constructed Ad5-Δ24RGD that incorporates the RGD motif in the fiber and a partial E1A deletion. The virus showed enhanced cytotoxicity of prostate cancer cells in vitro and in vivo over a single-targeted control (Suzuki et al., 2001). Further, the virus was shown to replicate in clinical specimens of ovarian cancer and prolong the survival of mice with intra peritoneal disease (Bauerschmitz et al., 2002). The same E1A deletion was also incorporated to Ad5.pK7-Δ24 with polylysine motifs in the C-terminus, and a therapeutic benefit was seen following both intratumoral and intravenous delivery (Ranki et al., 2007). Also, an E1B-55kD-deleted oncolytic Ad has been modified with the pK residues at the C-terminus of the fiber for glioma treatment (Shinoura et al., 1999).

One promising oncolytic Ad vector has been Ad5/3-Δ24. The chimerism incorporates the Ad3 knob and 24 bp deletion of E1A (Kanerva et al., 2003). It has shown increased cytotoxicity and transduction capacity of many tumor types with enhanced antitumor efficacy in vivo (Kangasniemi et al., 2006; Sarkioja et al., 2006; Guse et al., 2007; Raki et
al., 2008). The aforementioned vectors Ad5/3-Δ24, Ad5.pK7-Δ24 and Ad5-Δ24RGD have been able to kill cancer-initiating cells that have been considered resistant to standard treatments (Eriksson et al., 2007; Jiang et al., 2007).

The approach of double targeting has been taken a level further by including therapeutic and immunostimulatory agents into the Ad genome (Raki et al., 2007; Dias et al., 2010). These armed Ads have shown very promising results and have even been evaluated in patients with refractory disease (Cerullo et al., 2010; Koski et al., 2010; Pesonen et al., 2010).

3.4.4. Monitoring viral replication

To date, a challenge in many virotherapy approaches is monitoring the replication of the virus. The treatments are often aimed for patients with metastatic cancer; thus systemic application would also be needed and therefore evaluation of viral kinetics is critical. *Luciferase*, *LacZ* and *GFP* have been extensively used as transgenes to monitor biodistribution and activity of constructs. The expression can be detected from post mortem samples but also by non-invasive imaging of live animals. However, these techniques are often limited to preclinical evaluation and not amenable to human use.

For human applications, virus replication can be assayed through expression of soluble, biologically inactive marker proteins. As the virus replicates, the marker is produced at the tumor site and can be detected from the blood to assess the level of viral gene expression. Kanerva et al. showed that oncolytic Ad derived CEA could be detected from mice blood samples but failed to demonstrate a correlation between serum CEA levels and tumor size (Kanerva et al., 2005).

Human sodium/iodide symporter (hNIS) has been used in the context of Ad vectors to facilitate the accumulation of radiotracers for monitoring viral kinetics. Normally expressed on thyroid cells, hNIS imports iodine for intracellular concentration. Radioactive iodine, or similar molecules, have been used for decades for nuclear imaging of human malignancies. Based on tomography imaging, the localization of radiotracers is
feasibly detected and high sensitivity allows detection of even minor-scale accumulation. Boland and colleagues pioneered the Ad-driven NIS expression by inserting rat NIS in a first-generation Ad vector. They assessed the functionality and ability of the virus to direct iodine uptake in various tumor types and demonstrated the feasibility to image iodide accumulation in vivo (Boland et al., 2000). In a series of experiments Niu et al. showed a correlation between signal intensity and Ad-hNIS amount and a transient, but well defined transgene expression in cotton rat lungs (Niu et al., 2004; Niu et al., 2005). In their final experiment they showed that a conditionally replicating adenovirus could be visualized and also have a cytotoxic effect (Niu et al., 2006). Barton and collaborators constructed an oncolytic adenovirus encoding prodrug-converting enzymes and hNIS and evaluated the kinetics in dog prostate. They demonstrated the feasibility to image prostates daily and thus monitor dynamically the biodistribution of the vector (Barton et al., 2003). The construct was taken into a phase I clinical trial of clinically localized prostate cancer and in 7/12 patients the gene expression was detectable, though variable (Barton et al., 2008).

Monitoring viral replication and kinetics has gained a lot of attention in recent years. Despite promising preclinical studies, the translation of traceable vectors into the clinic has been low. Apart from a few exceptions, agents used in this regard have been replication defective with a very limited anticancer potential. Thus, there is a need for tumor selective, replication competent Ad vectors that can be tracked for their biodistribution, persistence and therapeutic effect.

4. Adenoviruses and radiation therapy

The rationale of combining adenoviruses with radiation therapy lies in observations that therapeutic effect of Ads alone has been quite limited in clinical trials. However, when combined to traditional treatments, such as chemotherapeutics and radiation, improved tumor responses have been detected (Raki et al., 2009). Most clinical trials using
oncolytic Ads for prostate cancer therapy have combined the experimental gene therapy to radiation. Many preclinical studies have presented data suggesting synergism between adenoviruses and radiation therapy, but mechanisms elucidating the molecular background have remained unclear.

4.1. Radiation therapy

The use of radiation therapy for cancer treatment dates back to the late 1890s. The effects of radiation therapy are mediated through ionizing radiation. Ionizing beams interact with intracellular molecules creating free hydroxyl radicals. These, together with the direct effect of ionizing beams on DNA, create single- and double-stranded DNA breaks. Several mechanisms exist to detect and repair the damages. These include molecular interactions aiming at cell cycle arrest to prevent the transfer of DNA damage to progeny cells, encaging the DNA repair machinery and increasing access sites to damaged DNA and finally the activation of cell-death pathways if the DNA cannot be repaired. A key regulator in the DNA repair machinery is the MRN complex (Mre11, Rad50 and NBS1) that functions by recognizing DSBs (Stracker et al., 2004) and initiating a signalling cascade of down-stream components such as ataxia-teleangiectasia mutated (ATM) and checkpoint kinases for checkpoint induction, cell cycle arrest and attempts to repair DNA (Figure 6). Nevertheless, when potent enough radiation doses are applied, damages cannot be compensated. Thus, the probability of the eradication of the cancer is dose dependent (Valicenti et al., 2000), but the risk and severity of adverse effects is also increased with higher irradiation doses.

Three sources of radiation therapy are mainly used. Delivery with external beams generated by a linear accelerator is referred to as external-beam radiation. In brachytherapy, radioactive sources are directly implanted into the tumor. In systemic radioisotope therapy orally or systemically delivered isotopes are carried to tumor tissue by specific transporters. In the context of prostate cancer, curative radiation therapy is
aimed for localized disease whereas adjuvant and palliative outcomes are expected in metastatic/recurrent disease (D'Amico et al., 2004; Pisansky, 2005).

4.1.1. External beam radiation

Conventional radiation therapy has been based on the delivery of radiation beams in a two-dimensional setting. Radiation beams are aimed at the tumor from multiple directions, but there is no variation of the delivered dose in different parts of the radiation target. As a disadvantage, some high-dose treatments are limited by the radiation toxicity of healthy tissues adjacent to the target tumor. Due to the location of the prostate, severe side effects of critical organs has restricted the delivery of optimal doses using conventional radiation therapy in prostate cancer treatment.

In contrast, 3D conformal radiation therapy has become a standard in the treatment of local and locally advanced prostate cancer (Käypähoito, 2007). The technique uses 3D modelling for dose calculation and typically a 70–80 Gy dose is delivered in fractions. The 3D conformal radiation therapy significantly reduces the acute and late radiation toxicity as compared to conventional therapy (Morris et al., 2005).

Intensity modulated radiation therapy (IMRT) is the most modern technology used in radiation oncology. It specially conforms to cancers surrounding critical tissues. In IMRT, a radiation dose is delivered according to the 3D shape of the tumor by modulating the radiation beam’s intensity. The radiation dose intensity is elevated near the gross tumor volume, while radiation among the neighbouring normal tissue is decreased or avoided completely.

4.1.2. Radionuclide therapy

Localized radiotherapy is achieved either with implantation of radioemitting seeds (brachytherapy) or by systemic delivery of radioactive isotopes. Radiation is delivered
selectively by the appropriate radiopharmaceutical, taking advantage of short-range α− or β-particles. In brachytherapy, iodine-125 ($^{125}$I) or palladium-103 ($^{103}$Pd) seeds are implanted into the prostate in ultrasound guidance. They emit a local radiation effect reducing the dose in adjacent tissues (Pisansky et al., 2008). In this setting, emitters of radiation may be left in the prostate or removed after a desired radiation dose is achieved. Brachytherapy is suitable for localized, low-risk prostate cancer but has no benefit for treating systemic prostate cancer. However, systemic $^{153}$Sm can be used to alleviate metastatic bone pain (Paes and Serafini, 2010).

The sodium/iodide symporter (hNIS) directs the uptake and concentration of iodine into thyroid cells. Its natural expression on the membrane of thyroid cells allows for radioiodide imaging and therapy of thyroid cancer. Thereby, thyroid cancer cells uptake radioiodine, which concentrates into cancer cells and delivers a local radiation effect. Since the discovery of the hNIS gene sequence, the experimental use of hNIS has been extended to treat tumors of non-thyroid origin. The hNIS cDNA can be delivered to tumors by a vector-system leading to the expression of hNIS and subsequent radionuclide uptake into transfected cells. Usually, radionuclides with short half-lives and low gamma-energy are used for imaging purposes ($^{123}$I, $^{125}$I and $^{99m}$Tc), whereas radionuclides with stronger radioactivity ($^{131}$I) are used for therapeutic effects. The hNIS-encoding Ad vectors have mostly been constructed for imaging purposes. However, to achieve a true therapeutic advantage, $^{131}$I has been applied with first-generation Ad vectors (Spitzweg et al., 2001; Gaut et al., 2004; Montiel-Equihua et al., 2008) and once with an oncolytic vector (Peerlinck et al., 2009).

4.2. Interaction between adenoviruses and radiation therapy

Synergism in cancer cell killing between Ads and radiation therapy has been proposed in numerous studies. Initially, it was suggested that the Ad E1A protein sensitizes cancer cells to DNA-damaging agents (Sanchez-Prieto et al., 1996). However, E1A mutated viruses have also shown increased antitumor effects when combined with radiation therapy (Lamfers et al., 2002; Liu et al., 2010). A common denominator of Ad infection
and radiation therapy seems to be the similarity between Ad genomes and dsDNA breaks. Ad infection results in accumulation of viral genomes into the nucleus and these linear dsDNA molecules can be misinterpreted as DSB by host cell (Weitzman et al., 2004). From the point of view of Ad replication, the DSB-initiated cascade, leading to a halt in the cell cycle, is unbeneifical. To prevent this, Ad E1B-55K, E4orf3 and E4orf6 act separately and in concert to degrade the components of MRN (Tauber and Dobner, 2001; Araujo et al., 2005; Schwartz et al., 2008). Moreover, radiation induces molecular changes that have been proposed to facilitate Ad transduction by upregulation of molecules involved in Ad internalization (Qian et al., 2005; Hingorani et al., 2008) and facilitate translation by activating pathways needed for efficient protein production (Hingorani et al., 2008).

The importance of synergy between Ads and irradiation lies in their distinct side effect profile and mechanism of action. When combining these agents, lesser side effects are expected due to the possible use of smaller effective doses while the efficacy is retained. Importantly, it has been suggested that the best efficacy is obtained with high radiation but low viral particle (vp)/cell doses (Lamfers et al., 2002; Hingorani et al., 2008; Liu et al., 2010).

**Figure 6. Relationship between Ad proteins and DSB repair complex.** The DSB is detected by the MRN-complex that activates ATM. Through multiple phosphorylation steps, checkpoint and G1/S arrest are induced. Ad proteins inhibit the MRN-complex.
5. Adenoviral gene therapy trials for prostate cancer

Over 40 gene therapy trials are ongoing for prostate cancer (http://www.clinicaltrials.gov/ct2/results?term=prostate+cancer+and+gene+therapy&rer=Open). For approaches using vectors, adenoviruses have been the most popular choice for delivery and, at present, six adenoviral gene therapy trials are ongoing for prostate cancer (http://www.cancer.gov/search/ResultsClinicalTrials.aspx?protocolsearchid=7964110). Various strategies including immuno-gene therapy, suicide gene therapy and oncolytic viruses as single agents or with other modalities have been evaluated in clinical trials (Freytag et al., 2007). Most of the trials have been conducted in patients with localized or locally recurrent prostate cancer after radiotherapy using intraprostatic instillation. Only one clinical trial has evaluated the systemic delivery (Small et al., 2006). Clinical benefits have been mostly observed with localized disease but the safety has been excellent and basically no dose limiting toxicities have been observed at any clinical stage (Freytag et al., 2007; Schenk et al., 2010).

5.1. Replication deficient adenoviruses in prostate cancer clinical trials

All clinical trials using first-generation Ad vectors have been I/II trials combating locally defined prostate cancer. Most of them have studied adenovirus-mediated prodrug therapy as an adjuvant to standard therapies (Table 3). In trials using Ad therapy as adjuvant for the primary treatment, clinical benefits have been hard to evaluate due to additional therapies, such as radical prostatectomy. In contrast, trials involving locally recurrent or metastatic disease have brought benefit for the participants as measured by serum PSA levels and PSA doubling time, even in long-term setting (Herman et al., 1999; Shalev et al., 2000; Miles et al., 2001; Nasu et al., 2007; Shirakawa et al., 2007). Though
the primary end points of these studies have not been the evaluation of efficacy, preliminary results are provided in Table 3.

The clinical trials have also provided evidence of tumor immunogenicity, an important aspect regarding antitumor effect. Both local and systemic immune responses have been detected and T- and B-cell infiltrations have been observed in post treatment biopsies. Onion et al. performed a detailed characterization of humoral and cellular immunoresponses in patients treated with Ad vector CTL102 and prodrug therapy. All patients demonstrated an immunological response against the vector, but importantly, also anti-tumor immune responses were shown in a minority of the patients.
### Table 3. Clinical trials for prostate cancer with replication deficient adenoviruses.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Implementation/ dose</th>
<th>Combined therapy</th>
<th>Phase</th>
<th>Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADV/HSV-tk</td>
<td>One intraprostatic injection/1x10^8 – 1x10^11 IU</td>
<td>GCV for 14 days</td>
<td>I</td>
<td>A decrease of &gt;50 % in PSA in 3 of 18 patients, lasting 6 weeks to 1 year.</td>
<td>(Herman et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Two or three intraprostatic injections every two weeks/ 5x10^{11} vp</td>
<td>Valganciclovir for 14 days after Ad vector injection</td>
<td>I/II</td>
<td>Good safety, mostly grade 1–2 adverse effects. Efficacy not reported.</td>
<td>(Teh et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>One to three cycles of intraprostatic injections. The study included 18 retreated</td>
<td>GCV or valganciclovir for 14 days after Ad vector</td>
<td>I/II</td>
<td>A mean of 28 % PSA reduction in 78 % of patients. A 3-fold mean prolongation of PSADT. Increases in blood cytotoxic CD8+ lymphocytes in high-dose treated patients. Significant correlation between the number of apoptotic cells and the density of CD8+T cells in tumor.</td>
<td>(Miles et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>patients from Herman et al. 1999 study/ 1x10^8 – 1x10^{11} IU</td>
<td>injection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>One intraprostatic injection/ 2x10^{10} or 2x10^{11} vp</td>
<td>GCV for 14 days after Ad vector injection, radical</td>
<td>I</td>
<td>No PSA responses. Increases in tumor infiltrating lymphocytes, no CPE observed in biopsies.</td>
<td>(van der Linden et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>One intraprostatic injection/ 1-4x10^{10} IU</td>
<td>GCV for 14 days after Ad vector injection, radical</td>
<td>I/II</td>
<td>Increases in tumor infiltrating and systemic CD8+ and macrophages.</td>
<td>(Ayala et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>One or two intraprostatic injection cycles/ 1x10^9 or 1x10^{10} IU</td>
<td>GCV for 14 days after Ad vector injection</td>
<td>I</td>
<td>A decrease in PSA in 63 % of patients, significant prolongation of PSADT. Increases in blood cytotoxic CD8+ lymphocytes.</td>
<td>(Nasu et al., 2007)</td>
</tr>
<tr>
<td>AdIL-12</td>
<td>One intraprostatic injection/1x10⁹ – 1x10¹⁰ pfu</td>
<td>Radical prostatectomy</td>
<td>I</td>
<td>33% PSA decrease at the lowest dose, initial PSA increases at higher doses.</td>
<td>(Trudel et al., 2003)</td>
</tr>
<tr>
<td>Ad-OC—hsv-TK</td>
<td>Two injection cycles into prostate and metastasis/5x10⁹ or 5x10¹¹ vp</td>
<td>Valaciclovir for 14 days after each Ad vector injection</td>
<td>I</td>
<td>No significant declines in PSA in most patients.</td>
<td>(Kubo et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Two injection cycles into prostate and metastasis/2.5x10⁹ or 2.5x10¹⁰ vp</td>
<td>Valganciclovir for 21 days after Ad vector injection</td>
<td>I/II</td>
<td>PSA response maintained for 1 year in 1 patient.</td>
<td>(Shirakawa et al., 2007)</td>
</tr>
<tr>
<td>CTL102</td>
<td>One or two intraprostatic injection cycles/1x10¹⁰ – 1x10¹²</td>
<td>Vector alone or radical prostatectomy with CB1954 and vector or CB1954 and vector</td>
<td>I/II</td>
<td>PSA responses in 84% of patients, some responses lasting over 1 year. PSADT increased in most of the patients.</td>
<td>(Patel et al., 2009)</td>
</tr>
</tbody>
</table>

CPE, cytopathic effect; GCV, ganciclovir; IU, infectious units; OC, osteocalcin; pfu, plaque forming unit; PSADT, PSA doubling time; vp, viral particles
5.2. Oncolytic adenoviruses in prostate cancer clinical trials

The observation of short lasting activity and limited clinical efficacy of replication deficient Ad vectors in clinical trials has shifted the field towards the use of oncolytic Ad vectors (Table 4). DeWeese et al. were the first to assess the safety of a replication competent Ad, CV706, in locally recurrent prostate cancer. CV706 has the E1A under the PSA-promoter for prostate specificity and lacks the E3 region (DeWeese et al., 2001). They demonstrated the safety of the treatment and provided some proof of efficacy. Subsequently, Freytag and colleagues introduced the first oncolytic Ad armed with a fusion suicide gene, Ad5-CD/TK-rep, lacking both E1B and E3 genes (Freytag et al., 2002; Freytag et al., 2003). This construct was evaluated with combined radiation therapy and a set of patients received also hormonal therapy, making the evaluation of viral contribution to the observed clinical benefits difficult. However, a cohort of patients was evaluable in a five-year follow-up where a significant increase in the PSA doubling time (PSADT) was observed (Freytag et al., 2007). The investigators postulate that the long-term benefit is linked to anti-tumor immunity evoked by their oncolytic construct, although immunological endpoints were not included in the trial. Later, Freytag et al. modified the first vector by improving the catalytic activity of the suicide fusion gene and included a region coding for excessive amounts of adenovirus death protein. The new, second-generation vector Ad5-γCD/mutTKSR39rep-ADP treatment was combined with prodrug therapies and IMRT and a set of patients were also on antihormonal therapy (Freytag et al., 2007). Again, the safety was good and surprisingly many participants had tumor-free specimens in the last biopsy.

Systemic delivery of an Ad vector has been applied in only one trial in the treatment of metastatic prostate cancer (Small et al., 2006). The vector, where E1A is placed under the control of the PSA enhancer and rat probasin promoter, was studied in a dose escalation study. The escalation was halted at $6 \times 10^{12}$ vp since two participants in this
cohort experienced asymptomatic grade 1–2 liver toxicity. Encouraging PSA responses were seen in 5 patients though no partial or complete responses were detected.

The latest phase I trial used Ad5-γCD/mutTK<sub>SR39rep</sub>-hNIS, similar to the previous construct, but expressing hNIS in place of ADP (Barton et al., 2008). The study aim was to evaluate quantification of the gene expression volume and examine the kinetics and persistence of reporter gene expression in the prostate. Transgene expression by SPECT imaging was detected in most of higher cohort patients with good safety. Also, a peak in transgene expression was detected 1–2 days post-treatment suggesting viral replication.

To summarize, the data obtained from clinical trials demonstrates safety and suggests clinical efficacy. Long-term follow-up would be required to compare the clinical benefits and survival of patients that have participated in these studies with patients treated with standard therapies, since non-randomized comparisons are subjected to many types of bias. Also, immunological endpoints should be assessed. To fulfil these expectations, the first, randomized, controlled phase III clinical trial has been initiated with Ad5-γCD/mutTK<sub>SR39rep</sub>-ADP. The trial compares the effects of Ad5-γCD/mutTK<sub>SR39rep</sub>-ADP combined to IMRT with IMRT only and will be completed by 2013 (http://www.cancer.gov/search/ViewClinicalTrials.aspx?cdrid=584489&version=HealthProfessional&protocolsearchid=7964110).
<table>
<thead>
<tr>
<th>Vector</th>
<th>Implementation/ dose</th>
<th>Combined therapy</th>
<th>Phase</th>
<th>Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV706</td>
<td>One intraprostatic injection of $1 \times 10^{11} - 1 \times 10^{13}$</td>
<td>-</td>
<td>I</td>
<td>5 PSA reductions of $\geq 50%$</td>
<td>(DeWeese et al., 2001)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep</td>
<td>One intraprostatic injection of $1 \times 10^{10} - 1 \times 10^{12}$ vp.</td>
<td>5-FC + valganciclovir</td>
<td>I</td>
<td>In 10/16 patients a transient decrease of $\geq 25%$ in PSA. In 5-year follow-up, PSADT almost doubled and initiation of androgen therapy was delayed by 2 years.</td>
<td>(Freytag et al., 2002; Freytag et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>One intraprostatic injection of $1 \times 10^{12}$ vp</td>
<td>5-FC + valganciclovir for 13 days post vector treatment + radiation therapy. Androgen deprivation for a small set of patients.</td>
<td>I</td>
<td>In 50% of patients, profound PSA declines were detected.</td>
<td>(Freytag et al., 2003)</td>
</tr>
<tr>
<td>CG7870</td>
<td>One intravenous injection of $1 \times 10^{10} - 6 \times 10^{12}$ vp.</td>
<td>-</td>
<td>I</td>
<td>A PSA decrease of $25-49%$ in 5/23 patients. 70% of patients had evidence of replication.</td>
<td>(Small et al., 2006)</td>
</tr>
<tr>
<td>Ad5-yCD/mut TK</td>
<td>One or two intraprostatic injection cycles/ $1 \times 10^{11}$ or $1 \times 10^{12}$ vp</td>
<td>5-FC + valganciclovir for 13 days post vector treatment + radiation therapy. A set of patients were on a simultaneous hormonal therapy.</td>
<td>I</td>
<td>7/8 evaluable patients were negative for adenocarcinoma at their last biopsy.</td>
<td>(Freytag et al., 2007)</td>
</tr>
<tr>
<td>SR39/ep-ADP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad5-yCD/mut TK</td>
<td>One intraprostatic injection/ $1 \times 10^{11}$ or $1 \times 10^{12}$ vp</td>
<td>5-FC + valganciclovir for three weeks + radiation therapy</td>
<td>I</td>
<td>No gene expression detected by nuclear imaging in patients receiving $1 \times 10^{15}$ vp. 78% of patients positive for gene expression in higher cohort group up to one week. Gene expression not detected outside prostate in any of the participants.</td>
<td>(Barton et al., 2008)</td>
</tr>
</tbody>
</table>

5-FC, 5-fluorocytosine; PSADT, PSA doubling time; vp, viral particles
AIMS OF THE STUDY

1. To evaluate the best transductional targeting strategy for prostate cancer and develop a new, capsid modified oncolytic adenovirus whose replication and persistence can be measured noninvasively by a secreted transgene product (I).

2. To combine oncolytic adenovirus treatment with radiation therapy, evaluate the optimal timing schedule and study the molecular mechanism leading to synergy of combination treatment (II).

3. To evaluate a new oncolytic adenovirus expressing hNIS for concentration and imaging of intratumoral iodide accumulation and for combination of locally targeted radiotherapy and oncolytic virotherapy (III, IV).

4. To evaluate a new oncolytic adenovirus expressing hNIS whose replication is tumor restricted by hTERT promoter and to study its systemic efficacy by SPECT/CT imaging (IV).
MATERIALS AND METHODS

The materials and methods are described in detail in the original publications.

1. Cell lines and fresh tissue samples

Table 1. describes the cell lines used in the studies. The specific culturing conditions are listed in the indicated original studies.

Table 1. Human cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>Embryonic kidney cells transformed with Ad5 E1.</td>
<td>ATCC(^1) or Microbix(^2)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>911</td>
<td>Embryonic retinoblasts transformed with Ad5 E1.</td>
<td>Gift from Dr. van der Eb(^3)</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>A549</td>
<td>Lung adenocarcinoma cells, high CAR expression</td>
<td>ATCC</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Androgen sensitive prostate cancer cells, isolated from lymph node metastasis</td>
<td>ATCC</td>
<td>I</td>
</tr>
<tr>
<td>22Rv1</td>
<td>Androgen insensitive prostate cancer cells</td>
<td>ATCC</td>
<td>III, IV</td>
</tr>
<tr>
<td>DU-145</td>
<td>Androgen insensitive prostate cancer cells, isolated from brain metastasis</td>
<td>ATCC</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>PC-3</td>
<td>Androgen insensitive prostate cancer cells, isolated from bone metastasis</td>
<td>ATCC</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>PC-3MM2</td>
<td>An aggressive subline of PC-3 cells</td>
<td>Gift from Dr. Isaiah J. Fidler(^4)</td>
<td>I, II, III, IV</td>
</tr>
</tbody>
</table>

\(^1\) American type culture collection (Manassas, VA, USA)  
\(^2\) Microbix (Toronto, Canada)  
\(^3\) University of Leiden (the Netherlands)  
\(^4\) MD Anderson Cancer Center (Houston, TX, USA)
Fresh prostate tissue samples were obtained from patients undergoing radical prostatectomy at Helsinki University Central Hospital. All patients signed an informed consent and the protocol was approved by the Surgical Ethics Committee of Helsinki University Central Hospital. Histopathological analyses were done by an experienced pathologist. After mechanically homogenizing the samples, they were cultured at 37°C and 5% CO₂ in Modified Eagles Medium (MEM) containing 20% fetal calf serum.

2. Viral constructs

Table 2 provides information on the adenovirus constructs used in this study. For large-scale production, all replication deficient adenoviruses were propagated in 293 cells and oncolytic vectors in A549 cells. The large-scale virus preps were purified by standard caesium chloride gradient centrifugation and vp/ml concentrations were determined by absorbance at 260 nm of purified virus. TCID₅₀-assay was used to determine the pfu/ml titer. All viruses were tested for the presence of inserted genes and respective capsid modifications, and for the absence of wild type contamination by PCR and sequencing.
Table 2. Adenoviruses used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>E1 region</th>
<th>E3 region</th>
<th>Fiber knob</th>
<th>Ref</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad300WT</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Wild type</td>
<td>ATCC(^1)</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Ad5luc1</td>
<td>Deleted, insertion of CMV driven luciferase transgene</td>
<td>Deleted</td>
<td>Wild type</td>
<td>(Krasnykh et al., 2001)</td>
<td>I</td>
</tr>
<tr>
<td>Ad5/3luc1</td>
<td>Deleted, insertion of CMV driven luciferase transgene</td>
<td>Deleted</td>
<td>Ad3</td>
<td>(Kanerva et al., 2002)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Ad5lucRGD</td>
<td>Deleted, insertion of CMV driven luciferase transgene</td>
<td>Deleted</td>
<td>RGD-4C motif in HI-loop</td>
<td>(Dmitriev et al., 1998)</td>
<td>I</td>
</tr>
<tr>
<td>Ad5/3-Δ24</td>
<td>24 bp deletion in Rb binding CR2 region</td>
<td>Wild type</td>
<td>Ad3</td>
<td>(Kanerva et al., 2003)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Ad5-Δ24RGD</td>
<td>24 bp deletion in Rb binding CR2 region</td>
<td>Wild type</td>
<td>RGD-4C motif in HI-loop</td>
<td>(Suzuki et al., 2001)</td>
<td>I</td>
</tr>
<tr>
<td>Ad5/3Δ24hCG</td>
<td>24 bp deletion in Rb binding CR2 region</td>
<td>hCGβ cDNA replaces gp19K/6.7K</td>
<td>Ad3</td>
<td>I, II</td>
<td>I, II</td>
</tr>
<tr>
<td>Ad5/3-Δ24-hNIS</td>
<td>24 bp deletion in Rb binding CR2 region</td>
<td>hNIS cDNA replaces gp19K/6.7K</td>
<td>Ad3</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Ad5/3-Δ24-Δgp19K</td>
<td>24 bp deletion in Rb binding CR2 region</td>
<td>gp19K/6.7K deleted</td>
<td>Ad3</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Ad5/3-hTERT-hNIS</td>
<td>Wild type, driven by hTERT-promoter</td>
<td>hNIS cDNA replaces gp19K/6.7K</td>
<td>Ad3</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>Ad5/3-hTERT-Δgp19K</td>
<td>Wild type, driven by hTERT-promoter</td>
<td>gp19K/6.7K deleted</td>
<td>Ad3</td>
<td>IV</td>
<td>IV</td>
</tr>
</tbody>
</table>

\(^1\) American Type Culture Collection (Manassas, VA, USA)
2.1. Construction of recombinant adenoviruses (I, III, IV)

Five novel oncolytic adenoviruses were constructed for the studies. All recombinant adenoviruses were created using a previously constructed pTHSN (Kanerva et al., 2005) as shuttle vector. Digestion of pTHSN with SunI/MunI creates a 945 bp deletion of gp19K/6.7K leaving the E3 region otherwise intact (Kanerva et al., 2005).

Ad5/3Δ24hCG (I) was constructed by inserting the 498 bp hCGβ cDNA fragment into SunI/MunI digested pTHSN. pTHSN-hCGβ and SrfI-linearized pAdEasy-1.5/3Δ24 (Kanerva et al., 2005) were electroporated into BJ5183 cells to create pAdEasy-1.5/3Δ24hCG by homologous recombination. The virus genome was released by PacI digestion of pAdEasy-1.5/3Δ24hCG and transfected to 911 cells, followed by standard plaque purification and large-scale virus production.

Ad5/3-Δ24-hNIS (III) was created by inserting a blunted hNIS cDNA fragment (gift from S. Russell, Mayo Clinic) into SunI/MunI digested and blunted pTHSN. The control virus Ad5/3-Δ24-Δgp19K (III) was created by digestion of pTHSN with SunI/MunI, leaving the gp19K/6.7K deletion intact. The resulting pTHSN-hNIS and pTHSN-Δgp19K were co-transfected with pAdEasy-1.5/3 into BJ5183 cells to obtain pAd5/3-ΔE1-hNIS or pAd5/3-ΔE1-Δgp19K, respectively. To insert E1A with 24 bp deletion, the plasmids were further recombined in BJ5183 cells with pShuttleΔ24 to obtain pAd5/3-Δ24-hNIS and pAd5/3-Δ24-Δgp19K, digested with PacI and transfected into 911 cells to create Ad5/3-Δ24-hNIS and Ad5/3-Δ24-Δgp19K.

To create Ad5/3-hTERT-hNIS and Ad5/3-hTERT-Δgp19K (IV), a shuttle vector for E1 was obtained by PCR amplifying the hTERT fragment from pBT255 (gift from Izumi Horikawa, National Cancer Institute). The hTERT fragment was digested with NotI/XhoI and inserted into NotI/XhoI digested pSE1AmpR to obtain phTERT-E1AmpR. The plasmid and pAdEasy-1.5/3-ΔA24 were linearized with Pmel and electroporated into BJ5183 for homologous recombination. The resulting plasmid was then further recombined with pTHSN-hNIS or pTHSN-Δgp19K to obtain phTERT-E1-5/3-hNIS and phTERT-E1-5/3-Δgp19K that were rescued into respective adenoviruses by linearization and transfection into 911 cells.
3. In vitro experiments

3.1. Transduction assays (I)

Cells were infected with luciferase-expressing, replication deficient adenoviruses for 30 min at RT, washed and supplemented with fresh 10 % GM. After 24 h incubation at 37°C, luciferase assay was performed (Luciferase Assay System, Promega, WI, USA). The protein content of cells was determined to normalize the luciferase activity for the amount of total protein.

3.2. Cytotoxicity assays (I, II, III, IV)

Cells were seeded at 10,000 cells/well and next day infected with oncolytic Ads for one or two hours at 37°C. In combination treatment with irradiation (II), a part of the cells was first irradiated and infected 24 h after irradiation. When first signs of complete CPE were observed, the cell viability was measured using MTS-assay (CellTiter 96 Aqueous One Solution cell proliferation assay, Promega).

For crystal violet experiment (I), cells were seeded at 50,000 cells/well, infected with respective oncolytic Ads for one hour, washed, and 5 % GM was added. Three to five days later, cells were fixed with 10 % formalin and stained with 1 % crystal violet.

3.3 hCGβ measurements (I)

Cells were infected with Ad5/3Δ24hCG for one hour at 37°C, washed and fresh 5 % GM was added. For tissue samples, the samples were homogenized to obtain approximately 50,000 cells that were infected with Ad5/3Δ24hCG for one hour at 37°C, washed and fresh 20 % GM was added. Samples of growth media were collected at indicated time points after infection and the cumulative amount of hCGβ was measured as previously described (Alfthan et al., 1992).
3.4. Irradiation experiments (II)

Cells were irradiated through a 1 cm thick plastic phantom with 1 cm of +37°C water on the bottom. Irradiation was performed with a linear accelerator (Clinac 600C/D, Varian Medical Systems, CA, USA) using a 6-MV photon beam and dose rate of ca. 4 Gy/min.

3.5. Microarray analysis (II)

Total RNA was extracted with TRIzol (Invitrogen, CA, USA) from cells irradiated with 4 Gy, and RNA was purified with RNeasy columns (Qiagen, CA, USA). RNA quality was analyzed using a 2100 Bioanalyzer (Agilent Technologies, CA, USA). Gene expression microarray analyses were carried out using one-cycle target labelling and Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, CA, USA). After hybridization, washing and staining, the arrays were scanned by GeneChip 7G Scanner and analyzed using Affymetrix GeneChipOperating (GCOS) 1.4 Software.

Genes with a fold-change at least three were considered as differentially expressed and analyzed for pathways using Signaling Pathway Impact Analysis—method (Draghici et al., 2007).

3.6. Western blots (II)

For all experiments, cells were irradiated with 4 or 10 Gy and a part of the cells were also infected with 100 vp/cell of Ad5/3Δ24hCG 24 h after irradiation. Protein complexes were detected using enhanced chemiluminescence (ECL™, Amersham Life Sciences).

Dynamin 2 and Mre11. Western blot analysis were done from 50–70 μg of protein isolated from lysated cells at baseline and at indicated intervals after irradiation. Primary mouse anti-Dynamin 2 antibody, dilution 1:250 (BD Biosciences Pharmingen, NJ, USA), or rabbit anti-Mre11 antibody, dilution 1:1,000 (Cell Signaling Technology, MA, USA), and secondary horseradish peroxidase-conjugated anti-mouse IgG, dilution 1:50,000
(Amersham Biosciences, NJ, USA), or anti-rabbit IgG, dilution 1:200,000 (Amersham Biosciences) were used to detect the respective proteins.

**Chk2-Thr68 and γH2AX-Ser139.** 25–50 μg of protein was isolated from total cell lysates, including nuclei. γH2AX-Ser139 was detected by a mouse antibody (Upstate/Millipore) using a concentration of 0,5 μg/ml and a horseradish peroxidase-conjugated anti-mouse secondary antibody, dilution 1:4,000 (DakoCytomation, Glostrup, Denmark). Chk2-Thr68 was detected by a rabbit antibody, dilution 1:800 (Cell Signaling Technology), and a biotinylated anti-rabbit antibody, dilution 1:2,000 (DakoCytomation) followed by incubation with a horseradish peroxidase-conjugated streptavidin (DakoCytomation).

### 3.7. Experiments detecting autophagy (II)

For all experiments, cells were irradiated with 10 Gy and 24 h later infected with Ad5/3Δ24hCG for one hour at 37°C.

**Flow-cytometry analysis.** When CPE was observed by microscopy, cells were trypsinized and stained with 1 μg/ml acridine orange (Sigma) for 15 min followed by brief centrifugation. Cells were resuspended in 1 x PBS. FL3-H emission from 20,000 cells was measured with a LSR flow cytometer (BD Biosciences).

**LC-3.** Prior to irradiation and infection, cells were transfected twice with pLC3-eGFP (gift from Ricardo Gargini, University of Madrid). When CPE was observed, cells were fixed with 4 % paraformaldehyde, mounted with Vectashield and imaged using an LSM Meta 510 confocal microscope (Carl Zeiss, Jena, Germany). LC-3 distribution was quantitated from cells containing more than 3 dot-formations and the number of these cells was normalized to untreated GFP-expressing cells and presented as ratio of positive cells to all cells.

**Electron microscopy.** Cells were cultured for 48 h after treatments, collected, fixed and dehydrated in series of ethanol and embedded in LX-112 resin. Cut sections (50–60
nm) were stained with uranyl acetate and lead citrate. Jeol JEM-1400 electron microscope (Jeol, Tokyo, Japan) was used for imaging.

3.8. hNIS RT-PCR (III, IV)

Cells were infected with 10 vp/cell and total RNA was collected 24 and 48 h later using RNeasy Kit (Qiagen). Amplification was done using One Step Reverse Transcription –PCR Kit (Qiagen) with 35 cycles and annealing at 54°C. The 453 bp hNIS product size was obtained using the following hNIS specific primers: forward 5’-CTTCTGAACTCGGTCTCAC-3’ and reverse 5’-TCCAGAATGTATAGCGGCTC-3’. For β-actin, a 482 bp PCR product size was obtained using primers specific for human β-actin (forward 5’-CGAGGCCAGAGCAAGACA-3’ and reverse 5’-CACAGCTTCTCTACATAATGTCACG-3’).

3.9. Iodide uptake (III, IV)

1x10^5 cells were infected with 10 vp/cell for two hours at 37°C. 24 h and 48 h later, cells were washed and (125I)NaI (MAP Medical Technologies Oy, Finland) was added for 20 min at RT. After washing twice, cells were lysed (Cell Culture Lysis Reagent, Promega) and radioactivity was quantified with a well counter connected with a multichannel analyzer Atomlab 950 (Biodex Medical Systems, NY, USA).

4. In vivo experiments

All the animal experiments were approved by the National Committee for Animal Experimentation in Finland (State Provincial Office of Southern Finland). For all experiments (I–IV), 6–8 week old nude/NMRI male mice were used (Taconic, Ejby, Denmark or Harlan, the Netherlands). Medetomidine-ketamine-0.9 % saline (1:2:7) anaesthesia was used for tumor cell inoculations, intra tumoral and intra venous virus delivery and radiographic imaging of mice.
For subcutaneous models (I–III), 5x10⁶ PC-3MM2 cells were inoculated into both flanks of mice and tumor growth was measured every other day using the formula ‘length x width² x 0.5’. The mice were killed when tumor diameter reached 10 mm (I) or 15 mm (II, III). Tumors were treated with intra tumoral injections of adenoviruses while untreated control groups received growth media.

The Intrapulmonary model of castration resistant prostate cancer metastatic to the lung (I, II, IV) was established by injecting 1.5x10⁶ PC-3MM2 cells in 200 μl of MEM into left lungs of anesthetized mice. Mice were put on their right side and injected between mid-axillary and mid-sternal line by conducting the tip of a 27 G needle at 5 mm depth and injecting the cell solution into the lung.

4.1 Antitumor effect of Ad5/3Δ24hCG and plasma hCGβ profile (I)

Subcutaneous tumors were inoculated into mice that were randomized into four treatment groups at treatment commencement, 1) mock, 2) wild type adenovirus, 3) Ad5/3-Δ24, 4) Ad5/3Δ24hCG. Each group contained five mice (tumor n=10). Mice received intratumoral injections of 3x10⁸ viral particles on days 5, 6, and 7 after cell inoculation. Five and ten days after the last virus injection, blood samples were taken from the great saphenous vein and analyzed for hCGβ.

In the survival experiment, intrapulmonary tumors were grown for 5 days and then mice were randomized into groups of 10 mice 1) mock, 2) Ad5/3-Δ24 and 3) Ad5/3Δ24hCG and treated intravenously with 2x10¹⁰ vp on days 5, 6, 10 and 11. Chest radiographs were taken on days 4, 9, 12 and 18, and plasma samples were collected for hCGβ measurement at indicated time points.
4.2. Combination with external beam radiation therapy (II)

In the subcutaneous model, three groups of 8 mice were treated with $2.35 \times 10^{10}$ vp of Ad5/3Luc1, Ad5/3Δ24hCG or GM on days 7 and 8 and $3 \times 10^{8}$ vp on days 10 and 11. In intrapulmonary model, mice (n=11/group) were randomized into groups receiving two cycles of $2 \times 10^{10}$ vp of Ad5/3Δ24hCG or GM intravenously. For both experiments, half of the mice in each group received external beam radiation therapy.

Radiation therapy was given 24 h before the viruses. Mice remained in standard plastic cages and received a whole body 2 Gy radiation therapy delivered with a linear accelerator (Clinac 600C/D, Varian Medical Systems).

4.3. Iodide uptake imaging in s.c. models of prostate cancer (III, IV)

Mice with four subcutaneous tumors were treated twice with $7 \times 10^{8}$ vp of hNIS expressing viruses (2 tumors), control viruses or saline. 24 h after the injections, mice received intravenously 2.0 MBq $^{[123]}$I NaI (MAP Medical Technologies Oy) and were imaged with γ-camera 0.5–13 h after iodide exposure.

4.4. Combination with radioiodide and biodistribution (III, IV)

In subcutaneous model (III), four treatment groups (n=6 mice/group) were established: 1) mock, 2) $^{131}$I only, 3) $7 \times 10^{8}$ vp of Ad5/3-Δ24-hNIS only or 4) $7 \times 10^{8}$ vp of Ad5/3-Δ24-hNIS and $^{131}$I. Tumors were injected on days 6 and 7, followed by intra peritoneal injection of 50 MBq $^{[131]}$I NaI on day 8.

For biodistribution of iodide (III), organs were collected, weighed and radioactivity measured with a γ-counter when mice were killed due to tumor size. Also, the whole body emitted radioactivity was measured from $^{131}$I treated mice.

In a survival experiment (IV) mice were randomized into four groups 1) mock, 2) $^{131}$I alone, 3) Ad5/3-hTERT-hNIS alone or 4) $^{131}$I + Ad5/3-hTERT-hNIS and received either
5x10^{10} vp or GM intravenously and 24 h later were injected with either 36 ± 5 MBq of [^{131}I]NaI or PBS intra peritoneally. The same dose of virus and iodide was repeated once a week for a total of 3 weeks.

24 hours after the first iodide injection (48 h after first virus injection), three mice from Ad5/3-hTERT-hNIS group were killed and their organs and tumors collected and weighed to measure radioiodide concentration for evaluation of iodide biodistribution.

4.5. SPECT/CT imaging (IV)

Mice were treated as in study IV survival experiment. SPECT/CT scans were performed a day after each intravenous Ad5/3-hTERT-hNIS or GM injection using multi-pinhole nanoSPECT/CT system (Bioscan, Washington DC, USA). CT images were obtained prior to SPECT scanning. Mice under isoflurane anaesthesia were injected intravenously 18 ± 2 MBq of [^{123}I]NaI and SPECT scans were run two hours later. All images were reconstructed with MEDISO software (Medical Imaging Systems, Kingsbury, England) and analyzed using In Vivo Scope software (Medical Imaging Systems).

5. Statistics (I–IV)

Cytotoxicity assays, tumor mean size, SDs, and SEs in all studies were calculated using Microsoft Excel.

In combination studies in vitro (II), Chou and Talalay’s median-effect method (Chou, 2010) was used to calculate combination index (CI) values. CI <1 indicates synergism, CI=1 additivity, and CI>1 antagonism. One sample t-test was performed to test statistical difference of mean CI-values. Iodide uptake analysis in vitro used one-way ANOVA with Bonferroni’s post-hoc test (III) or Student’s two-tailed t-test (IV).
Tumor size comparisons in subcutaneous models (I, II, III) were conducted with a repeated-measures linear model with PROC MIXED (SAS Version 9.0/9.1, NC, USA). This method models the changes in tumor size over time and allows a single predicted comparison between groups in all time points. The tumor volumes were log-transformed to normalize the data. Treatment group, time, and the interaction of treatment group and time were evaluated by F tests (I) or Tukey-Kramer model (III) and the differences in mean values with t-tests.

Survival analyses were conducted by plotting Kaplan-Meier curves, and the comparisons between groups were done pairwise by the log-rank procedure with SPSS 11.5 (SPSS Inc., Chicago, IL). For survival experiment in study II, Cox regression models were used to assess differences in survival between the 4 groups with the Ad5/3Δ24hCG + 2 Gy as the reference group.

For all tests, a p value of <0.05 was considered statistically significant.

6. Ethical considerations

**Human tissue samples.** The protocol was approved by the Surgical Ethics Committee of Helsinki University Central Hospital and fulfilled the legal requirements for medical research involving human samples. All samples were processed anonymously, used only for the intended purpose and discarded afterwards. The patients did not experience any harm due to tissue sampling since they were ongoing surgery for medical purposes. All patients signed an informed consent.

**Animal models.** All experimental procedures were approved by the National Committee for Animal Experimentation in Finland (State Provincial Office of Southern Finland). The well-being of mice was checked daily. Food and water were available ad libitum. Each cage was supplemented with disposable paper pulp housing and aspen shavings for nesting. If any signs of discomfort or impending death (weight loss > 20 %, immobility, recession) emerged, mice were sacrificed immediately. Subcutaneous tumors were allowed to reach a maximum of 10 mm (I) or 15 mm (II, III) before mice were sacrificed. Also, if the tumors became necrotic, the mice were sacrificed.
RESULTS AND DISCUSSION

1. Improving prostate cancer cell transduction and oncolysis by serotype 3 targeted adenovirus (I)

The ability of unmodified adenoviruses to enter target cells is highly dependent on the presence of CAR receptor and the gene expression from Ad vectors is dictated by this entering capacity. Lack or down-regulation of CAR has been reported for prostate cancer and the loss of CAR expression implicates emergence of metastatic disease (Okegawa et al., 2000). For prostate cancer treatment, these associations are problematic and need to be overcome to achieve efficient tumor transduction and subsequent therapeutic effect.

1.1. Serotype 3 targeted adenovirus improves transduction and oncolysis of prostate cancer cells (I)

The first aim was to study how capsid modifications affect the gene transfer of reporter expressing, non-replicating adenoviruses. The gene transfer was analyzed in one hormone sensitive (LNCaP) and three castration resistant (PC-3, DU-145 and PC-3MM2) prostate cancer cell lines. Two different knob modifications were compared to the wild type. Ad5lucRGD binds to the cell surface integrins that are important in adenoviral internalization after initial binding to CAR. Ad5/3luc1 harbours the serotype 3 knob and uses a CAR-independent entry pathway. In all cell lines, the modified viruses exhibited increased infectivity as compared to Ad5 wild type. Ad5lucRGD showed only modest increase in DU-145 and LNCaP cells, while in PC-3MM2 cells it was superior to other viruses at lower concentrations. However, Ad5/3luc1 mediated luciferase activity was remarkably higher than Ad5lucRGD and resulted in up to 83- and 16-fold increases in PC-3 and PC-3MM2 cell lines (Figure 1, panels A–D in I). The switch from Ad5 knob to Ad3 knob has been reported to increase the infectivity about 100-fold in ovarian and renal cell cancer (Kanerva et al., 2002; Guse et al., 2007) while being ineffective in gastric
carcinoma (Kangasniemi et al., 2006). Thus, the infectivity enhancement was not as potent as in ovarian and renal cancer, but proved to be well beyond that of Ad5. From our results it could be estimated that about 50 times smaller doses of 5/3-chimera could be used to obtain similar gene transfer rates than with an unmodified vector.

Correlation of infectivity enhancement to oncolytic potential was tested with cytotoxicity assays. Oncolytic viruses harbouring the respective capsid modifications and a 24 bp deletion in E1A for selective replication in cells deficient of the pRb/p16-pathway showed improved cell killing as compared to wild type Ad5. Of the tested modified viruses, Ad5/3-Δ24 was the most oncolytic (Figure 1, panels E–H in I). We believe that the capsid modification improved the efficacy of these viruses since dl922-947 (similar to Ad5/3-Δ24 but without knob modification) has shown poor cell killing effects in vitro in a previous study evaluating E1A/E1B-deleted Ads against prostate cancer (Satoh et al., 2007).

When translated into clinical use, it would be advantageous to monitor viral replication noninvasively. Secreted markers encoded from viral transgenes could be used to adjust input virus doses by measuring transgene concentration in blood or urine samples. Detection of markers whose expression would be linked to viral replication would help in evaluating the virus persistence in vivo. Therefore, Ad5/3Δ24hCG was constructed (Figure 2 in I). Ad5/3Δ24hCG is based on Ad5/3-Δ24 (Kanerva et al., 2003) and its novelty lies in its transgene. It carries the human chorionic gonadotropin β-chain (hCGβ) in the deleted gp19K/6.7K under the native E3-promoter and thus the secretion of hCGβ is coupled to viral replication. The gp19K/6.7K region was chosen for its previously described ability to support transgene expression and for the dispensable nature of the gp19K/6.7K product. The replication dependent secretion was previously shown by Kanerva et al. who used a similar construct coding for secretable hCEA (Kanerva et al., 2005).

The oncolytic potency of Ad5/3Δ24hCG was tested in prostate cancer cells and more effective (PC-3) or similar (DU-145, LNCaP) cell killing was detected in comparison to Ad5 wild type (Figure 2B in I). With PC-3MM2 cells, Ad5/3Δ24hCG was less effective than wild type at some doses. From an evolutionary point-of-view, we believe that wild type Ads
are by nature optimized for efficient replication and cell lysis. Thus, these results were encouraging since improved cell killing efficacy over wild type Ad was obtained with tumor-targeted Ads.

1.2. Therapeutic efficacy of Ad5/3Δ24hCG in in vivo models of prostate cancer (I)

The antitumor efficacy of Ad5/3Δ24hCG was evaluated in two xenograft models of human prostate cancer. First, the ability to retard the growth of subcutaneous PC-3MM2 tumors was tested. PC-3MM2 cells were chosen for their aggressive nature and rapid growth that mimics metastatic, castration resistant prostate cancer. No previous studies have evaluated this cell line in the context of adenoviruses in vitro or in vivo. Ad5/3Δ24hCG treatment resulted in statistically significant tumor growth inhibition (p < 0.05) when compared with the untreated group. In comparison to other oncolytic adenoviruses, the antitumor efficacy was similar. However, the advantage of using Ad5/3Δ24hCG in place of other oncolytic viruses lies in its capability to secrete hCGβ.

Most previous studies evaluating oncolytic Ads for prostate cancer treatment have used subcutaneous models of androgen sensitive, PSA-secreting LNCaP cell line (Rodriguez et al., 1997; Dilley et al., 2005; Cheng et al., 2006). Although useful for direct tumor treatment and response evaluation by PSA-measurements, these models rarely present the clinical cases of advanced prostate cancer where the tumor is androgen-insensitive and aggressive, metastatic and sometimes with multiple locations. Therefore, a model of intrapulmonary PC-3MM2 tumors representing metastatic disease was evaluated for the first time. Mice bearing intrapulmonary tumors received two cycles of intravenous Ad5/3Δ24hCG and the treatment responses could be detected with chest radiographs (Figure 5 in I). No toxicity was seen after virus delivery.

Ad5/3Δ24hCG treatment led to statistically significant survival when compared with the untreated mice (p = 0.002). Ad5/3Δ24hCG and the positive control Ad5/3-Δ24 were equally effective in prolonging the survival (Figure 6K in I) as could be predicted from the in vitro results. Importantly, despite the aggressive nature of the model, survival enhancement was clearly suggesting the effectiveness of Ad5/3Δ24hCG in treatment of
castration resistant, metastatic prostate cancer. However, all mice died eventually due to tumor burden. This cell line or this model had not been used previously, so the data obtained from these studies is unique and comparisons to previous results in the treatment of prostate cancer with oncolytic Ads are not unequivocal. However, since none of the mice survived, the therapeutic efficacy of Ad5/3Δ24hCG should be improved when treatment of refractory prostate cancer is considered. This could be achieved by combining Ad5/3Δ24hCG with conventional therapies such as chemo- and radiation therapy for synergistic antitumor effect. Two previous studies have shown that oncolytic viruses with radiation therapy increase the potency of the treatment (Lamfers et al., 2002; Dilley et al., 2005) and similar data has been obtained when oncolytic Ads have been combined to chemotherapy (Raki et al., 2008). It also seems likely that the administration needs optimizing of both the schedule and the dosing to achieve improved antitumoral effect.

1.3. Correlation of hCGβ production and oncolysis in vitro and in vivo (I)

Another aim was to monitor viral replication by measuring hCGβ concentration from growth media of infected prostate cancer cells and simultaneously monitor the oncolytic and replicative effect. No base-line secretion of hCGβ was detected from the uninfected prostate cancer cells. In contrast, increasing hCGβ concentrations were measured from infected cells. Most well-defined increases were seen with 10–1,000 vp/cell consistent with the oncolytic effect that was measured by crystal violet staining (Figure 3 in I). The secreted hCGβ concentrations were high and correlated also with the amount of newly produced virions (Figure 4 A in I).

The hCGβ production in vitro from tissue samples of normal and cancerous prostatic tissue was analyzed. Infection of fresh prostate cancer tissue resulted in increased accumulation of hCGβ over time while normal prostate sample produced very low amounts of hCGβ and only at later time points (Figure 4, panels D–E in I). This highlights the tumor selectivity of the virus in these important clinical samples. The low hCGβ production from normal prostate tissue at 72 hours may reflect the adaptation to in vitro
conditions that require the upregulation of growth signalling pathways and thus have low permissivity for Ad replication. Overall, it could be concluded that hCGβ secretion correlates with cytopathic effect and viral replication *in vitro*.

The feasibility of measuring hCGβ from mice sera samples was tested in both animal models simultaneously with tumor growth and survival evaluations. In the subcutaneous model, blood samples obtained five days post virus treatment showed high concentrations while samples on day ten contained lesser amounts of hCGβ suggesting early initial replication of the virus. The hCGβ increase was due to transgene expression since mice treated with other viruses failed to show any increase in blood concentrations. However, smaller concentrations of hCGβ on day ten suggest that the virus was no longer replicating efficiently. PC-3MM2 cells form very tight tumors that are scarcely vascularised and this might restrict the intratumoral spread of the input virus. Alternatively, the rapid growth pattern of these tumors may involve murine-derived stromal components that do not allow human Ad replication (Blair et al., 1989).

The systemic delivery of Ad5/3Δ24hCG resulted in three distinct patterns of hCGβ expression (Figure 6, panels A–J in I). Most of the mice demonstrated a two-peak response consistent with the viral dosing. Some mice had only an initial response while two mice did not seem to respond. The secretion pattern or amount of hCGβ did not correlate with survival. The most likely explanation for missing second peak and low overall values in some mice is the poor vasculature and intratumoral barriers prohibiting viral entry and intratumoral dissemination. Since human adenoviruses do not replicate productively in murine tissues the virus is presumably cleared through the reticuloendothelial system after initial systemic exposure (Alemany et al., 2000). Thus, although hCGβ could be detected from blood samples, the outcome of mice could not be predicted from the amount or pattern of hCGβ secretion.

Ad5/3Δ24hCG is based on a similar construct Ad5/3-Δ24-hCEA (Kanerva et al., 2005). The comparison of *in vivo* marker secretion -results shows similar expression patterns. However, with Ad5/3-Δ24-hCEA it was possible to follow the animals longer and many of them remained alive at the end of the experiment, but this might reflect the discrepancy between the tumor models more than the difference between the viruses. The
advantage of using hCGβ over hCEA is that both intact hCG and hCGβ are normally secreted by placental trophoblasts during early pregnancy and are consequently undetectable in men. Also, many carcinomas have been reported to secrete or express CEA and this feature could potentially obscure the separation between tumor-secreted and Ad-derived CEA. Free hCGβ has no known biological function (Stenman et al., 2004) and thus could be safely used. Finally, an advantage lies in the size of the transgene, since hCGβ is about 500 bp whereas hCEA is three times larger. Bulky transgenes have been shown to slow down the replication of transgenic Ads (Bett et al., 1993).

2. Combining oncolytic adenoviruses and external beam radiation therapy

Radiation therapy is a widely used approach in treating prostate cancer. However, it is not applicable in metastatic disease since various body parts need to be exposed to radiation. Consequently, the use of radiation therapy in metastatic prostate cancer is limited to palliative care. This implicates that metastasis are not insensitive to radiation.

At the cellular level, ionizing radiation reacts with water molecules creating reactive oxygen species that disturb DNA structure by creating single- and double-strand breaks. The DSBs are considered to be the major mediators of radiation induced cytotoxicity. The DNA damages induce a multistep signalling cascade that leads to cell cycle arrest and to attempts to repair the defects. The capacity of tumor cells to repair genetic material might be limited and accumulating damage leads to attenuated cellular division and ultimately to cell death. Further, even when functional and corrective mechanisms exist, the higher radiation doses increase the probability of the eradication of the cancer (Valicenti et al., 2000). However, the risks and severity of adverse effects are also increased with higher doses. Previous studies have suggested a synergistic effect between adenoviruses and radiation therapy (Lamfers et al., 2002; Dilley et al., 2005). The synergism could allow the use of smaller radiation doses for enhanced antitumor effect while reducing side effects to normal tissues.
2.1. Oncolytic adenovirus 24 h after radiation therapy results in synergistic cell killing

Although synergistic interactions between Ads and radiation therapy have been reported, the timing of combination had not been systematically evaluated before. As with some chemotherapeutic agents and Ads, scheduling of the treatment may be important since the treatment may result in antagonistic effect instead of synergism when the order of administration is changed.

To determine the interactions between Ad5/3Δ24hCG and radiation, prostate cancer cells were infected with variable doses of the virus or irradiation or both. The cytotoxic agents were dosed at different time points and the cell viability was evaluated by MTS tests. Synergism was assessed using Chou and Talalay’s median effect that creates combination indexes (CI). A CI value < 1 indicates synergy, a CI equal or close to 1 indicates additive effect and a CI > 1 stands for antagonism. The tested cell lines PC-3MM2 and DU-145 are relatively radiation resistant. However, a synergistic effect was detected in both cell lines when virus was given 24 h after irradiation, the CI-index being 0.525 for PC-3MM2 cells and 0.664 for DU-145 (Figure 1 a and b, in II). When the virus was administered 24 h before, an antagonistic effect was observed in PC-3MM2 cells but the synergism was retained in DU-145. The synergistic effects were more pronounced at low viral concentrations. In DU-145 cells, radiation only at 15 Gy resulted in 74 % cell survival, while 1 vp/cell of only Ad5/3Δ24hCG led to survival in 88 % of the cells. When combined, the cell survival was 42 %. In PC-3MM2 cells, the respective survival rates were 31 %, 67 % and in combination therapy 6.4 %. These results suggest even more pronounced efficacy than those reported by Lamfers et al. (Lamfers et al., 2002) and the observation could be explained by better transduction efficacy due to 5/3 modification, albeit higher radiation doses were used in our study.

To verify that the synergism would not be confined to Ad5/3Δ24hCG only, other oncolytic viruses were tested with radiation therapy. At all irradiation and viral doses, oncolytic Ads showed significantly (p < 0.005) enhanced cell killing when compared to radiation with replication deficient virus Ad5/3luc1 when oncolytic Ads were administered 24 h after irradiation (Figure 1, c–f in II). When a smaller radiation dose (4
Gy) was used, transgene-void Ad5/3-Δ24 and wild-type adenovirus were more efficient in cancer cell killing than Ad5/3Δ24hCG. However, when a more potent radiation dose was used (15 Gy) the differences were minimal. This reflect two facts: Ad5/3Δ24hCG might be slower in replication (as seen in Figure 2B in I) and when radiation doses increase, the effect of oncolysis has a lesser contribution than radiation to the survival.

Hingorani et al. have reported the upregulation of Ad-derived transgene expression in cells infected with replication-deficient Ads and irradiated at various time-points after infection (Hingorani et al., 2008). They found most profound enhancement of transgene expression 24 h after irradiation. Although they used a replication-deficient Ad, our results indirectly support their findings.

2.2. Combination of Ad5/3Δ24hCG and radiation therapy in vivo (II)

Tumor growth inhibition and survival in mice were evaluated to test whether the synergistic interaction of Ad5/3Δ24hCG and radiation therapy functions also in vivo. In the subcutaneous model, three treatment groups were established: mock, Ad5/3Luc1 and Ad5/3Δ24hCG. Within each treatment group half of the mice received 2 Gy irradiation before intratumoral virus injections. The tumor growth was slowest in the group receiving combination therapy and by day 11 tumor size was significantly (p < 0.05) smaller in this group versus virus alone (Figure 2a in II). From this point on, the tumor growth was still hindered in the combination group but could not be compared to the control group from which many mice were killed due to tumor size (data not shown).

The effect of combination treatment on mice survival was tested in an orthotopic model of aggressive prostate cancer metastatic to the lung. Treatment with 2 Gy radiation therapy or Ad5/3Δ24hCG alone resulted in mean survivals of 25.9 and 20.2 days, respectively. When combination treatment was applied, the mean survival increased to 30.2 days which was significantly longer than survival in the virus only-treated group, p<0.02 (Figure 2b in II). Even though synergistic interactions were not measured using the CI-indexes, these results suggest that treatment combinations are more beneficial than either treatment alone.
As in study I, similar patterns of hCGβ production were measured. Only the initial hCGβ peak was detected and the amount of secreted hCGβ did not correlate with survival. It has been previously shown that radiation increases transgene expression, but the studies have been mostly performed with replication-deficient constructs where transgene expression originates from E1 (Qian et al., 2005; Hingorani et al., 2008). Moreover, these studies have concentrated on the immediate effects of radiation and no long-term expression-enhancement has been studied. In this regard it was interesting that at later time points in our study (days 17 and 21) the combination treated mice had higher hCGβ levels than mice in the virus-only group (data not shown).

2.3. Molecular background for synergistic interactions

Synergistic interactions between replication deficient adenoviruses and radiation therapy have been published before (Hingorani et al., 2008; Hingorani et al., 2008). In this context, radiation induced dsDNA breaks and subsequent signalling through the mitogen activated protein kinase (MAPK/ERK) pathway have been shown to enhance the transgene expression. Also, upregulation of Dynamin 2, an important mediator of Ad internalization, has been reported following radiation (Qian et al., 2005). In the context of glioma, many groups have shown increased apoptosis when Ads and radiation therapy have been combined (Idema et al., 2007; Yacoub et al., 2008). However, putative molecular interactions between oncolytic Ads and radiation therapy leading to synergy have remained unclear.

As increased transgene expression post-radiation has been reported with replication deficient Ads, we assessed whether the gene expression of oncolytic Ads would lead to enhanced replication potency after radiation therapy. Treatment with 4 Gy irradiation did not affect the viral yield from Ad5/3Δ24hCG infected prostate cancer cells (Figure 2d in II). This suggested that the enhanced efficacy might be due to adenovirus potentiating the effects of radiation because it seemed that replication was not enhanced. Induction of apoptosis was assessed by measuring the cleavage rate of caspase-3. Ad5/3Δ24hCG or irradiation alone or in combination did not inflict significant differences in apoptosis
(Figure 3a in II). Also, the levels of Dynamin 2 were assessed by Western blots of cells treated with irradiation. The results did not suggest increase in virus uptake (Figure 3b in II), because no Dynamin 2 increase after irradiation was observed. This was a surprising finding, since Qian et al. (Qian et al., 2005) quite clearly demonstrated an increase in Dynamin 2 levels after irradiation, which, in their experiments with replication-deficient Ad, led to higher transgene expression. The differential results might be explained by the differences between cancer types (colon vs. prostate) and different radiation doses (8 Gy vs. 4 Gy). Taken together, we concluded that upregulation of Dynamin 2, apoptosis or increased viral replication do not explain the synergy.

Gene profiling of irradiated cells was performed to reveal significant genetic changes or affected cellular pathways that would facilitate Ad-induced cytotoxicity (Supporting information table 1 in II). In microarray analysis, some individual genes regulating DNA synthesis, metabolism and replication were downregulated. This was an interesting finding, since supposedly this would negatively affect Ad replication. The individual genes were mapped into pathways to generate a more comprehensive understanding of genetic changes. In both cell lines, pathways involved in processing and initiating defensive responses to pathogens were disturbed (Supporting information table 2 in II), This might be important in in vivo interactions. However, microarray analysis did not reveal any significant explanations for synergy in vitro.

The DNA double-strand breaks induced by radiation are recognized by the cellular DNA repair machinery. The MRN complex (Mre11, Rad50 and NBS1) is critical in recognizing these breaks and initiating responses leading to activation of repair processes (Stracker et al., 2004). ATM is a downstream target protein for MRN and further regulates the phosphorylation of proteins in the cascade leading to cell cycle arrest and DNA repair (Carson et al., 2003). Ad proteins have been shown to inhibit the cellular double-strand break repair machinery (Stracker et al., 2002; Araujo et al., 2005) by binding to proteins of the MRN complex leading to their degradation (Tauber and Dobner, 2001; Araujo et al., 2005). Particularly, Mre11 is a key target of Ad proteins E4orf3 and E4orf6 (Stracker et al., 2002).
Therefore, the levels of Mre11 were assessed in PC-3MM2 and DU-145 cells and a clear decrease was found in combination treated cells (Figure 3c in II). Abrogation of ATM activity was confirmed by Western blots of the phosphorylated form of Checkpoint kinase 2 (Chk2-Thr68), a downstream target of ATM. Chk2-Thr68 levels were reduced in combination treated cells versus radiated cells (Figure 3d in II). Ultimately, we assessed the levels of phosphorylated histone H2AX (γH2AX) that is a marker of DNA injury and DSB. Albeit being a downstream target of Chk2, γH2AX can be phosphorylated by ATR in ATM-compromised cells (Nichols et al., 2009). Elevated γH2AX was detected in combination-treated cells suggesting increased detection of abnormal DNA, including double-strand breaks and viral genomes (Figure 3e in II).

Taken together, these data indicate that the dsDNA genomes resulting from Ad replication are recognized by the DNA signalling pathway, which results in induction of the double strand break repair response. However, Ad proteins counteract this mechanism to maximize productive Ad replication, so that their own genomes would not be misinterpreted as DSB. In cells that have sustained radiation injury, these Ad proteins inhibit double strand break repair and thus DNA damage accumulates.

These results are supported by a publication from Hingorani et al. that came out in the vicinity of our publication (Hingorani et al., 2008). They elegantly demonstrated that by using DSB inhibitors – acting similarly to Ad inhibitory proteins – with radiation therapy, they could increase the transgene expression from replication-deficient Ads. This finding warrants future studies of oncolytic Ads and irradiation, combined to DSB-repair inhibitors, for enhancing the cell killing effect even further.

2.4. Induction of autophagy by oncolytic adenovirus (II)

Increased cell death in combination-treated cells could not be explained by apoptosis. Further, the mechanisms of cytopathic effect (CPE) are poorly defined. We hypothesized that cellular death could be explained by autophagy in apoptosis-resistant cells. Autophagy is an evolutionarily conserved intracellular lysosomal pathway that is
responsible for degradation of long-lived proteins and cell organelles (Blommaart et al., 1997). Not only it is induced by cellular starvation, but also viral infection or radiation can result in autophagy (Paglin et al., 2001).

Microtubule-associated protein 1 light chain (LC3) plays a key role in the formation of autophagosomes and is the only protein that is reliably associated with completed autophagosomes. A typical finding for autophagy is a punctuate distribution of LC3 (Klionsky et al., 2008). Autophagy was assessed by transfecting cells with a plasmid expressing an eGFP-LC3 fusion protein. Then, cells were irradiated and 24 h later infected with 100 vp/cell of Ad5/3Δ24hCG. At the appearance of CPE, 48 h after infection, LC3 showed a punctuate pattern in the combination-treated cells. A lower degree of autophagy was seen in cells treated with virus or radiation only (Figure 4a in II). Quantitation of punctuate distribution did not reveal statistical significance, though a greater number of cells presenting the punctuate pattern of autophagy were detected in combination-treated cells.

Autophagy is associated with the cytoplasmic accumulation of acidic vesicular organelles that can be detected with an acridine orange (AO) staining (Klionsky et al., 2008). In FACS analysis of the stained cells, 0.43 % and 0.03 % of mock and virus infected PC-3MM2 cells were found positive for AO, respectively. Irradiated cells showed 1.83 % positivity, while the combination treatment increased the positive population to 6.3 % of the total, which is a 15-fold increase compared to mock treatment (Figure 4c in II).

Finally, electron microscopy confirmed the deposition of autophagic vacuoles. Albeit radiation therapy or Ad5/3Δ24hCG alone resulted in the formation of autophagocytic vesicles, the number was greater in combination treated cells (Figure 5 in II).

3. Tropism-modified oncolytic adenoviruses expressing hNIS (III, IV)

The systemic nature of disseminated prostate cancer would recall for irradiation of wide body areas if the synergistic effects of oncolytic adenoviruses and radiation therapy would be applied in a conventional setting. This approach is not applicable due to poor
benefit–risk ratio rising from irradiation of normal tissues. However, local, targeted radiotherapy can be used through intrinsic transporters capable of transporting systemically administered radionuclides. A widely used approach utilizes human sodium/iodide symporter (hNIS), a transmembrane protein that normally localizes in the basolateral membranes of thyroid cells and actively transports iodine (I\(^\text{-}\)) into the cells for thyroid hormone synthesis. The ability to accumulate I\(^{\text{-}}\) via hNIS has for decades provided the basis for diagnostic scintigraphic imaging and radioiodide therapy of thyroid carcinoma, where radioactive iodide molecules are used to image or internally radiate cancer cells of thyroid origin.

In the context of modified adenoviruses, hNIS has been used as a transgene to monitor viral spread and persistence (Merron et al., 2007; Barton et al., 2008). Although hNIS has already been used for clinical and preclinical imaging, no previous studies have assessed the utility of radioiodide for enhancing the therapeutic efficacy of oncolytic adenoviruses. In studies III and IV, hNIS was inserted in the E3 region of tumor-targeted adenoviruses for imaging viral localization, monitoring viral persistence and allowing for locoregional radiation therapy.

Four new oncolytic adenoviruses containing the Ad5 fiber shaft and the Ad3 knob were constructed. Ad5/3-Δ24-hNIS and Ad5/3-hTERT-hNIS have the hNIS cDNA replacing the deleted E3 gp19K/6.7K. Their control viruses Ad5/3-Δ24-Δgp19K and Ad5/3-hTERT-Δgp19K have no insertions in the deleted region. In Ad5/3-Δ24-hNIS and Ad5/3-Δ24-Δgp19K, a 24-bp deletion in CR2 of the adenoviral E1A gene confers replication selectivity to Rb-p16 pathway mutant cells. In Ad5/3-hTERT-hNIS and Ad5/3-hTERT-Δgp19K, the E1A is under the control of hTERT promoter. hTERT is expressed in rapidly replicating cells for de novo synthesis of telomeres and its activity has been confirmed in many tumors in contrast to normal tissues where it is very seldom activated (Kim et al., 1994; Meyerson et al., 1997).
3.1. hNIS expression and functionality in vitro (III, IV)

To ensure the transgene expression in infected cells, castration resistant prostate cancer cell lines were infected with the four new viruses. The mRNA of infected cells was then isolated and subjected to RT-PCR to assess the mRNA levels of the transgene. Both at 24 h and 48 h post infection, Ad5/3-Δ24-hNIS and Ad5/3-hTERT-hNIS infected cells featured the hNIS amplification product while cells infected with control viruses remained negative for hNIS (Figures 2 in III and 1A in IV).

Since the transgenic mRNA expression does not confer to the functionality of hNIS, the ability of infected cells to transport and concentrate iodine was evaluated. 24 h and 48 h post infection the cells were exposed to $^{125}$I and analyzed for radioactivity. In all cell lines infected with Ad5/3-Δ24-hNIS and Ad5/3-hTERT-hNIS, iodine uptake was significantly higher than in mock treated cells. The highest increases were seen in 22Rv1 and PC-3MM2 cells with both viruses. In PC-3 and DU-145 cells, a more modest but still significantly enhanced accumulation was seen in comparison to mock-treated cells (Figures 3 in III and 1B in IV).

3.2. Oncolytic efficacy of hNIS expressing adenoviruses (III, IV)

The replication potency and oncolysis of the new viruses were assessed with MTS-assays. The hNIS expressing vectors were compared to oncolytic control vectors Ad5/3-Δ24-Δgp19K and Ad5/3-Δ24 or Ad5/3-hTERT-Δgp19K and Ad5 wild type. Ad5/3Luc1 served as a replication deficient control whereas Ad5/3-Δ24 is an isogenic control to Ad5/3-Δ24-hNIS with an intact $E3$. Ad5/3-Δ24-hNIS and Ad5/3-hTERT-hNIS showed efficient cell killing in all prostate cancer cell lines. For PC-3MM2 cells, Ad5/3-Δ24-hNIS at 1 vp/cell and Ad5/3-hTERT-hNIS at 10 vp/cell were sufficient to cause almost complete cytolysis (Figure 4 in III and Figure 2 in IV). Importantly, Ad5/3-hTERT-hNIS was significantly more oncolytic than wild type Ad5 in PC-3MM2, PC-3 and DU-145 cell lines (Figure 2 in IV).

In most of the cell lines, the control viruses, Ad5/3-Δ24-Δgp19K and Ad5/3-hTERT-Δgp19K, were more efficient in prostate cancer cell killing than the similar hNIS-
containing constructs. This may be explained by the larger genome size of transgene expressing viruses which has been reported to affect the oncolytic speed (Bett et al., 1993), also in a very similar hNIS-expressing construct by Merron et al. (Merron et al., 2007). The hNIS transgene is about 2.2 kbp in size but with the 1 kbp gp19K deletion, the genome size remains under the critical 105 % (Bett et al., 1993). On the other hand, slower replication and oncolysis can also be a benefit in the context of these constructs, since it prolongs the transgene-expression time and thus could provide a wider time-window for radioiodide administration.

3.3. Intratumoral delivery and iodide uptake in vivo (III, IV)

The natural existence of hNIS on most thyroid cancer cells has permitted the use of radioactive iodide for systemically administered local radiotherapy. Because Ad5/3-Δ24-hNIS and Ad5/3-hTERT-hNIS are tumor-selective viruses, replication and subsequent hNIS expression is only expected in tumor cells. To evaluate the ability of hNIS expressing adenoviruses to direct the iodide uptake into tumors of non-thyroid origin in vivo, a model of subcutaneous tumors was established. Each mouse was bearing four PC-3MM2 tumors at backside. The two lowermost tumors were injected twice with hNIS-expressing viruses and the upper tumors with control viruses. 24 h later, mice received intravenously 123I followed by a series of γ-camera imaging. The results show that Ad5/3-Δ24-hNIS and Ad5/3-hTERT-hNIS express functional hNIS in vivo and the iodide accumulation is tumor specific (Figure 7). Further, with both viruses a plateau of 123I was achieved at two hours and the tumors could be imaged still at 13 h post iodide-injection (Figures 5A and B in III and 3A in IV). The iodide accumulation into thyroid and stomach is explained by the endogenous expression of mouse NIS in these organs. Excretion of iodide through the urine accounts for bladder visualization (Zuckier et al., 2004). It would have been both important and interesting to follow the capability of the tumors to accumulate iodide for a longer time. Merron et al. have published a very similar construct to Ad5/3-hTERT-hNIS and they reported a total decline in radioiodide signal 5 days after i.t. administration of the virus, even though initial strong signals were detected.
(Merron et al., 2007). Unfortunately, animal regulations did not allow us to prolong imaging experiments beyond 13 h.

To quantify $^{123}$I biodistribution, organs and tumors from mice treated with Ad5/3-$\Delta$24-hNIS were collected after imaging and their radioactive content was measured (Figure 5C in III). As expected, the thyroid and stomach together captured $\sim$30 % of the injected dose. Nevertheless, Ad5/3-$\Delta$24-hNIS–treated tumors accumulated $>$6 % of the total iodide dose. Iodide uptake remained $<$1 % of the initial dose in other organs including mock or Ad5/3-$\Delta$24-Δgp19K-treated tumors. From these values a kinetics model for $^{123}$I could be calculated revealing that a dose $\sim$2.2 Gy/g of tumor tissue is achieved. Interestingly, this dose is close to the daily fraction of radiation therapy for prostate cancer ($\sim$2 Gy), when fractionated radiotherapy is used.

A large amount of infectious virus was present in tumors, whereas only trace amounts were seen in the thyroid and stomach with TCID$_{50}$ assay (Supplementary Figure S2 in III). This confirmed that Ad5/3-$\Delta$24-hNIS was replicating in tumors and that thyroid and stomach accumulation was reflecting the endogenous NIS expression. However, our human-specific adenovirus constructs were not expected to replicate in mouse cells; only the tumor was human-cell derived sustaining full viral replication.

3.4. Therapeutic efficacy of intratumoral Ad5/3-$\Delta$24-hNIS combined to radioiodide therapy (III)

Ad5/3-$\Delta$24-hNIS antitumor efficacy was evaluated in mice bearing subcutaneous PC-3MM2 tumors. Six mice per group (12 tumors per group) received Ad5/3-$\Delta$24-hNIS or GM intratumorally on 2 consecutive days followed by a 50 MBq intra peritoneal injection of $^{131}$I or saline. The growth of tumors treated with only Ad5/3-$\Delta$24-hNIS, was significantly slower than those treated with the mock or $^{131}$I alone (p < 0.05 for both). When mice received both Ad5/3-$\Delta$24-hNIS and $^{131}$I, tumor sizes were significantly smaller than in any other group, all p < 0.001 (Figure 6 in III). Organs collected from animals, killed due to their tumor size, showed that radioactivity declined over time and biodistribution of $^{131}$I was not different in mice receiving iodide only or iodide with
**Figure 7.** hNIS expressing viruses mediate iodide uptake in vivo. Mice bearing subcutaneous prostate cancer tumors were injected intratumorally with indicated viruses and 24 h later with $^{123}$I. The image shows iodide uptake detected with a $\gamma$-camera at 13h post $^{123}$I. Th, thyroid; S, stomach; B, bladder.

Ad5/3-Δ24-hNIS (Supplemental figure 3 in III). Since we were the first to combine oncolytic adenoviruses and therapeutic radioidide, the results cannot be compared to any previous studies. However, if compared with study II that used Ad5/3Δ24hCG and external beam radiation, a better tumor control was achieved by combining oncolytic viruses with $^{131}$I. For example on experiment day 10, the tumors in the combination group were 2.4-fold of initial size in study II while the respective number in study III was 1.6. This difference might be explained by more accurate radiation targeting in study III using $^{131}$I, where an exact dose/mice is calculated instead of external, approximated radiation dose. Interestingly, we showed in study II that synergy is obtained when virus is given after radiation therapy, but in study III radioiodide was administered after virus injections still leading to good tumor control. This might be explained by the accumulation of iodide in to the tumor and mediating its ionizing effects for a longer time.
period than external radiation therapy. In this regard, as the replicative cycles of the adenovirus proceed, the effects of radiation are antecedent and might be potentiated.

### 3.5. Therapeutic efficacy of systemic Ad5/3-hTERT-hNIS combined to radioiodide therapy (IV)

We were also the first to evaluate the treatment efficacy of intravenously administered oncolytic adenovirus with $^{131}$I. For evaluation of systemic Ad5/3-hTERT-hNIS therapy, nude mice, bearing aggressive, intrapulmonary PC-3MM2 prostate cancer tumors, were injected intravenously with Ad5/3-hTERT-hNIS and the next day intraperitoneally with therapeutic radionuclide $^{131}$I. This treatment cycle was repeated weekly for three weeks. Significant survival enhancements were seen in the Ad5/3-hTERT-hNIS + $^{131}$I group where the p-value compared to mock was <0.001 and to $^{131}$I alone, 0.007 (Figure 3B in IV). Thus a survival enhancement could be detected even when Ad5/3-hTERT-hNIS was administered systemically. The effects of combination treatment with systemically administered oncolytic adenovirus in an aggressive pulmonary model of prostate cancer were also evaluated in study II. In these two studies, it could be estimated that ~0.5 - 1 vp/ tumor cell dose is delivered in vivo. Since some of the initial dose is trapped to the liver, we could achieve treatment efficacy with surprisingly low vp-doses. For some reason, the intraperitoneally injected iodide might not have been as accessible to the intrapulmonary tumors as it was for subcutaneous tumors, as the median survival did not vary between external and internal radiation groups (27 days in II and 28 days in IV). Alternatively, the difference can be explained by better anti-tumor efficacy of Ad5/3-hTERT-hNIS alone with radiation having little effect. This is supported by the fact that there was no statistical significance between virus alone- and combination-groups.

The difference between Ad5/3-Δ24-hNIS and Ad5/3-hTERT-hNIS lies in the E1A region: the former has a native E1A promoter with a 24-bp deletion in E1A for replication in cells with a dysfunctional pRb-pathway, while the latter has an hTERT promoter driving the native E1A expression. Since E1A is a key regulator of Ad replication, the pRb and hTERT status of PC-3MM2 cells might render either construct above the other in terms of
efficacy. However, PC-3MM2 cells were not analysed for these features, since strong previous data has shown that both of these mutations are present in the majority of cancer cells (Kim et al., 1994; Sherr, 1996).

3.6. SPECT/CT imaging of Ad5/3-hTERT-hNIS in vivo (IV)

Recent developments in SPECT have rendered the modality a powerful tool in preclinical imaging. SPECT has been successfully employed in the visualization of various transgenes (Waerzeggers et al., 2009), but its utility over other imaging modalities has been especially suitable for hNIS imaging, where selective, readily available and inexpensive probes, \(^{131}\text{I}, 123\text{I}\) and \(^{99m}\text{Tc}\), can be employed (Merron et al., 2010). Importantly, SPECT imaging can be combined with simultaneous CT-scanning to obtain important anatomical information. The data can be converted into 3D format for detailed toporegional localization of the emitting radionuclide.

To study the feasibility of hNIS-mediated imaging, a similar setting to the survival experiment was employed. 24 h after each virus administration, mice were given an intravenous bolus of \(^{123}\text{I}\) followed by a SPECT/CT scan and quantitation of the \(^{123}\text{I}\) signal. In some mice, CT scans revealed a progressive congestion of the lungs in function of time, as in our first study using the intrapulmonary model (Figure 5 in I). After the first virus injection, only little hNIS expression was detected but after 2 more rounds of treatment, tumors could be imaged (Figures 4a and 5a in IV). Tumor size may have also contributed to detectability, and smaller tumors, especially in the Ad5/3-hTERT-hNIS + \(^{131}\text{I}\) group, might not have been easily detected for low \(^{123}\text{I}\) accumulation. However, in comparison to mice not treated with the virus, the difference in tumor signal was clear (Figure 5B in IV). The data suggest that intravenous injection of Ad5/3-hTERT-hNIS allows tumor transduction, virus replication, hNIS expression and radioiodide accumulation to such degree that it can be detected with SPECT/CT.

The iodide uptake into the thyroid was also evaluated and provided important information about the thyroid response to radioiodide therapy, since all mice treated with \(^{131}\text{I}\) showed diminished uptake by the thyroid during the 2\(^{nd}\) and 3\(^{rd}\) weeks of
treatment (Figure 4B in IV). The normal thyroid can be protected from radioactive iodine by extra iodide supplementation prior to the therapy since exogenous potassium iodide effectively protects the thyroid for 24 h when administered up to two hours prior to $^{131}$I (Verger et al., 2001; Takamura et al., 2004). If the same timing of Ad5/3-hTERT-hNIS and $^{131}$I would apply in a clinical setting, potassium iodide administered 4–6 hours after the virus injection would saturate the thyroid without hampering the later, transgene mediated iodide transportation in the tumor. Alternatively, excess thyroxin prior to radioiodide treatment dampens the radioiodide transport into the thyroid. The possibility to employ thyroxin even in a preclinical animal model was shown by Cho et al. who used a lentiviral vector expressing hNIS to treat glioma (Cho et al., 2002). This study revealed non-existent thyroidal accumulation of radioiodide after a thyroxin supplemented diet whereas the tumor could take up iodide normally. In the case of our study, thyroidal blocking could have also benefited the iodide accumulation into the tumor since most of the radioiodiode was taken up by the thyroid. Thus, a changed bioavailability might not only protect the thyroid but also improve imaging data.

Autopsies performed at the end of the imaging experiment revealed larger tumor masses than what were observed by SPECT imaging. Thus, the virus was not simultaneously replicating in the entire tumor and only the part of the tumor that sustained active replication of the virus was positive for hNIS expression. One plausible explanation is the heterogeneity of the tumor mass allowing for differential hTERT activation and consequent expression of hNIS within the tumor. More likely, the virus propagation from circulation to tumors was hindered by boundaries set by vasculature and intratumoral structure. Merron et al. used an otherwise similar Ad construct, but without the 5/3 modification, in a subcutaneous model of colon carcinoma (Merron et al., 2007). They reported early virus replication and visualization, but the signal was aborted five days after intratumoral virus injection. They suggested likewise that the remaining cancer cells might be inaccessible to the virus or not permissive for viral replication. However, as this result was obtained in two independent studies, it would be interesting to study if the promoter has any effect on these findings.
Other putative explanations exist for the weaker-than-expected signal from the tumors. First, Ad5/3-hTERT-hNIS is optimized for oncolysis and strong oncolysis can be achieved with already small vp-doses (Figure 2 in IV). In a situation where oncolysis occurs rapidly, cells have limited time to produce hNIS and concentrate radiotracers, before the cell is lysed. In this regard, the high oncolytic potency of Ad5/3-hTERT-hNIS may work against the imaging. Thus, low oncolytic potency may be useful for expression of transgenes expressed on the cell surface. Consequently, slower virus replication may allow for better imaging of membrane associated proteins. Second, high levels of early transgene expression may be preferable over later replication-coupled transgene expression. In this regard, the use of a constitutively active promoter, such as that of CMV, could be more beneficial for hNIS expression. The use of other promoters would unfortunately hamper the proposed tumor specificity of the construct. Also, a rapid radioiodide efflux from tumor cells expressing exogenous hNIS has been proposed (Haberkorn, 2001), which may affect the radioiodide concentration even in the presence of hNIS. Finally, the sensitivity of SPECT may require relatively high levels of transgene expression that might not have been obtained at a high-enough degree.

4. Limitations of animal models

Translation of in vitro results to clinical procedures is a multistep process requiring animal testing for safety and efficacy evaluation. Thus far, the animal models for testing Ad constructs have mostly used mice. However, a limiting factor in studying replication and tumor specificity of oncolytic adenoviruses is the attenuated replication in mouse tissues (Blair et al., 1989) and consequently mouse-cancer models cannot be used to study the efficacy of oncolytic adenoviruses. Often, the efficacy of Ad-vectors is evaluated in immunodeficient mice which permit the growth of human xenograft tumors. However the lack of a functioning immune system prevents the researchers to estimate how oncolytic Ads might function in immune-competent humans. The immunological interactions are important since the majority of humans have existing
anti-Ad antibodies, which affect the bioavailability, clearance and toxicity of the vectors (Yang et al., 1995).

Animal experiments in this thesis used immunodeficient mice for their above-mentioned ability to grow human prostate cancer tumors. Taken into account the limitations, we cannot clearly demonstrate the tumor-specific replication of our constructs. For Ad5/3Δ24hCG (I), the tumor specificity was shown in clinical specimens of prostate cancer. A caveat of this approach is the rapid acclimatization of clinical specimens into in vitro conditions if specific procedures are not applied.

For study III using Ad5/3-Δ24-hNIS we aimed to show tumor specificity by analyzing mice organs for Ad presence. Although this method provides information on virus biodistribution (Ads can enter mouse cells) it does not highlight whether the construct would have replicated in the tissue if the tissue was human. Importantly, one must be careful when choosing the method for biodistribution analysis. qPCR is sensitive but reflects the amount of vector that has entered the tissue without providing information on functionality of the virus. On the other hand, TCID<sub>50</sub> is not as sensitive (or specific) but provides information on functional - and thus clinically important - virus particles.

In study IV, we aimed to show the tumor specificity of the virus by demonstrating that a SPECT-signal is detected from the tumor in the lungs. A dilemma arises from the human specificity of the hTERT-promoter. Even if mouse tissues would allow human Ad replication, the species specificity of hTERT hinders the toxicity studies on murine normal cells. However, hTERT cancer specificity has been studied in many previous studies comparing the replication/transgene expression of hTERT-driven adenoviruses in normal and cancerous tissues or by studying the hTERT expression in different clinical samples of cancer (Kim et al., 1994; Nakayama et al., 1998; Wirth et al., 2003; Su et al., 2004; Fujiwara et al., 2007; Huang et al., 2008). A proof-of-concept for the safety in humans has come from a number of phase I clinical trials evaluating Telomelysin, an Ad where the hTERT-promoter element drives the expression of E1A and E1B (Nemunaitis et al., 2010). Taken together, the safety has been shown in humans but the tumor-specificity of Ad5/3-hTERT-hNIS could further be verified with more delicate methods such as using a
similar construct with a mouse TERT-promoter, albeit the replication would still be partly attenuated in mice.

The safety of hNIS constructs has also been evaluated in humans. Barton et al. used an oncolytic Ad coding for hNIS and prodrug-converting enzymes in the treatment of prostate cancer (Barton et al., 2008). The patients in this study received intra prostatic injections of the Ad construct and were subsequently imaged throughout several days using $^{99m}$TcO$_4$. The signals obtained were constricted to the prostate suggesting an organ-confined expression pattern, even in the presence of viral particles in some of the serum samples. Of note, the construct used by Barton et al. is without cancer-specificity and expresses hNIS under a constitutively active CMV-promoter. Because this construct was tumor-confined, we believe that our constructs would be as well, at least if used intratumorally.

In recent years, the use of Syrian hamsters has gained interest since these animals are both semi-permissive for human Ad replication and immunocompetent (Thomas et al., 2006). Albeit bringing some improvements for the study of oncolytic Ads in animals, recent unpublished data suggest that Syrian hamsters are more suitable for evaluation of vectors with an unmodified capsid and less suitable for Ad5/3 since the distribution of Ad3 receptor in Syrian hamsters is unknown. Also other species, such as dogs and baboons have been evaluated as animal models for oncolytic Ads, without any further success (Hemminki et al., 2003).
SUMMARY AND CONCLUSIONS

This study evaluated modified oncolytic adenoviruses for the treatment of castration resistant prostate cancer. In principle, oncolytic adenoviruses were combined with radiation therapy. The feasibility of monitoring viral replication and kinetics by different marker genes was evaluated.

A part of the first aim was to evaluate different capsid modifications to study their effect on prostate cancer infection. This aim was accomplished by examining the transduction and oncolytic capacity of capsid-modified adenoviruses in prostate cancer cells. Based on these results, a new oncolytic adenovirus, Ad5/3Δ24hCG, was constructed. Ad5/3Δ24hCG is serotype 3-receptor targeted, replicates selectively in cells with a dysfunctional Rb/p16 pathway and expresses hCGβ from partially deleted E3. Incorporation of the serotype 3 knob enhanced the transduction capacity over wild type Ad in all tested cell lines implicating that entry to prostate cancer cells can be improved by modifying the Ad5 knob. hCGβ production was found to be in concordance with viral replication and oncolysis in vitro and the virus showed tumor selectivity in fresh human prostate tissue samples. Ad5/3Δ24hCG suppressed the tumor growth in mice and significantly prolonged the survival of mice with aggressive, metastatic disease. Measurements of serum hCGβ suggested viral replication, but no correlation to survival was found. Thus it could not be concluded that hCGβ measurements in vivo would predict the treatment outcome.

The cytotoxicity of Ad5/3Δ24hCG combined with radiation therapy was assessed in aim II and found to be synergistic. The optimal timing schedule for combination therapy was found when oncolytic Ads were given 24 h after irradiation. In vivo, the combination therapy hindered tumor growth and significantly prolonged the survival of mice bearing aggressive, metastatic prostate cancer tumors. The second aim included the screening for molecular mechanisms behind the expected synergy. While changes in gene expression, increased apoptosis, Dynamin 2 levels or viral replication did not seem to account for the synergism, the inhibition of the DSB-repair machinery by adenoviral proteins was found to explain, at least in part, these observations. Finally, the
accumulating DNA damage resulted in increased cell death by autophagy. These findings set the background for further studies on radiotherapy and oncolytic adenoviruses.

The established synergy between oncolytic adenoviruses and radiation was then used in construction of a new oncolytic adenovirus, Ad5/3-Δ24-hNIS for local delivery of systemically applicable radioactive isotopes. In the third study of the thesis, Ad5/3-Δ24-hNIS was shown to mediate the uptake of radioiodide into prostate cancer cells. The iodide uptake could be imaged by γ-camera in vivo, which together with biodistribution analysis suggested tumor specificity of the virus. In a murine model, viral oncolysis together with radioiodide resulted in better antitumor efficacy than either agent alone. Previous studies have featured hNIS-armed oncolytic adenoviruses for imaging purposes, but this study was the first to evaluate the combination of oncolytic adenoviruses with therapeutic radioiodide.

The hNIS-based approach was re-evaluated in the last study, where hTERT, a tumor specific promoter, was placed to drive the expression of E1A in Ad5/3-hTERT-hNIS. One aim was to evaluate the anti-tumor efficacy by the hTERT promoter instead of the 24-bp deletion used in the previous studies. It was shown that this virus efficiently killed prostate cancer cells and encoded for a functional hNIS in vitro. Ad5/3-hTERT-hNIS was studied by systemic administration in vivo together with radionuclide therapy and imaging experiments. The first round of systemic treatment with Ad5/3-hTERT-hNIS did not reveal a distinguishable signal from intrapulmonary tumors, but subsequent administrations resulted in clearly detectable tumors over time. However, results from imaging experiments revealed that hNIS expression remained quite low and the oncolytic effect of virus was presumably more contributing to antitumor efficacy. The imaging data obtained from the study corroborated the tumor selectiveness of the virus by hTERT promoter. This study demonstrated that Ad5/3-hTERT-hNIS was suitable both for detection of viral kinetics and for treatment of prostate cancer with radioiodide therapy. Nevertheless the approach needs improvements.

In conclusion, our results suggest useful combination effects between adenoviral oncolysis and radiation therapy. These findings show that improved means for longitudinal analysis of viral kinetics and persistence can be coupled to therapeutic use
and may help to detect *in vivo* obstacles that otherwise might be difficult to analyze. Also, side effects of the combination treatments are non-overlapping, which might improve efficacy without increasing toxicity. Using virus-mediated hNIS expression, the approach can be extended to treatment of metastatic prostate cancer through transgene mediated uptake of radioactive compounds. The findings of this thesis set the background for clinical translation for the treatment of castration resistant prostate cancer. However, in radiation oncology, therapeutic doses are commonly delivered in fractionated doses. This approach was not evaluated in the presented studies, but could show useful benefits in a clinical setting. In the future, the combination of local delivery of targeted adenoviruses and radiation therapy might improve the outcome of patients with refractory disease. However, further short-term and long-term studies are needed to evaluate the safety and efficacy of the approach before starting any clinical use.
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REFERENCES


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Rommelaere, J., Geletneky, K., Angelova, A. L., Daeffler, L., Dinsart, C., Kiprianova, I.,
therapeutics. Cytokine Growth Factor Rev 21, 185-95.

Rots, M. G., Elferink, M. G., Gommans, W. M., Oosterhuis, D., Schalk, J. A., Curiel, D. T.,
system to evaluate specificity of replicating and non-replicating gene therapy


Sanchez-Prieto, R., Quintanilla, M., Cano, A., Leonart, M. L., Martin, P., Anaya, A. and
Ramon y Cajal, S. (1996). Carcinoma cell lines become sensitive to DNA-damaging
agents by the expression of the adenovirus E1A gene. Oncogene 13, 1083-92.

163-70.

Sangro, B., Mazzolini, G., Ruiz, J., Herrera, M., Quiroga, J., Herrero, I., Benito, A., Larrache,
adenovirus encoding interleukin-12 for advanced digestive tumors. J Clin Oncol
22, 1389-97.

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

Oncolytic virotherapy for prostate cancer by E1A, E1B mutant adenovirus.
Urology 70, 1243-8.

Schenk, E., Essand, M., Bangma, C. H., Barber, C., Behr, J. P., Briggs, S., Carlisle, R., Cheng,

present in malignant prostatic epithelium and is associated with prostate cancer,

Schroder, F. H., Hugosson, J., Roobol, M. J., Tammela, T. L., Ciatto, S., Nelen, V.,


Steegenga, W. T., Riteco, N. and Bos, J. L. (1999). Infectivity and expression of the early adenovirus proteins are important regulators of wild-type and DeltaE1B adenovirus replication in human cells. Oncogene 18, 5032-43.


