

# ADENOVIRAL GENE THERAPY FOR NON-SMALL CELL LUNG CANCER

Merja Särkioja

# ADENOVIRAL GENE THERAPY FOR NON-SMALL CELL LUNG CANCER

Merja Särkioja

Cancer Gene Therapy Group,  
Molecular Cancer Biology Program

and

Transplantation Laboratory, Haartman Institute and  
Finnish Institute for Molecular medicine

University of Helsinki

and

Helsinki University Central Hospital  
Finland

*Academic Dissertation*

*To be publicly discussed with permission of  
the Faculty of Medicine of the University of Helsinki,  
in Haartman Institute Lecture Hall 1, Haartmaninkatu 3, Helsinki,  
on the 3<sup>rd</sup> of Oct 2008, at 12 noon.*

Helsinki 2008

## SUPERVISED BY

Res. Professor Akseli Hemminki, M.D., PhD.

Docent Anna Kanerva M.D., PhD.

Cancer Gene Therapy Group  
Molecular Cancer Biology Program and Transplantation Laboratory, Haartman Institute, Finnish  
Institute for Molecular Medicine, University of Helsinki and  
Helsinki University Central Hospital  
Finland

## REVIEWED BY

Docent Anna-Liisa Levonen M.D., PhD.

A.I. Virtanen Institute for Medical Sciences  
University of Kuopio  
Finland

and

Docent Maija Lappalainen M.D.  
Department of Virology  
Helsinki University Central Hospital  
Finland

## OFFICIAL OPPONENT

Professor Richard Vile PhD.  
Department of Molecular Medicine  
Mayo Clinic College of Medicine  
Rochester, Minnesota  
United States of America

ISBN 978-952-92-4405-8 (paperback)

ISBN 978-952-10-4938-5 (pdf)

<http://ethesis.helsinki.fi>

Helsinki 2008  
Yliopistopaino

*To Meri-Vuokko and Petteri*

# CONTENTS

<b>CONTENTS</b> .....	<b>5</b>
<b>LIST OF ORIGINAL PUBLICATIONS</b> .....	<b>10</b>
<b>ABBREVIATIONS</b> .....	<b>11</b>
<b>ABSTRACT</b> .....	<b>13</b>
<b>1 INTRODUCTION</b> .....	<b>15</b>
<b>2 LUNG CANCER</b> .....	<b>16</b>
2.1 Molecular mechanism of NSCLC .....	17
2.1.1 Tumor suppressors.....	17
2.1.2 Oncogenes.....	18
<b>3 CANCER GENE THERAPY</b> .....	<b>19</b>
<b>4 ADENOVIRAL CANCER GENE THERAPY</b> .....	<b>20</b>
4.1 Adenoviruses.....	20
4.2 Transductional targeting.....	23
4.2.1 Adapter molecule-based targeting .....	24
4.2.2 Genetic targeting.....	25
4.3 Transcriptionally targeted adenoviruses .....	28
<b>5 ONCOLYTIC ADENOVIRUSES</b> .....	<b>31</b>
5.1 Oncolytic adenoviruses combined with targeting moieties.....	33
5.2 Armed oncolytic adenoviruses.....	34
5.3 Oncolytic adenoviruses in combination with conventional therapies .....	35
5.4 Cells as carriers of oncolytic adenoviruses .....	35

<b>6</b>	<b>CLINICAL TRIALS WITH ONCOLYTIC ADENOVIRUSES.....</b>	<b>36</b>
<b>7</b>	<b>ADENOVIRUSES AND IMMUNE RESPONSE .....</b>	<b>39</b>
7.1	Manipulation of adenoviruses to circumvent immune response .....	40
	<b>AIMS OF THE STUDY.....</b>	<b>42</b>
	<b>MATERIALS AND METHODS .....</b>	<b>43</b>
<b>1</b>	<b>CELL LINES, HUMAN MESENCHYMAL STEM CELLS (MSCS), AND PRIMARY NSCLC (I-IV).....</b>	<b>43</b>
<b>2</b>	<b>ADENOVIRAL VECTORS AND REPLICATING ADENOVIRUSES (I- IV) 44</b>	
2.1	Construction of Cox-2 and 3'UTR element-expressing plasmids (III) .....	46
2.2	Construction of non replicating adenoviruses .....	48
2.3	<i>In vitro</i> transfection of plasmids (III) .....	48
2.4	High titer production of viruses (I-IV) .....	48
<b>3</b>	<b><i>IN VITRO</i> STUDIES .....</b>	<b>48</b>
3.1	Adenovirus-mediated gene transfer assays (I-III) .....	48
3.2	Determination of receptor expression by flow cytometry (II) .....	49
3.3	Cytotoxicity assay (I, II) .....	49
3.4	Semiquantitative polymerase chain reaction (III) .....	49
3.5	Assessment of neutralizing antibody (NAb) titers (IV) .....	50
3.6	Effect of NAbs on gene transfer (IV).....	50
<b>4</b>	<b><i>IN VIVO</i> STUDIES.....</b>	<b>50</b>
4.1	Mice and mice models used (I-IV).....	50
4.2	Biodistribution assay (I, IV).....	51
4.3	Therapeutic assay (I, II) .....	51

4.4	<i>In vivo</i> transfection of plasmids and infection of viruses (III) .....	52
4.5	Noninvasive <i>in vivo</i> imaging (I-III) .....	52
4.6	Induction of NAbs (IV).....	52
4.7	Effect of NAbs on gene transfer (IV).....	52
<b>5</b>	<b>STATISTICS (I-IV).....</b>	<b>53</b>
	<b>RESULTS AND DISCUSSION .....</b>	<b>54</b>
<b>1</b>	<b>MODIFIED ADENOVIRUSES SHOW ENHANCED GENE TRANSFER TO NSCLC CELL LINES, MSCS, AND PRIMARY LUNG CANCER TISSUES <i>IN VITRO</i> (I, II) .....</b>	<b>54</b>
<b>2</b>	<b>BIODISTRIBUTION OF CAPSID-MODIFIED ADENOVIRUSES (I)..</b>	<b>55</b>
<b>3</b>	<b>CAPSID-MODIFIED ADENOVIRUSES DISPLAY IMPROVED CELL KILLING EFFICACY IN NSCLC <i>IN VITRO</i> AND <i>IN VIVO</i> (I).....</b>	<b>56</b>
<b>4</b>	<b>MSCS TARGET LUNGS <i>IN VIVO</i> (II) .....</b>	<b>57</b>
<b>5</b>	<b>ONCOLYTIC ADENOVIRUSES AND MSCS LOADED WITH ONCOLYTIC ADENOVIRUSES SHOW THERAPEUTIC EFFICACY IN AN ORTHOTOPIC MURINE MODEL OF ADVANCED LUNG AND BREAST CANCERS (II) .....</b>	<b>59</b>
<b>6</b>	<b><i>IN VIVO</i> MONITORING OF ONCOLYTIC ADENOVIRUSES (I, II) ..</b>	<b>59</b>
<b>7</b>	<b>COX-2 AND CMV PROMOTER ARE INDUCED FOLLOWING ADENOVIRUS INFECTION, BUT THE COX-2 3'UTR ELEMENT CAN RETAIN SPECIFICITY <i>IN VITRO</i> AND <i>IN VIVO</i> (III) .....</b>	<b>60</b>
<b>8</b>	<b>SWITCHING THE ADENOVIRAL FIBER RETAINS VIRAL GENE TRANSFER IN THE PRESENCE OF NABS (IV).....</b>	<b>63</b>
	<b>SUMMARY AND CONCLUSIONS .....</b>	<b>66</b>
	<b>ACKNOWLEDGEMENTS.....</b>	<b>68</b>

**REFERENCES..... 70**





## LIST OF ORIGINAL PUBLICATIONS

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

- I** Särkioja M, Kanerva A, Salo J, Kangasniemi L, Eriksson M, Raki M, Ranki T, Hakkarainen T, Hemminki A. Noninvasive imaging for evaluation of the systemic delivery of capsid-modified adenoviruses in an orthotopic model of advanced lung cancer. *Cancer* 2006; 107:1578-88.
- II** Hakkarainen T, Särkioja M, Lehenkari P, Miettinen S Ylikomi T, Suuronen R, Desmond RA, Kanerva A, Hemminki A. Human mesenchymal stem cells lack tumor tropism but enhance the antitumor activity of oncolytic adenoviruses in orthotopic lung and breast tumors. *Hum Gene Ther* 2007; 18: 627-41.
- III** Särkioja M, Hakkarainen T, Eriksson M, Ristimäki A, Desmond RA, Kanerva A, Hemminki A. The cyclo-oxygenase 2 promoter is induced in nontarget cells following adenovirus infection, but an AU-rich 3' untranslated region destabilization element can increase specificity. *J Gene Med* 2008; 10: 744-53.
- IV** Särkioja M, Pesonen S, Raki M, Hakkarainen T, Salo J, Ahonen MT, Kanerva A, Hemminki A. Changing the adenovirus fiber for retaining gene delivery efficacy in the presence of neutralizing antibodies. *Gene Therapy* 2008; 15: 921–29.

## ABBREVIATIONS

5-FU	5-fluorouracil
AAV	adeno associated virus
ACE	angiotensing-converting enzyme
Ad3	adenovirus serotype 3
Ad5	adenovirus serotype 5
BAd3	bovine Ad type 3
CAR	coxsackie-adenovirus receptor
CD	cytosine deaminase
CDK	cyclin dependent kinase
CEA	carcinoembryogenic antigen
CMV	cytomegalovirus
Cox-2	cyclooxygenase 2
CXCR4	chemokine receptor
DC	dendritic cells
EGF	epidermal growth factor
Ep-CAM	epithelial cellular adhesion molecule
Fab	antibody fragment
FGF	fibroblast growth factor
FLAG	Asparagin, Tyrosine, Lysine, Asparagin, Asparagin, Asparagin, Asparagin Lysine
GCV	Ganciclovir
GM	growth media
HD vector	helper-dependent vector
hMSC	human mesenchymal stem cell
HSPG	heparan sulfate proteoglycan
HSV-tk	herpes simplex virus type 1 thymidine kinase
hTERT	human telomerase reverse transcriptase
i.ha	intra hepatic artery
i.p	intraperitoneal
i.t	intratumoral
i.v	intravenous
IFN	interferon
INK4	inhibitors of cyclin-dependent kinase CDK4
kb	kilobase
kD	kiloDalton
KS	kaposi carcinoma
LacZ	$\beta$ -galactosidase
luc	firefly luciferase
MAP	mitomycin C+doxorubicin + cisplatin
MAPK	mitogen-activated protein kinase
Mdr-1	multiple drug resistance 1

MSC	mesenchymal stem cell
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl-2H tetrazolium,inner salt
Nab	neutralizing antibody
NF-κB	nuclear factor-κB
NGR	asparagine-glycine-arginine
NRP	human lung surfactant protein D
NSCLC	Non-small cell lung cancer
orf	open reading frame
PEG	polyethylene glycol
Pfu	plaque forming unit
pK	polylysin
PKR	protein kinase R
PLGA	polyethylene glycol-cationic lipid and poly (lactic-glycolic) acid
PSA	prostate-specific antigen
Rb	retinoblastoma
RGD-4C	arginine-glycine-aspartatic acid
sCAR	soluble form of CAR
SCCA2	squamous cell carcinoma antigen
SCCHN	squamous cell carcinoma of the head and neck
SCLC	small cell lung cancer
SIGYLPLP	Seriini-Isoleucine-Glycine-Tyrosine-Leucine-Proline-Leucine-Proline
SLP1	secretory leukoprotease inhibitor 1
TAG-72	tumor associated glycoprotein 72
TCID <sub>50</sub>	tissue culture infective dose <sub>50</sub>
TK/GCV	thymidine kinase ganciclovir
TSP	tumor/tissue specific promoter
TTS	dual promoter with hTERT and human surfactant protein A1 promoters.
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UTR	untranslated region
VA RNA	virus-associated RNA
vp	viral particles

## ABSTRACT

Adenoviral gene therapy is an experimental approach to cancer refractory to standard cancer therapies. Adenoviruses can be utilized as vectors to deliver therapeutic transgenes into cancer cells, while gene therapy with oncolytic adenoviruses exploits the lytic potential of viruses to kill tumor cells. Although adenoviruses demonstrate several advantages over other vectors - such as the unparalleled transduction efficacy and natural tropism to a wide range of tissues - the gene transfer efficacy to cancer cells has been limited, consequently restricting the therapeutic effect. There are, however, several approaches to circumvent this problem.

We utilized different modified adenoviruses to obtain information on adenovirus tropism towards non-small cell lung cancer (NSCLC) cells. To enhance therapeutic outcome, oncolytic adenoviruses were evaluated. Further, to enhance gene delivery to tumors, we used mesenchymal stem cells (MSCs) as carriers. To improve adenovirus specificity, we investigated whether widely used cyclooxygenase 2 (Cox-2) promoter is induced by adenovirus infection in nontarget cells and whether selectivity can be retained by the 3' untranslated region (UTR) AU-rich elements. In addition, we investigated whether switching adenovirus fiber can retain gene delivery in the presence of neutralizing antibodies.

Our results show that adenoviruses, whose capsids were modified with arginine-glycine-aspartic acid (RGD-4C), the serotype 3 knob, or polylysins displayed enhanced gene transfer into NSCLC cell lines and fresh clinical specimens from patients. The therapeutic efficacy was further improved by using respective oncolytic adenoviruses with isogenic 24bp deletion in the E1A gene. Cox-2 promoter was also shown to be induced in normal and tumor cells following adenovirus infection, but utilization of 3'UTR elements can increase the tumor specificity of the promoter. Further, the results suggested that use of MSCs could enhance the bioavailability and delivery of adenoviruses into human tumors, although cells had no tumor tropism *per se*. Finally, we demonstrated that changing

adenovirus fiber can allow virus to escape from existing neutralizing antibodies when delivered systemically.

In conclusion, these results reveal that adenovirus gene transfer and specificity can be increased by using modified adenoviruses and MSCs as carriers, and fiber modifications simultaneously decrease the effect of neutralizing antibodies. This promising data suggest that these approaches could translate into clinical testing in patients with NSCLC refractory to current modalities.

# 1 Introduction

Lung cancer is one of the most common cancers and the leading cause of cancer-related mortality worldwide. It can be divided into two types: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Non-small cell lung cancer can be subdivided further into squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma and it accounts for approximately 80% of all lung cancers. A paucity of effective treatments exists for NSCLC patients. Treatments include chemotherapy and surgery, but in most cases the cancer is at advanced stage when it is detected and these treatments are not curative.

Consequently, there is a need for new therapies (Ramalingam and Belani 2008).

A promising treatment alternative for many types of cancers refractory to current modalities is adenoviral gene therapy. Adenoviruses are excellent vectors for gene therapy since they can infect many types of cells, can be grown to high titers, do not integrate into host genome, and, above all, they have low pathogenicity for humans (Glasgow *et al.* 2004). Importantly, there are encouraging results in recent randomized trials concerning glioma, prostate, hepatocellular, and head and neck cancers (Immonen *et al.* 2004; Li *et al.* 2007; Shirakawa *et al.* 2007). However, three potential limitations may have an impact on the efficacy of adenoviruses: 1) Adenoviruses do not transduce efficiently clinically relevant cancer cells, since they exhibit variable expression of adenovirus primary receptor coxsackie adenovirus receptor (CAR), 2) Adenoviruses do not have a natural preference to replicate only in tumor cells, 3) Replication may also be inefficient in large tumor masses, which are very complex in structure, and 4) Adenoviruses have a strong immunogenicity that especially affects systemic re-administration of viruses.

To circumvent some of these problems, adenoviruses can be retargeted to cancer cells by improving their abilities to attach to heterologous receptors other than CAR, which can enhance gene transfer to the desired tissues (Bauerschmitz *et al.* 2002). Further, adenovirus replication can be controlled at transcriptional level by specifically engineered deletions in viral genes or tissue-specific promoters controlling crucial replication regulations, such as E1A, resulting in viruses that replicate only in cells where promoters are expressed

(Saukkonen and Hemminki 2004). Replication can also be controlled via posttranscriptional regulation with elements affecting replication at the mRNA level (Ahmed *et al.* 2003; Gahery-Segard *et al.* 1998). To avoid neutralizing antibodies (NAbs) raised by adenoviruses upon re-exposure, different serotypes of adenoviruses can be used, (Hashimoto *et al.* 2005), alternatively, even small changes in the Ad5 fiber knob can allow the virus to escape from pre-existing capsid-specific NAb (Gahery-Segard *et al.* 1998; Rahman *et al.* 2001). Further, using carriers such as mesenchymal stem cells (MSCs), which are not recognized by NAbs, can enhance gene transfer and antitumor efficacy (Studený *et al.* 2004).

We used capsid-modified adenoviruses to determine the adenovirus tropism towards NSCLC. In addition, we tested whether adenoviruses could be further targeted by regulating gene expression posttranscriptionally. Also, we present some unpublished data suggesting that although gene expression can be beneficially modulated with post-transcriptional control elements in the context of plasmids, the effect was not powerful enough to be useful in the context of recombinant adenoviruses. We also studied the possibility of utilizing MSCs as carriers for oncolytic adenoviruses, thereby combining MSCs with the anti-tumor effect provided by oncolytic adenoviruses. Finally, we investigated whether the changing adenovirus fiber can retain gene transfer efficiency in the presence of adenovirus neutralizing antibodies.

## **2 Lung cancer**

Lung cancer is the second (among men) and fourth (among women) most common cancer in Finland, with 2140 new cases in 2006 (Finnish Cancer Registry; [www.cancerregistry.fi](http://www.cancerregistry.fi)). The most common histological type of NSCLC is adenocarcinoma, followed by squamous-cell carcinoma and large-cell carcinoma. The prognosis of NSCLC is poor because the disease is often at an advanced stage when detected. The overall 5-year survival rate is only 8% for men and 12% for women.

Treatment of NSCLC remains a challenge. Surgical resection at an early stage represents the only treatment associated with a high likelihood of attaining 5-year survival. However,



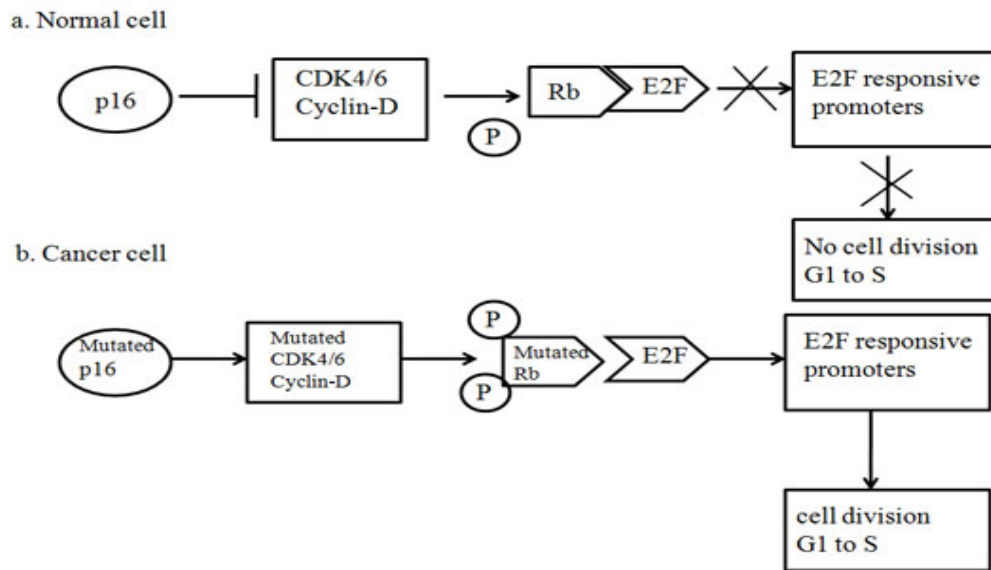
since the NSCLC is often detected at a later stage, combination therapies with radio- and chemotherapy are used.

## **2.1 Molecular mechanism of NSCLC**

Development of cancer, including NSCLC, involves accumulation of multiple molecular abnormalities over a long period of time. These include chromosomal abnormalities, inactivation of tumor suppressor genes, activation of oncogenes, expression of hormone receptors and, production of growth factors. The most common genetic alterations involve mutations in the p53/p14ARF, p16/retinoblastoma (Rb) and in K-ras pathways. These mutations involving tumor suppressor genes may be a rate-limiting event in the development of cancer (Brambilla *et al.* 1999).

### **2.1.1 Tumor suppressors**

p53 is a tumor suppressor gene that is mutated in 2/3 human lung cancers, and it is commonly seen in squamous cell carcinomas. In normal cells, when DNA damage occurs, p53 can halt the cell cycle at the G1 checkpoint, preventing the cycle from proceeding to the S phase until the DNA is repaired or it can induce the cell to apoptosis. In cancer cells, by contrast, when p53 is mutated, the control is lost. The p16/Rb pathway regulates entry and progression through the cell cycle. Disruption in one or more factors in this pathway can lead to tumor genesis. p16 and pRb proteins are critical members of the Rb pathway, and they are mutated in 80% of NSCLC (Belinsky *et al.* 1998; Tanaka *et al.* 1998). In normal cells, when cell cycle protein p16 is needed, it functions as an inhibitor of the CDK4/6 cyclin-D complex. This leads to dephosphorylation of protein Rb, which in turn cannot release transcription factor E2F that binds to DNA to stimulate the synthesis of proteins necessary for cell division. No transcription of the genes necessary for the cell cycle to progress from G1 to S phase, therefore occurs. If CDK4/6-cyclin D complex is not under inhibition, it increases phosphorylation of the retinoblastoma protein (pRb), which can then release E2F and transcription will occur and the cell cycle continues from G1 to S phase. In tumor cells with defects in p16 or Rb function or a CDK4/6 cyclin D complex with increased activity, E2F is constitutively active (Jakubczak *et al.* 2003) (Figure 1.).



**Figure 1. p16/Rb pathway. a)** In normal cells, when p16 is needed, it inhibits CDK4/6 Cyclin-D complex that dephosphorylates protein Rb which prevents Rb to interact with E2F and the cell cycle is arrested to G1. **b).** In cancer cells, the E2F transcription factor is constantly active and defects in p16, CDK4/6 Cyclin-D complex or pRb, don't affect on cell division.

### 2.1.2 Oncogenes

Oncogenes are typically mutated genes that have an important role in cell signaling pathways. They encode proteins that control cell proliferation, apoptosis, or both. They can be activated by structural alterations resulting from a mutation or a gene fusion (Konopka *et al.* 1985; Tsujimoto *et al.* 1985). The most important abnormalities detected are mutations involving the *ras* family of oncogenes. The *ras* oncogene family has three members: H-*ras*, K-*ras*, and N-*ras*. These genes encode a protein on the inner surface of the cell membrane with GTPase activity and may be involved in signal transduction. K-*ras* mutations are most commonly seen in 30% in adenocarcinomas of the lung (Westra *et al.* 1993).

### 3 Cancer gene therapy

Cancer is a complex disease requiring combinations of several therapeutic modalities to treat it effectively. Gene therapy was originally devised to treat disease by replacing a lost or defective gene. Now it has broadened to encompass a variety of approaches of introducing genetic materials into cells for therapeutic purposes. These strategies can be roughly categorized into two main categories: immunologic and molecular (Heo DS 2002). One of the frequently used genetic immunotherapy strategy involves the transfer of the genes of the immune-stimulant molecules such as cytokines such as *interleukin 12* (IL12) gene to boost T-cell mediated immune response against cancer (Shi *et al.* 2002; Barajas *et al.* 2001). Other approaches includes the manipulation of antigen presenting cells such as dendritic cells to enable them of more active tumor antigen presentation in the tumor tissue (Vollmer *et al.* 2002; Vulink *et al.* 2008) and direct genetic vaccination by the antigen-encoding genes (Hanke *et al.* 2002; Grim *et al.* 2000).

Molecular approaches involves suicide conversion gene therapy, use of anti-oncogenes to inhibit oncogene activity, utilization of tumor suppressor genes, anti-angiogenesis gene therapy, transfer the drug resistance genes to overcome the dose-limiting toxicity of traditional chemotherapy, the use of anti-viral vectors to deliver anticancer reagents to the tumor tissue and also adenovirus gene therapy that will be discussed in the chapters 4-6. One of the most investigated suicide gene/prodrug systems is the herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV) system where non-toxic prodrug GCV is converted to the toxic form. The efficiency of the system has been demonstrated successfully in patients with malignant glioma in a clinical trial where the mean survival of patients was prolonged by 81% when treated with TK/GCV therapy after surgery (Immonen *et al.* 2004).

Anti-oncogenes are mostly targeted against *bcl-2*, *c-myc* genes and *K ras* gene family. Treatments with anti-oncogenes have resulted in reduction in tumor growth both *in vitro* and *in vivo* with melanoma and colon cancer (Hu *et al.* 2001; Duggan *et al.* 2001; Chana *et al.* 2002; Tokunaga *et al.* 2000).

Successful transfection of the functional *wt p53* into cancer cells has showed therapeutic outcomes in patients. When combined with radiation, no viable tumor was observed in

63% of patients after three month of the completion of the therapy (Swisher *et al.* 2003). The main targets for anti-angiogenesis cancer therapy includes inhibiting angiogenic inducers (namely, vascular endothelial growth factor and angiopoietin) or introducing angiogenic inhibitors such as angiostatin, endostatin, Interleukin-12, and p53 (Chen *et al.* 2001).

One of the main problems with traditional chemotherapy is the toxicity to normal cells. To protect cells and to enable the use of larger drug doses, drug resistance genes such as multiple drug resistance gene-1 (*Mdr-1*), can be transferred into the hematopoietic progenitors (Licht and Peschel 2002; Koc *et al.* 1999).

To improve carriers capacity to deliver anticancer reagents, as well as synthetic oligonucleotides, antibodies and RNA, in addition to therapeutic genes, nonviral vectors have been developed. Mostly used carriers include liposomes and polymers that can be packed with the DNA of desired anticancer agent. Molecules are delivered into cells by endocytosis (Wheeler *et al.* 1996; Sudimak *et al.* 2000; Qian *et al.* 2002).

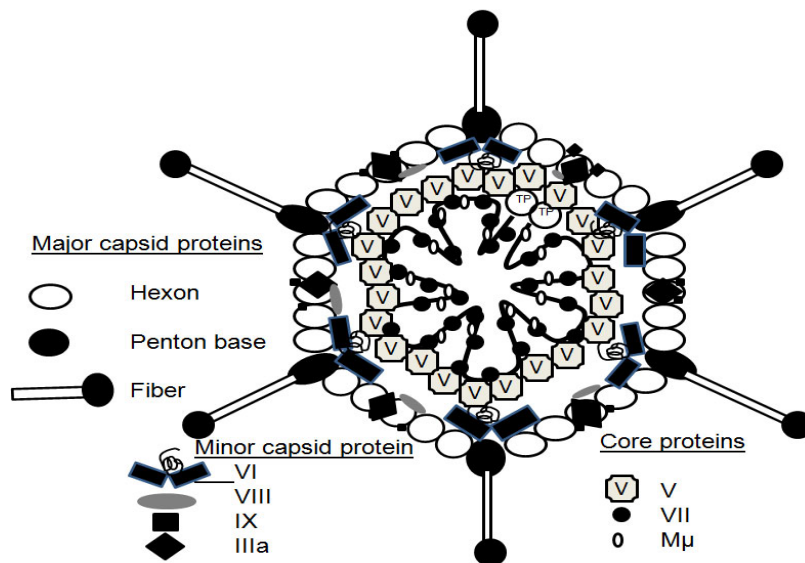
## **4 Adenoviral cancer gene therapy**

Adenoviruses are probably the most studied viral gene transfer vectors, but other viruses are used as well. Numerous cancer gene therapy studies have been conducted with retroviruses (Cole *et al.* 2005; Thanarajasingam *et al.* 2007), lentiviruses, vaccinia viruses, adeno-associated virus (AAV), and herpes simplex virus to mention but a few (Kirn *et al.* 2007; Mueller and Flotte 2008; Pellinen *et al.* 2004; Tyler *et al.* 2008).

### **4.1 Adenoviruses**

Adenoviruses are nonenveloped DNA viruses. They have a characteristic morphology with an icosahedral capsid, which consists of three major proteins: hexon (II), penton base (III), and a knobbed fiber (IV), along with minor proteins: VI, VIII, IX, IIIa, and IVa2 (Stewart *et al.* 1993). Highly basic protein VII, small peptide *mu*, and a terminal protein (TP) are associated with DNA. Protein V provides a structural link to the capsid via protein VI (Figure 2). Thus far, at least 51 serotypes of human adenovirus have been identified and

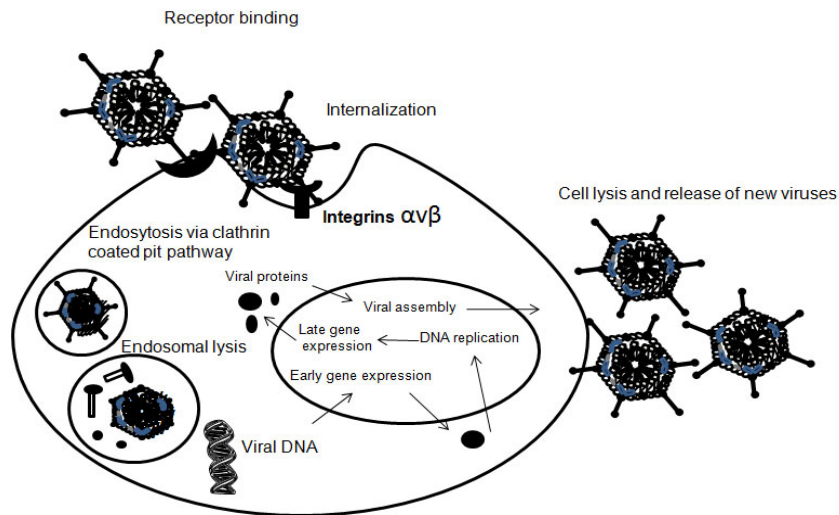
classified into six distinct subgroups, A-F (De Jong *et al.* 1999). Adenovirus serotypes 1-7 cause the most common infections in humans (Arstila 2004). In the context of gene therapy, the most studied adenovirus serotype 5 (Ad5) belongs to subgroup C.



**Figure 2. Structure of adenovirus 5 virion.** [modified from the figure in (Russell 2000)].

The adenovirus infection cycle can be divided into two phases, early and late, occurring before and after virus DNA replication. The first phase covers the entry of the virus into the host cell (Figure 3), followed by the passage of the virus genome into the nucleus and selective transcription and translation of early viral genes. Early genes are needed for the transcription and translation of late genes, which lead to the assembly of structural proteins and maturation of the infectious virus. Entry of the virus into the host cell is mediated through specific receptors on the target cell. The virus binds to a receptor via the knob portion of the fiber. The receptor for adenoviruses belonging to subgroup A-F, but excluding subgroup B, is known to be CAR, at least *in vitro*. (Roelvink *et al.* 1998). This traditionally accepted mechanism of cell entry is now known to be more complex at least *in vivo* after systemic delivery and it is not even known in humans. More intense research

is required to reveal virus-host interactions in terms of viral components, blood cell and serum interactions of adenoviral vectors and mechanisms behind the adenovirus tropism (Baker 2007a and b; Nicklin *et al.* 2005; Waddington *et al.* 2008). Some adenovirus serotypes seem to have additional binding specificities, suggesting that other receptors besides CAR may be needed for the entry (Segerman *et al.* 2000). After the interaction of the virus with the receptor, the entry proceeds via clathrin-mediated endocytosis. The key step is the interaction of the penton base arginine-glycine-aspartic acid (RGD) motif with host cell  $\alpha_v\beta$  integrins, leading to endosome formation and internalization. After internalization, adenovirus DNA is transported to the nucleus, which is followed by early gene protein synthesis. The early gene region consists of four genes: E1, E2, E3, and E4. E1 gene products can be subdivided further into E1A and E1B. E1A proteins modulate cellular metabolism in a way that makes cell more susceptible to virus replication. They interfere with, for instance, the cell division processes and the regulation of NF- $\kappa$ B, p53, and pRb proteins. E1B functions cooperatively with E1A by inhibiting apoptosis. E2 is also subdivided into two separate transcription regions: E2A and E2B. Their gene products (including DNA polymerase) are required for DNA replication. E3 gene products are, on the other hand, responsible for defending against the host immune system, enhancing cell lysis, and releasing of virus progeny. E4 gene products mainly facilitate virus RNA metabolism, promote virus DNA replication, and prevent host protein synthesis. Transcription of late genes leads to production of the virus structural components and the encapsidation and maturation of virus particles in the nucleus (Russell 2000).



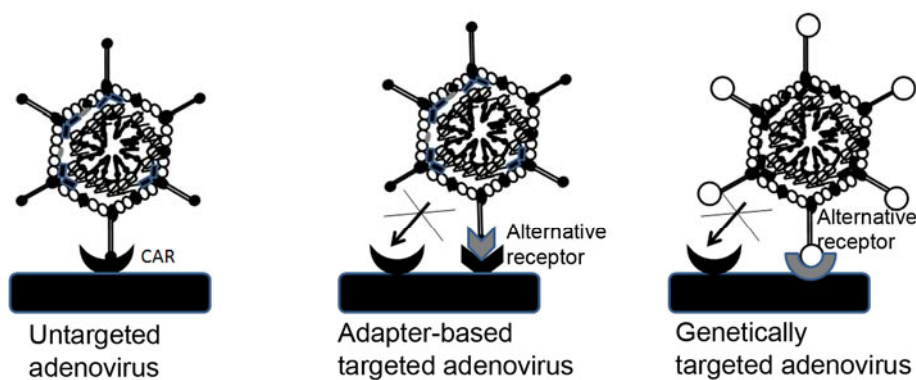
**Figure 3. Schematic illustration of the adenovirus 5 infection pathway.** Cell entry is initiated by binding of the fiber knob to its primary receptor CAR, which is followed by clathrin-mediated endocytosis. Interaction of the penton base RGD motif with host cell integrins ( $\alpha\beta$ ) is needed for the process to proceed. After endosomal lysis, viral DNA is transported to the nucleus, where viral genes are expressed.

Adenoviruses might be useful gene transfer vehicles for treatment of various diseases (Bainbridge *et al.* 2008; Hedman *et al.* 2003; Immonen *et al.* 2004; Li *et al.* 2007; Shirakawa *et al.* 2007). However, clinical trials have shown that uncontrolled, high-dose gene transfer and expression could have side effects (Raper *et al.* 2003). Viruses should therefore be targeted to the preferred tissue on a transductional or transcriptional level. The former implies restriction of viral entry to target cells, whereas the latter involves selective gene expression in the cells of interest.

## 4.2 Transductional targeting

Adenoviruses display unparalleled transduction efficacy and natural tropism to a wide range of epithelial tissues. Receptor recognition could be one of the most important factors involved in cell tropism. Increased CAR expression appears to have a growth inhibitory effect on some cancer cell lines, while loss of CAR expression correlates with tumor

progression and advanced disease (Okegawa *et al.* 2001; Bauerschmitz *et al.* 2002; Miller *et al.* 1998a; Cripe *et al.* 2001; Dmitriev *et al.* 1998). In addition, CAR has been suggested to play a role in cell adhesion, and its expression may be cell-cycle dependent (Cohen *et al.* 2001). As efficient gene transfer is one of the key issues for successful gene therapy, low expression level of CAR on tumor cells causes a major challenge (Okegawa *et al.* 2001; Seidman *et al.* 2001). To circumvent deficiency of CAR, viruses can be retargeted by improving their abilities to attach to heterologous receptors. There are two distinct approaches to retarget adenoviruses transductionally: adapter molecule-based targeting and targeting via genetic manipulation of the adenovirus capsid (Figure 4).



**Figure 4. Transductional targeting of adenoviruses.** Transductional targeting can be used to target adenovirus to alternative cellular receptors instead of its primary receptor CAR. Targeting can be based on use of adapter molecules or genetically modified fibers that allow the virus to bind alternative receptors.

#### 4.2.1 Adapter molecule-based targeting

The idea behind adapter molecule-based targeting is to use adapter molecules that crosslink the adenovirus vector to alternative cell surface receptors, bypassing the native CAR-based tropism (Figure 4). The majority of currently used adapter molecules are bispecific, which both ablates the native CAR binding and formats a novel tropism to cellular receptors, highly expressed on cancer cells. Various adapter molecules from



bispecific antibodies, chemical conjugates between antibody fragment (Fab) and cell selective ligand (ex. folate), Fab antibody and peptide ligand conjugates to recombinant fusion proteins that incorporate Fabs and peptide ligands have been used successfully. The first *in vitro* study of adenovirus targeting via adapter molecule was done by Douglas *et al* (Douglas *et al.* 1996). They targeted to the folate receptor, overexpressed on many malignant cells with anti-knob neutralizing Fab-folate conjugate. A similar targeting system was used with FGF receptor-positive Kaposi's sarcoma (KS) cells *in vitro*, where basic fibroblast growth factor (FGF2) was fused to anti-knob Fab (Goldman *et al.* 1997). Other Fab-ligand conjugates have been targeted to epithelial cellular adhesion molecule (Ep-CAM) (Haisma *et al.* 1999) , tumor-associated glycoprotein (Tag-72) (Kelly *et al.* 2000), epidermal growth factor (EGF) receptor (Miller *et al.* 1998b), and CD 40 cell marker (Tillman *et al.* 1999), with promising results.

An interesting alternative method to the chemical conjugates was developed utilizing a truncated soluble form of CAR (sCAR) fused to EGF (Dmitriev *et al.* 2000). Approaches resulted in increased reporter gene expression in many cancer cell lines overexpressing either EGF receptor or CD40 *in vitro*. When targeted to the EGF receptor, up to a 9-fold increase in gene transfer was seen compared with nontargeted adenovirus.

The above approaches have also been tested *in vivo*. A bispecific antibody was used to target adenovirus to the angiotensin-converting enzyme (ACE) expressed in the pulmonary capillary endothelium. Systemic delivery of adenovirus resulted in a 20-fold increase in the lungs compared with the untargeted control. Moreover, adenovirus gene transfer to liver decreased 83% (Reynolds *et al.* 2000). Another unique lung-targeting approach was developed by Everts *et al*, where sCAR was fused with with a single-chain anti-CEA antibody (MFE-23). sCAR-MFE targeted adenovirus decreased liver gene expression by > 90 % and increased transduction to the CEA-positive epithelial tumor cells after systemic delivery (Everts *et al.* 2005; Li *et al.* 2007).

#### **4.2.2 Genetic targeting**

The advantage of the adapter-based targeting approach is the wide range of ligands that can be incorporated into fusion molecules, but there are also several drawbacks. The structure itself is complex, which results in creation of a heterogeneous population of

virions, and the stability of the complexes in the human circulation is not very well characterized. This might be disadvantageous in human trials. Therefore, one-component systems where adenovirus capsid proteins are genetically manipulated have been created. Overall, there are three basic ways to modify adenovirus fiber to redirect its tropism: 1) so-called fiber pseudotyping, 2) ligand incorporation into the fiber knob, and 3) 'de-knobbing' of the fiber coupled with ligand addition.

Understanding of adenovirus infection biology indicates that genetic modifications are usually located in the fiber, the primary capsid determinant of adenoviral tropism. Many clinically relevant cancer cell types are refractory to adenovirus infection due to variable or aberrant expression of the primary receptor CAR (Bauerschmitz *et al.* 2002; Cripe *et al.* 2001; Hemminki *et al.* 2002; Miller *et al.* 1998a; Okegawa *et al.* 2000; Seidman *et al.* 2001). Genetic replacement of fiber knob was first accomplished by Krasnykh *et al.* (Krasnykh *et al.* 1996). Adenovirus serotype 5 knob was replaced by the knob from serotype 3, which uses a different receptor than Ad5 in cell entry. Ad3 belongs to subgroup B and primary receptors for adenoviruses belonging to the group have recently been suggested, including CD46, a complement regulatory protein expressed on the surface of nucleated human cells (Liszewski *et al.* 1991), and CD80 and CD86, costimulatory molecules present on mature dendritic cells and B lymphocytes and involved in stimulating T-lymphocyte activation (Short *et al.* 2004). The receptor for adenovirus 3 is still unknown, although there are suggestions that it could be on one of the above receptors or some coreceptor or combination of receptors (Sirena *et al.* 2004; Tuve *et al.* 2006a; Sirena *et al.* 2004). Ad5/3 chimera has displayed enhanced CAR independent transduction on variety of neoplastic cells (Krasnykh *et al.* 1996; Davidoff *et al.* 1999; Kanerva *et al.* 2002b). Promising results with Ad5/3 chimeras have encouraged many researchers to evaluate other serotype chimeras. Ad5/35, Ad5/11, and Ad5/7 have shown enhanced transduction refractory to the CAR in many types of cells with clinical importance and reduced localization to the liver (Shayakhmetov *et al.* 2000; Stecher *et al.* 2001; Gall *et al.* 1996; Mizuguchi & Hayakawa 2002; Schoggins *et al.* 2005).

The fiber knob region is the major attachment site for the primary receptor at least *in vitro*. Consequently, modification of the knob region has become a major research focus for targeting gene delivery. Studies have exploited two locations within the knob, C-terminus

and HI loop, which can be manipulated without losing function of the fiber. Since the C-terminus is solvent-exposed, Wickham *et al.* added a polylysine tail to mediate adenovirus binding through heparan sulfate proteoglycans (HSPGs), yielding promising results (Wickham *et al.* 1997; Kangasniemi *et al.* 2006; Ranki *et al.* 2007a).  $\alpha_v\beta$  integrin-binding arginine-lysine-aspartic acid (RGD) motifs have also successfully been incorporated into the C-terminus (Wickham *et al.* 1997). Another promising knob location, the HI-loop, which is exposed outside and can tolerate rather large (up to 100 amino acids) peptide insertions, is located between  $\beta$ -strands H and I in the knob monomer. FLAG peptide was introduced to the site first by Krasnykh *et al.*, and other retargeting motifs in the HI loop have been evaluated. RGD-4C peptide in the HI loop is widely used with enhanced infectivity to wide range of tumor cells *in vitro* and *in vivo* (Cripe *et al.* 2001; Dmitriev *et al.* 1998; Hemminki *et al.* 2001; Kanerva *et al.* 2002b; Kangasniemi *et al.* 2006; Ranki *et al.* 2007a). Other peptide that has provided cancer selective targeting when inserted into the HI loop includes asparagine-glycine-arginine (NGR) (Mizuguchi *et al.* 2001). Wu *et al.*, combined a polylysine tail in the C-terminus and a RGD-4C motif in the HI loop, and enhanced gene transfer was achieved with several CAR-deficient cell lines, as well as with human pancreatic islet cells (Wu *et al.* 2002). Some studies have also used both genetic fiber modification and an adapter-based approach where incorporating proteins with immunoglobulin binding domains into the C-terminus or the HI loop resulted in enhanced gene transduction in cells containing the Fc region of Ig (Henning *et al.* 2005; Korokhov *et al.* 2003; Volpers *et al.* 2003). Since the ligand incorporation methods described above do not necessarily ablate CAR binding, a native fiber has been replaced by a knobless fiber and simultaneous adding of targeting ligand to the fiber has resulted in a more targeted adenovirus (Falgout and Ketner 1988; Von Seggern *et al.* 1999). However, the major barrier to this approach is that the knob region contains a stabilization element for the fiber element. To circumvent the problem, external trimerization elements, such as the bacteriophage T4 fibritin or the neck region of human lung surfactant protein D (NRP), have been used (Hedley *et al.* 2006; Krasnykh *et al.* 2001; Magnusson *et al.* 2001). Li *et al.* described a novel trimerization element within the adenovirus fiber protein containing an N-terminus tail and shaft repeats

that was able to form stable virions and serve as a platform for the generation of tissue-specific adenovirus vectors (Li *et al.* 2006).

The need for targeting adenoviruses has led to searches for other capsid proteins that could be manipulated such as hexon, penton, and pIX. Due to the location of the hexon protein, it is an attractive site for incorporating targeting ligands. Hexon hypervariable regions have a loop that protrudes outside the capsid and Vigne *et al.* exploited the possibility to attach an RGD motif to this loop. Notably, the stability of the virion was unaffected and enhanced gene delivery to non CAR cells was achieved (Vigne *et al.* 1999). The RGD motif in the penton base was replaced with a peptide that targeted adenoviruses to the metastatic melanomas and glioblastomas (Wickham *et al.* 1995). Recently, pIX was found to be a versatile capsid location suitable for both targeting and imaging motifs (Dmitriev *et al.* 2002; Le *et al.* 2004).

### **4.3 Transcriptionally targeted adenoviruses**

To achieve more tumor cell-selective adenoviruses, gene expression can be regulated transcriptionally. Regulation is usually mediated by tumor-specific promoters. Transcriptional targeting of adenovirus transgene expression or replication restricts the transcription unit expression to the cells where the promoter being used is active. A number of tumor, or tissue-specific promoters have been applied in cancer gene therapy (Glasgow *et al.* 2004; Saukkonen & Hemminki 2004). Table 1 summarizes some of these.

**Table 1. Selected tumor-specific promoters used in adenoviral gene therapy.**

Promoter	Transgene(s)	Disease application	Reference
<b>CEA</b>	LacZ	Gastric cancer	(Brand <i>et al.</i> 1998;
	HSVtk	Lung cancer	Osaki <i>et al.</i> 1994).
<b>SLPI</b>	HSVtk	Ovarian cancer	(Barker <i>et al.</i> 2003a).
<b>Cox-2</b>	HSVtk	Gastric cancer	(Yamamoto <i>et al.</i> 2001).
	Luciferase	Pancreatic cancer	(Wesseling <i>et al.</i> 2001).
	HSVtk	Ovarian cancer	(Casado <i>et al.</i> 2001).
	HSVtk		(Sarkioja <i>et al.</i> 2008).
Luciferase	Lung cancer	(Sarkioja <i>et al.</i> 2008).	
<b>Midkine (MK)</b>	Luciferase	Pancreatic cancer	(Wesseling <i>et al.</i> 2001).
	HSVtk		(Wesseling <i>et al.</i> 2001).
<b>hTERT</b>	E1A_IRES_E1B	Lung cancer	(Hashimoto <i>et al.</i> 2008).
	GFP/TRAIL	Breast cancer	(Lin <i>et al.</i> 2002).
<b>E2F</b>	E1A	Lung cancer	(Tsukuda <i>et al.</i> 2002).
		Ovarian cancer	(Tsukuda <i>et al.</i> 2002).
<b>CXCR4</b>	E1A	Lung cancer	(Zhu <i>et al.</i> 2007b).
	E1A	Breast cancer	(Rocconi <i>et al.</i> 2007).
	E1A	Renal cancer	(Haviv <i>et al.</i> 2004).
<b>SCCA2</b>	Luciferase	Lung cancer	(Oshikiri <i>et al.</i> 2006).
<b>TTS</b>		Lung cancer	(Fukazawa <i>et al.</i> 2004).

Abbreviations used: CEA, carcinoembryonic antigen; SLP1, secretory leukoprotease inhibitor; Cox-2, cyclooxygenase 2; hTERT, human telomerase reverse transcriptase; CXCR4, chemokine receptor; SCCA2, squamous cell carcinoma antigen; TTS, dual promoter with hTERT and human surfactant protein A1 promoters.

Carcinoembryonic antigen (CEA) promoter was one of the first tissue-specific promoters explored. CEA is expressed in many types of cancers. Herpes simplex virus *thymidine kinase* (HSVtk) expression driven by the CEA promoter left CEA-negative cell lines resistant to ganciclovir (GCV) prodrug therapy, while CEA-positive A549 cells were 1000-fold more sensitive (Osaki *et al.* 1994). Further, Hashimoto *et al.* used human telomerase reverse transcriptase promoter (hTERT)-driven adenovirus E1A and E1B genes connected to the internal ribosome entry site (IRES) to target lung cancer cells *in vitro* and *in vivo*, which led to a 50% reduction in cell viability in normal cells versus lung cancer cells (Hashimoto *et al.* 2008). In addition, E2F, CXCR4, SCCA2, and TTS have been used to target adenoviruses to lung cancer tissue, with promising results. E2F transcription factors are critical regulators of cell growth and are often overexpressed in cancer cells because of frequent aberrations in the pRb/E2F/p16(INK4a) pathway. As a result, a majority of tumor cells exist in a high proliferative state with E2F constantly active, making it a good candidate promoter for targeting adenovirus (Tsukuda *et al.* 2002). Chemokine receptor CXCR4 is highly expressed in lung cancer and CXCR4 promoter-driven RGD-modified adenovirus showed a 10-fold increase in replication in lung cancer tissue slices, in contrast to a 10-fold decrease in the human liver slices (Spano *et al.* 2004; Zhu *et al.* 2007b).

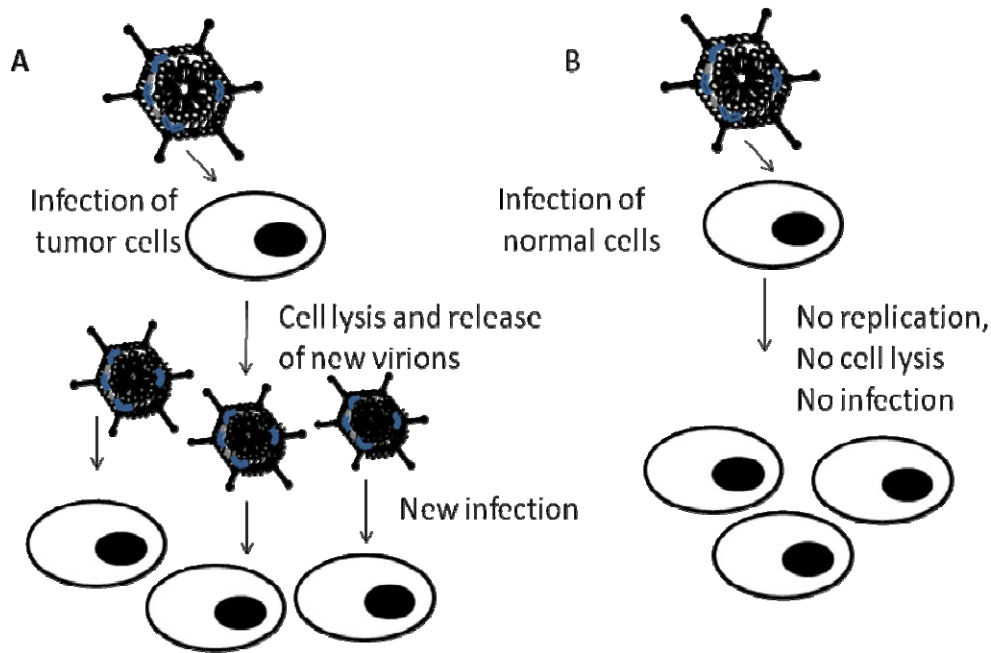
In addition to tissue-specific promoters, adenoviral transgene expression or replication can also be controlled at a posttranscriptional level. Eukaryotic mRNAs contain 3'-untranslated regions (UTRs), the AU- rich elements of which are involved in posttranscriptional control of gene expression. These elements are frequently present in 3-UTRs of many proto-oncogene and cytokine mRNAs (Dixon *et al.* 2000; Krays *et al.* 1989). The usual physiological function of the elements is to rapidly downregulate mRNA transcripts when the threat (e.g. infection) has passed. Ahmed *et al.* used CMV promoter-driven adenovirus, where the E1A was under the regulation of the 3'UTR element from the cyclooxygenase 2

(Cox-2) gene, allowing RAS/P-(MAPK)-specific stabilization on the mRNA (Ahmed *et al.* 2003).

Tumor-specific promoters and other regulatory elements have the potential to increase specificity and decrease toxicity of adenoviral gene therapy. However, adenoviruses targeted only with promoters or regulators are dependent on CAR for entry. Combining transductional and transcriptional targeting is an effective way to achieve a synergistic effect on gene transfer (Barker *et al.* 2003b; Reynolds *et al.* 2001).

## **5 Oncolytic adenoviruses**

Although nonreplicating first-generation adenoviruses have provided high *in vitro* and *in vivo* transduction rates and good safety data, clinical trials have suggested that the single-agent antitumor effect may not be sufficient for all treatment approaches. Advanced human solid tumor masses are larger and more complex than xenograft tumors, which restrict sufficient access of virions to tumor cells. Necrotic, hyperbaric, hypoxic, and stromal areas can for instance, be problematic. To overcome this problem, oncolytic adenoviruses have been developed. Their antitumor effect is based on oncolysis caused by replication *per se*, whereas normal tissues are spared due to low replication (Figure 5). Hypothetically, the replication would continue until all cancer cells are lysed, and released viruses would even disseminate into the bloodstream and infect distant metastasis.



**Figure 5. Oncolytic adenoviruses.** A) Infection of tumor cells results in viral replication, cell lysis, and release of new viruses. B) Normal cells are spared due to lack of replication.

Adenovirus early 1 (E1) gene is responsible for replication and most development of oncolytic adenoviruses have focused on the genetic engineering of E1 genes. There are two approaches that have been used to restrict viral replication to target cells and to spare normal tissue. The first published oncolytic adenovirus, d11520, later called ONYX-015 has two mutations in the E1B gene coding the E1B-55kD protein and is very similar to the H101, the first licensed oncolytic agent (Bischoff *et al.* 1996; Xia *et al.* 2004b). Adenovirus E1B-55kD protein binds to tumor suppressor p53 to inactivate it for induction of S-phase-like state, which is required for viral replication. Further, it is responsible for forming a different complex with E4orf6 that shuts off host mRNA nuclear export and host protein synthesis. It also facilitates the nuclear localization of transcription factor YB1 to activate the E2 late promoter (Holm *et al.* 2002). Tumor cells often have an inactivated p53-p14ARF pathway, and thus, viruses should only replicate in these cells. However some tumor cells seem to fail to support E1B-55kD deleted adenoviral replication.



Recently O'Shea *et al* suggested that it may be due to failure in late mRNA export that prevents host protein shutoff (O'Shea *et al.* 2005).

Another type of Ad contains a 24 bp deletion in constant region 2 (CR2) of the *E1A* gene. This domain is responsible for binding the Rb tumor suppressor/cell cycle regulator protein, thereby allowing adenovirus to induce an S-phase-like state. As a result, viruses with the  $\Delta 24$ -E1A deletion have a reduced ability to overcome the G1-S checkpoint and replicate efficiently only in cells where this interaction is not necessary, e.g. in tumor cells defective in the *Rb-p16* pathway. (Heise *et al.* 2000b; Fueyo *et al.* 2000; Suzuki *et al.* 2001).

A third type of deletion used to confer cancer selectivity to adenovirus is the deletion of virus-associated (VA) RNA genes. These small type I and II RNAs inactivate the protein kinase R (PKR). PKR plays a major role in the interferon (IFN) antiviral cell defense, and by binding to PKR, VA-RNAs counteract this IFN response to adenovirus, and conversely, adenoviruses defective in VA-RNAs are blocked by IFN via protein translation inhibition. However, many cancer cells present a truncated IFN pathway, and adenoviruses with VA RNAs deleted retain replication in these cells, but not so in normal cells (Cascallo *et al.* 2003; Cascallo *et al.* 2006).

In addition, viral replication can be restricted to target cells by using tumor-specific promoters (TSPs). TSPs are usually added to control *E1A*, but alternatively, other genes such as *E4* can also be regulated. Various promoters have been used and some selected ones are presented in Table 1.

## **5.1 Oncolytic adenoviruses combined with targeting moieties**

To improve adenovirus-based replication in the context of clinically applicable cancer gene therapy, it is essential to increase targeting to tumor cells and decrease targeting to the liver due to possible toxicity. Adenovirus 5-based oncolytic viruses may fall victims to variable CAR expression, and a number of strategies exists to improve Ad5 based viral tropism.

Previously described transductional targeting methods have been combined with oncolytic viruses with good results. Serotype chimeric Ad5/3- $\Delta 24$  has shown increased therapeutic efficacy at least in ovarian, kidney, breast, and gastric cancers (Kanerva *et al.* 2003; Guse *et al.* 2007b; Kangasniemi *et al.* 2006; Ranki *et al.* 2007a). Ad5- $\Delta 24$ RGD, which has RGD

modification in the HI loop also has shown efficient cell killing in numerous cancer cells, including ovarian cancer, glioma and osteosarcoma (Lamfers *et al.* 2002; Suzuki *et al.* 2001; Witlox *et al.* 2004). Further, Ranki *et al.* added seven polylysins to the C-terminus of the Ad5 $\Delta$ 24 creating Ad5.pK7- $\Delta$ 24, which displayed enhanced oncolysis of breast cancer cells *in vitro* and *in vivo* (Ranki *et al.* 2007).

Recent studies have utilized double, or even triple-targeted adenoviruses to achieve more specificity. Combining a transductional targeting moiety with a tumor-specific promoter has resulted in a pancreatic and ovarian cancer specific adenovirus featuring Ad3 knob and Cox-2 promoter driving E1A with a 24 bp deletion in the CR2 (Bauerschmitz *et al.* 2006; Ramirez *et al.* 2008). Guse *et al.* used many double- and triple-targeted adenoviruses with Ad3 knob, RGD, and pK7 motifs combined with Cox-2 promoter driving CR1 and/or CR2 deletions with promising results in ovarian and renal cancer xenografts (Guse *et al.* 2007a).

## **5.2 Armed oncolytic adenoviruses**

Despite successful targeting and specific replication of oncolytic adenoviruses in the tumor tissue, clinical trials with early generation adenoviruses have shown that complete eradication of solid tumor masses rarely occurs. This might be due to intratumoral complexities such as stromal matrix and other extracellular barriers within the tumor tissue (Cheng *et al.* 2007). A useful approach for improving the potency of replicating agents is to arm viruses with therapeutic transgenes, e.g. genes encoding prodrug-converting enzymes (Hermiston and Kuhn 2002). Common approaches include HSV-TK and *Escherichia coli* cytosine deaminase (CD), which convert nontoxic prodrugs [ganciclovir (GCV) and 5-fluorocytosine, respectively] into cytotoxic metabolites. The activated drugs can spread into surrounding cells creating a so called bystander effect.

Reports combining a TK/GCV system with replicating adenoviruses have been rather controversial. Some studies have demonstrated that GCV enhances oncolytic potency of adenoviruses expressing HSV-tk (Nanda *et al.* 2001; Raki *et al.* 2007; Wildner *et al.* 1999), in contrast to others where oncolytic potential was not improved possibly due to the inhibition of viral replication by GCV (Hakkarainen *et al.* 2006; Lambright *et al.* 2001; Morris & Wildner 2000; Wildner & Morris 2000). Double suicide therapy with both HSV-

tk and CD genes has proven to be rather efficient, especially when combined with radiotherapy (Freytag *et al.* 1998; Rogulski *et al.* 2000; Wu *et al.* 2005).

### **5.3 Oncolytic adenoviruses in combination with conventional therapies**

Combining adenoviral gene therapy with traditional therapies is a powerful tool to achieve enhanced or even synergistic cell killing and antitumor efficacy. The mechanism of the additive or synergistic effect of combined therapy is not yet thoroughly known, although a few hypotheses have been tendered. Firstly, expression of adenoviral E1A protein may sensitize cells to chemotherapy or radiotherapy-mediated cell killing (Duque *et al.* 1998; Sanchez-Prieto *et al.* 1996). Secondly, chemotherapeutic agents may enhance the level of virus replication. Finally, chemotherapeutic agents may affect the receptor status of the target cells or each agent may work independently within the tumor tissue.

There are a number of studies where oncolytic adenoviruses have been combined with chemo- or radiotherapy, and some of them have even proceeded to clinical testing. ONYX-015 was the first virus to undergo clinical trials combined with chemotherapy. ONYX-015 was combined with 5-fluorouracil (5-FU) and cisplatin to treat squamous cell carcinoma of the head and neck carcinoma (Xia *et al.* 2004a; Khuri *et al.* 2000a). ONYX-015 was also combined with gemcitabine (pancreatic cancer) (Hecht *et al.* 2003), mitomycin C+doxorubicin + cisplatin (MAP) (advanced sarcoma) (Galanis *et al.* 2005a), and carboplatin + taxol (lung metastasis) (Nemunaitis *et al.* 2001), among other but a few studies. Chemo- and radiotherapies were combined with HSV-tk and CD-expressing adenovirus to treat prostate cancer with good safety data (Freytag *et al.* 2003b); these treatments may provide a potential long-term benefit for the patients (Freytag *et al.* 2007).

### **5.4 Cells as carriers of oncolytic adenoviruses**

Although infectivity and specificity of adenoviruses can be enhanced, obstacles remain that may hinder the bioavailability of viruses, especially after intravenous administration. Despite retargeting, the majority of systemically administered adenoviruses are taken up by Kupffer cells in the liver, decreasing the amount of available virus (Alemany *et al.* 2000). In addition, adaptive and innate immune responses may eliminate therapeutic

viruses (Bessis *et al.* 2004). Further, tumors are known to be very heterogeneous in structure, containing hypoxic, necrotic, and stromal areas, which may hinder spreading of the adenovirus within the tumor. A potential solution to solve these problems is the use of cells as viral carriers (Sanz *et al.* 2005; Raykov and Rommelaere 2008; Fritz and Jorgensen 2008). This might have advantages over systemic viral administration, as attenuated neutralization and improved viral targeting could result in smaller viral doses administered to patients, which may in turn reduce the systemic side effects. However, the use of cells as agents for systemic virus delivery depends on **1**) their ability to be transduced with adenovirus, **2**) their ability to allow the replication and release of oncolytic adenoviruses and **3**) their ability to home specifically to tumor. There are several cell types that have been used as carriers of viruses such as antigen-specific T-cells (Cole *et al.* 2005; Yotnda *et al.* 2004), macrophages (Griffiths *et al.* 2000), endothelial progenitor cells (Jevremovic *et al.* 2004), non antigen specific T-cells (Ong *et al.* 2007) and mesenchymal stem cells (MSCs)(Dvorak 1986; Studeny *et al.* 2004a).

MSCs are precursor cells that originate either from e.g. bone marrow or fat tissue, and they can differentiate into adipocytes, chondrocytes, osteoblasts, myoblasts, and tenocytes (Prockop 1997). One function of the MSCs is to repair injured tissue, and it is also hypothesized that the tumor environment resembles injured tissue, and therefore, circulating MSCs would home to tumors. (Dvorak 1986; Studeny *et al.* 2002; Studeny *et al.* 2004a). In some studies adenoviruses have been targeted to tumor tissue using MSCs as carriers. Nakamura *et al.* targeted adenoviral vector coding human interleukin-2 within MSCs with an augmented antitumor effect and prolonged survival of glioma-bearing rats (Nakamura *et al.* 2004). Stoff-Khalili *et al.* used hMSCs to target oncolytic adenovirus in to lung metastases of breast cancer which reduced the growth of lung metastases *in vivo* (Stoff-Khalili *et al.* 2007a).

## **6 Clinical trials with oncolytic adenoviruses**

Cancer is a complex disease that often eludes successful treatment due to its propensity to evolve or adapt in the face of current therapeutic regimes. Viral oncolytic therapy has been

under investigation as a novel anticancer strategy and the first cancer trial with wild type adenovirus was done as early as 1950s. Since the late 1990s oncolytic adenoviruses have been utilized to treat phase I-III cancer patients. The first oncolytic adenovirus used in a clinical trial was ONYX-015, which has a 55 kb deletion in the *E1b* gene, and thus, has tumor selectivity to cancer cells with mutant p53. The virus established a clinical proof-of-concept for oncolytic virotherapy in a first phase I trial in which the virus was directly injected into head and neck tumors with 14% regression rates (Ganly *et al.* 2000; Nemunaitis *et al.* 2000). When ONYX-015 was combined with cisplatin and 5-FU in the phase II trial, approximately 65% of patients showed tumor regression (Khuri *et al.* 2000b; Nemunaitis *et al.* 2000). A phase III trial was done in China with a closely related virus H101 and investigators reported 79% response rate in combination-treated patients versus 40% in controls without virus treatment ( $p < 0.001$ ) (Xia *et al.* 2004b) and later the virus got a official selling permit in China.

Another oncolytic adenovirus used in clinical trials is CV706. CV706 is a prostate-specific antigen (PSA)-selective, replication-competent adenovirus that showed in the phase I study good safety data and intraprostatic replication (DeWeese *et al.* 2001). In addition, another prostate specific, intravenously administered virus cg 7870 has been used in phase I study to treat hormone refractory metastatic prostate cancer with promising safety data (Small *et al.* 2006). Overall, trials with oncolytic adenoviruses have reported good safety data, but only marginal efficacy. Selected oncolytic adenovirus trials are summarized in Table 2.

For a more detailed listing of gene therapy trials, see

<http://www.abedia.com/wiley/index.html>.

**Table 2. Clinical trials with oncolytic adenoviruses.**

Virus/treatment	Genetic modification	Phase	Route	Max.dose	Disease	Beneficial/total	Reference
ONYX-015	E1b-55 kD deletion	I	i.t.	$1 \times 10^{11}$ pfu	SCCHN	2/22	(Ganly <i>et al.</i> 2000).
ONYX-015	<i>E1b</i> -55 kD deletion	I	i.t.	$1 \times 10^{11}$ pfu	Pancreatic cancer	0/23	(Mulvihill <i>et al.</i> 2001).

ONYX-015	<i>E1b</i> -55 kD deletion	I	i.v.	2x10 <sup>13</sup> vp	Cancer metastatic to lung	0/10	(Nemunaitis <i>et al.</i> 2001).
ONYX-015	<i>E1b</i> -55 kD deletion	I	i.p.	1x10 <sup>11</sup> pfu/d x 5d	Ovarian cancer	0/16	(Vasey <i>et al.</i> 2002).
ONYX-015+ 5-FU+ leucovorin	<i>E1b</i> -55 kD deletion	I	i.ha.	2x10 <sup>12</sup> vp	Colorectal cancer metastatic to liver	1/11	(Reid <i>et al.</i> 2001).
ONYX-015	<i>E1b</i> -55 kD deletion	I	i.t.	1x10 <sup>10</sup> pfu	Glioma	3/24	(Chiocca <i>et al.</i> 2004).
ONYX-015+ etanercept	<i>E1b</i> -55 kD deletion	I	i.v.	1x10 <sup>12</sup> pfu	Advanced ca.	0/9	(Nemunaitis <i>et al.</i> 2007).
CV 706	PSA promoter-enhancer contr. E1A	I	i.t.	1x10 <sup>13</sup> vp	Prostate cancer	5/20	(DeWeese <i>et al.</i> 2001).
Ad5- CD/TKrep + GCV/ 5-FU + radiation	<i>E1b</i> -55 kD deletion + TK/CD	I	i.t.	1x10 <sup>12</sup> vp	Prostate cancer	15/15	(Freytag <i>et al.</i> 2003a).
ONYX-015 + 5-FU	<i>E1b</i> -55 kD deletion	I-II	i.t. i.ha. i.v.	3x10 <sup>11</sup> pfu	HCC and colorectal cancer metastatic to liver	3/16	(Habib <i>et al.</i> 2001).
ONYX-015	<i>E1b</i> -55 kD deletion	II	i.t.	2x10 <sup>11</sup> vp X 10d	SCCHN	5/40	(Nemunaitis <i>et al.</i> 2001).

ONYX-015 + cisplatin and 5-FU	<i>E1b-55 kD</i> deletion	II	i.t.	1x10 <sup>10</sup> vp/d X 5d	SCCHN	19/37	(Khuri <i>et al.</i> 2000a).
ONYX-015 + Gemcitabine	<i>E1b-55 kD</i> deletion	I-II	i.t.	2x10 <sup>11</sup> vp/wk 8 cycles	Pancreatic cancer	2/21	(Hecht <i>et al.</i> 2003).
ONYX-015	<i>E1b-55 kD</i> deletion	II	i.v.	2x10 <sup>12</sup> vp/ 2 weeks	Metastatic colorectal cancer	0/18	(Hamid <i>et al.</i> 2003).
CG7870	Rat probasin controlling <i>E1A</i>	I-II	i.t.	1x10 <sup>13</sup> vp	Prostate cancer	10/20	DeWeese 2003
H101 + cisplatin/ adriamycin + 5-FU	<i>E1b-55 kD</i> deletion	III	i.t.	1.5x10 <sup>12</sup> vp/d X 5d	SCCHN	71/160	(Xia <i>et al.</i> 2004b).
ONYX-015 + MAP chemotherapy	<i>E1b-55 kD</i> deletion	I-II	i.t.	5x10 <sup>10</sup> pfu	Sarcoma	1/6	(Galanis <i>et al.</i> 2005b).

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

## 7 Adenoviruses and immune response

Effective readministration of adenovirus might be important factor in achieving full potential of adenoviral gene therapy for treating many diseases. More than 50 different

serotypes of adenoviruses are known to infect humans. Due to the ubiquitous nature of adenoviruses, a majority of the human population has been exposed to them (also to Ad5), leading to the development of adenovirus-specific neutralizing antibodies (NAbs). A high neutralizing antibody titer might not be a limiting factor in local treatment (Cichon *et al.* 2001; Hodges *et al.* 2005), but it can decrease the bioavailability of systemically administered adenoviruses. NAbs can be induced against all adenoviral capsid proteins, such as hexon, penton and fiber and there may even be synergy between them (Gahery-Segard *et al.* 1998; Rahman *et al.* 2001). Strategies that are being examined to circumvent immune response to adenovirus vectors include immunosuppression, immunomodulation, serotype switching, use of targeted adenovirus vectors, microencapsulation of adenovirus vectors, use of helper-dependent (HD) adenovirus vectors, and development of nonhuman adenovirus vectors.

### **7.1 Manipulation of adenoviruses to circumvent immune response**

Following intravenous inoculation, adenoviruses are generally taken up by Kupffer cells in the liver, and by macrophages in the spleen leading to rapid induction of an innate immune response. The innate immune response is activated following recognition of molecular patterns on the adenovirus capsid by receptors on macrophages and dendritic cells (DCs), resulting in activation of multiple signaling pathways, such as mitogen-activated protein kinase (MAPK) and nuclear factor (NF)- $\kappa$ B pathways, that augment expression of several proinflammatory cytokines and chemokines (Bruder and Kovesdi 1997; Lieber *et al.* 1997; Muruve *et al.* 1999). The use of such immunosuppressive agents as cyclosporine and cyclophosphamide to block antibodies raised against adenovirus enhances the duration of transgene expression (Smith *et al.* 1996). Since macrophages in the liver activate the immune response, there are approaches where depletion of macrophages and dendritic cells has resulted in increased transgene expression (Ranki *et al.* 2007b; Schiedner *et al.* 2003). Further, alteration of the immunodominant epitopes of adenovirus capsid can also help to evade immune response. Covalent attachment of polymers, such as polyethylene glycol (PEG), to the adenovirus capsid has been shown to inhibit antibody-mediated virus neutralization but there is evidence that it also decreases tumor transduction of the virus (Croyle *et al.* 2002; Croyle *et al.* 2005).



Adenoviral serotypes are classically determined based on neutralization assay and it is therefore logical that different serotype can overcome NAb. In some studies, serotype switching in vector construction has helped to overcome immune response (Kass-Eisler *et al.* 1996; Mastrangeli *et al.* 1996). Especially the use of subgroup B adenoviruses, such as Ad3, Ad11 and Ad35 has been promising since these are not dependent on CAR-mediated internalization and can evade pre-existing Ad5 immunity (Barouch *et al.* 2004; Stone *et al.* 2005; Vogels *et al.* 2003; Kanerva *et al.* 2002b). The adenoviral fiber knob has been shown to induce DC activation and maturation (Molinier-Frenkel *et al.* 2003), and therefore, fiber knob modifications to incorporate cellular ligands with novel cell-binding capacity might confer targeting and be one way to decrease vector immunogenicity (Nanda *et al.* 2005) Adenoviral microencapsulation with, for instance, polyethylene glycol-cationic lipid and poly (lactic-glycolic) acid (PLGA) copolymer has been demonstrated to guard adenovirus vectors from neutralizing antibodies and to be capable of effective transduction and gene expression on target cells (Beer *et al.* 1998; Chillon *et al.* 1998; Steel *et al.* 2004). Lastly, utilization of helper dependent (HD) adenoviruses has resulted in controversial results in evading immune response. HD adenoviral vectors lack all coding sequences of the adenovirus genome, except the packaging sequence and inverted terminal repeats, have been shown to elicit minimal cell-mediated immune response (Morsy *et al.* 1998) but led to acute toxicity accompanied by activation of the innate immune response after systemic administration in baboons (Brunetti-Pierrri *et al.* 2004).

To extend the range of Ad vectors that can be used to evade human adenovirus neutralizing immune response, a number of nonhuman adenoviruses, such as bovine Ad type 3 (BAd3), canine Ad type 2, porcine Ad type 3, ovine Ad ,and chimpanzee Ad have been exploited for gene transfer, without hindrance from cross-neutralizing effects (Bangari & Mittal 2004; Farina *et al.* 2001; Hemminki *et al.* 2003; Hofmann *et al.* 1999; Mittal *et al.* 1995).

## **Aims of the study**

- 1.** To determine the best receptor binding motif for NSCLC cell lines and fresh NSCLC primary tissue samples *in vitro* and *in vivo* and to evaluate gene transfer and antitumor efficacy of oncolytic adenoviruses through non-CAR receptors. In addition, the biodistribution, and the oncolytic potency of adenoviruses were evaluated *in vivo* with noninvasive imaging. **(I)**.
- 2.** To examine the capacity of capsid-modified adenoviruses to infect and replicate in MSCs. Further, biodistribution, tumor-homing ability, and tumor-killing efficacy of systemically delivered, virus-loaded MSCs in orthotopic lung and breast cancer tumor models were evaluated **(II)**.
- 3.** To investigate induction of the Cox-2 promoter both in normal and tumor cells after adenovirus infection and to explore the utility of AU-rich elements for regaining promoter selectivity **(III)**.
- 4.** To evaluate whether changing the adenovirus fiber knob could allow the virus to overcome pre-existing adenoviral NABs in the context of low or high NAb titer, in mice with advanced orthotopic NCSLC, and in human clinical samples of both cancerous and nonmalignant lung tissue **(IV)**.

## Materials and methods

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

### 1 Cell lines, human mesenchymal stem cells (MSCs), and primary NSCLC (I-IV)

**Table 3. Human cell lines used in studies I-IV.**

Cell line	Description	Source	Study
<b>293</b> ATCC CRL-1573	Transformed embryonic kidney cells	ATCC (Manassas, VA)	I,III
<b>NCI-H460</b> HTB-177	Lung large-cell carcinoma	ATCC	I
<b>NCI-H661</b> HTB-183	Lung large-cell carcinoma	ATCC	I
<b>LNM35/EGFP</b>	Lung large-cell carcinoma expressing enhanced green fluorescent protein	Takashi Takahashi (Honda Research Institute, Japan)	I,II, III, IV
<b>SW900</b> HTB-59	Lung squamous cell carcinoma	ATCC	I
<b>Ma44-3</b>	Lung squamous cell carcinoma	Kazuya Kondo (University of Tokushima, Japan),	I
<b>NCI-H520</b> HTB-182	Lung squamous cell carcinoma	ATCC	I
<b>Calu-3</b> HTB-55	Lung adenocarcinoma	ATCC	I
<b>NCI-H23</b> CRL-5800	Lung adenocarcinoma	ATCC	I

<b>A549</b> CCL-185	Lung adenocarcinoma	ATCC	I,III,IV
<b>201T</b>	Lung adenocarcinoma	Jay D. Hunt (Louisiana State University, New Orleans, LA),	I
<b>M4A4-LM3</b>	Breast cancer	S.Goodison (University of Florida, FL)	II
<b>Hs173we</b> CRL-7834	Human fibroblast	Gerd Bauerschmitz, (University of Duesseldorf, Germany)	III

Fresh NSCLC primary tissue samples were collected after signed informed consent from patients diagnosed with advanced lung cancer at the Helsinki University Central Hospital. The study was approved by the hospital Ethics Committee (I). Bone marrow-derived human mesenchymal stem cells were isolated as described previously (Leskelä *et al.* 2003). Adipose stem cells were obtained from human subcutaneous and intraperitoneal adipose tissues (II).

## 2 Adenoviral vectors and replicating adenoviruses (I-IV)

**Table 4. Replication deficient and oncolytic adenoviruses used in this study**

<b>Virus</b>	<b>EIA</b>	<b>Transgene</b>	<b>Capsid modification</b>	<b>Target receptor</b>	<b>Source</b>	<b>Study</b>
<b>Adluc1</b>	Deleted	<i>Luciferase</i>	-	CAR	(Dmitriev <i>et al.</i> 1998).	I,II, IV
<b>A5/3luc1</b>	Deleted	<i>Luciferase</i>	Serotype 3 knob	Unknown and CD46	(Kanerva <i>et al.</i> 2002a).	I, IV
<b>Ad5lucRGD</b>	Deleted	<i>Luciferase</i>	RGD motif in the HI	$\alpha_v\beta$ integrins	(Dmitriev <i>et al.</i>	I,II, IV

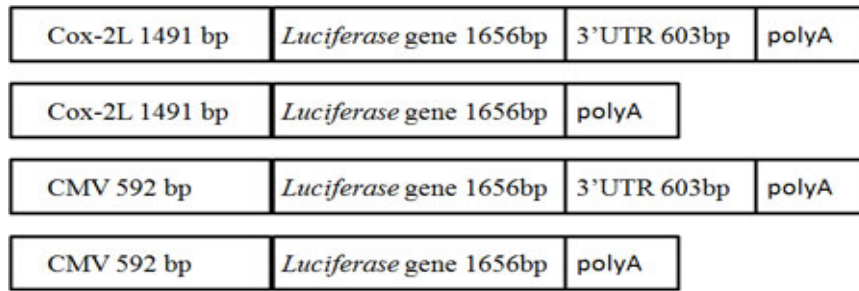
			loop	and CAR	1998).	
<b>Ad5(GL)</b>	Deleted	<i>Luciferase</i> and <i>GFP</i>	-	CAR	(Wu <i>et al.</i> 2002).	I, II, IV
<b>Ad5.pK7(GL)</b>	Deleted	<i>Luciferase</i> and <i>GFP</i>	7 polylysins in the C- terminus	Heparan sulfates and CAR	(Wu <i>et al.</i> 2002).	I, II, IV
<b>Ad5.RGD.pK7(GL)</b>	Deleted	<i>Luciferase</i> and <i>GFP</i>	RGD motif in the HI loop, seven polylysins in the C- terminus	$\alpha_v\beta$ integrins, Heparan sulfates, CAR	(Wu <i>et al.</i> 2002).	I, II
<b>Ad5.LacZ</b>	Deleted	$\beta$ - <i>galactosidase</i>	-	CAR	(Sarkar <i>et al.</i> 2005).	I
<b>Ad5.pK7LacZ</b>	Deleted	$\beta$ - <i>galactosidase</i>	seven polylysins in the C- terminus	Heparan sulfates, CAR	(Sarkar <i>et al.</i> 2005).	II
<b>Ad5F/K21</b>	Deleted	$\beta$ - <i>galactosidase</i>	21 polylysins in the C- terminus	Heparan sulfates, CAR	(Sarkar <i>et al.</i> 2005).	I
<b>Ad5Cox2Lluc</b>	Deleted	Cox-2 promoter <i>luciferase</i>	-	CAR	This study unpublished data	
<b>Ad5Cox2LlucUL</b>	Deleted	Cox-2 promoter <i>Luciferase</i> , 3'UTR	-	CAR	This study unpublished data	
<b>Ad300wt (Ad5 wild-type)</b>	Intact	-	-	CAR	ATCC	I,II, III
<b>Ad5<math>\Delta</math>24E3+</b>	24-bp deletion in CR2	-	-	CAR	(Suzuki <i>et al.</i> 2001).	I, II, III

<b>Ad5/3 -Δ24</b>	24-bp deletion	-	Serotype 3 knob	Unknown, CD46	(Kanerva <i>et al.</i> 2003).	I, II, III
<b>Ad5- Δ24RGD</b>	24-bp deletion	-	RGD motif in the HI loop	$\alpha_v\beta$ integrins,	(Suzuki <i>et al.</i> 2001).	I, II
<b>Ad5.pK7-Δ24</b>	24-bp deletion	-	seven polylysins in the C- terminus	Heparan sulfates,	(Ranki <i>et al.</i> 2007).	I, II

## 2.1 Construction of Cox-2 and 3'UTR element-expressing plasmids

### (III)

In the plasmids pShuttleCox-2Luc and pShuttleCox-2Luc + 3'-UTR, the Cox-2L promoter (-1492/+59) was amplified by PCR from full-length Cox-2 containing pGL3/Cox-2 using forward primer 5'-AAAAGATCTGAGGTACCTGGT-3' to place a *BglIII* site at the 5'-end of Cox-2L and reverse primer 5'-AAAAGCTTCGCTGCTGAGGA-3' to place a *HindIII* site at the 3'-end. A 603-bp long 3'-UTR fragment from Cox-2 was amplified from pZeo/Luc3 + 3'-UTR using forward primer 5'-AAAATCTAGAAAGTCTAATGATC-3' and reverse 5'-AAAATCTAGAACTTTAAG-3' to place *XbaI* at both ends. The Cox-2L promoter and 3'-UTR were cloned into luciferase-containing plasmid pGL3basic. The final constructs: pShuttleCox-2Luc and pShuttleCox-2Luc + 3'-UTR were created by digesting pGL3Cox-2Luc and pGL3Cox-2Luc + 3'-UTR with *KpnI* and *Sall* and subcloning into pShuttle. pShuttleCMVluc and pShuttleCMVluc + 3'-UTR were also created: the luciferase gene and 3'-UTR were digested with *KpnI* and *Sall* from pGL3 basic and inserted into pShuttleCMV. All plasmids were purified by agarose gel electrophoresis.



**Figure 6. Schematic presentation of the plasmids constructed in this study:**

pShuttleCox-2Luc + 3'-UTR, pShuttleCox-2Luc, pShuttleCMVluc + 3'-UTR and pShuttleCMVluc.

**Table 5. The plasmids used in this study**

Plasmid	Description	Source	Used in
<b>pGL3basic</b>	pGL3 cloning vector	Promega, Madison, WI	III
<b>pGL3/Cox-2</b>	pGL3 vector containing full-length Cox-2	Ari Ristimäki, University of Helsinki, Finland	III
<b>pZeo/Luc3 + 3'-UTR</b>	pZeo/Luc3 vector containing full-length 3'UTR	Ari Ristimäki	III
<b>pShuttleCMV</b>	pShuttle vector containing CMV promoter	Stratagene, La Jolla, CA	III
<b>pShuttleCox-2Luc</b>	pShuttle vector containing Cox-2L promoter driving luciferase	This study	III
<b>pShuttleCox-2Luc + 3'-UTR</b>	pShuttle vector containing Cox-2L promoter driving luciferase and 3'UTR element	This study	III
<b>pShuttleCMVluc</b>	pShuttle vector containing CMV promoter driving luciferase	This study	III
<b>pShuttleCMVluc + 3'-UTR</b>	pShuttle vector containing CMV promoter driving luciferase and 3'UTR element	This study	III
<b>pAdEasyI</b>	<i>E1/E3</i> deleted Ad5 genome	This study unpublished data	

## **2.2 Construction of non replicating adenoviruses**

Previously described plasmids pShuttleCox-2luc and pShuttleCox-2luc+3'UTR were digested with *PmeI* and homologous recombination was done with AdEasy I plasmid. Resulting plasmid was further digested with *PacI* and transfected with 293 cells.

## **2.3 *In vitro* transfection of plasmids (III)**

$1.5 \times 10^5$  cells were seeded into 12-well plates 17–20 h prior to transfection; 1  $\mu$ g of each plasmid were transfected with Fugene 6 reagent (Roche, Basel, Switzerland) in a ratio of 3:2 (Fugene 6/plasmid) following the manufacturer's instructions.

## **2.4 High titer production of viruses (I-IV)**

Replication-deficient and oncolytic adenoviruses were propagated in 293 and A549 cells, respectively. Viruses were purified by standard cesium chloride gradient techniques, and quality control was performed by PCR. Viral particles (vp) were determined by spectrophotometry, infectious units are expressed as the median tissue culture infective dose<sub>50</sub> (TCID<sub>50</sub>) (Svensson *et al.* 1999).

## **3 *In vitro* studies**

### **3.1 Adenovirus-mediated gene transfer assays (I-III)**

Cells and tissue samples were infected with Ad5luc1, Ad5(GL), Ad5/3luc1, Ad5lucRGD, Ad5.pK7(GL), or Ad5.RGD.pK7(GL) for 30min and 1 hour respectively. Luciferase activity was determined 24 h postinfection with a luciferase assay system (Promega) according to the manufacturer's protocol. Values are presented relative to syngeneic control viruses [Ad5luc1 and Ad5(GL)], which were assigned a value of 1. For immunohistochemistry, NCI-H460 cells were infected in chamber slides (Nunc, Naperville, IL). The next day, the cells were fixed with 10% formalin and permeabilized with 0.1% Tween 20/phosphate-buffered saline (PBS). Blocking was done with 1% bovine



serum albumin (BSA)/PBS for 30 min at room temperature. Antihexon was used as a primary antibody at a concentration of 1.5 mg/ml (LabFrontier, Seoul, Korea). Texas Red-labeled secondary antibody (AbCAM, Cambridge, UK) was used at a concentration of 2 mg/ml. Cells were stained with Hoechst dye (Oriola, Espoo, Finland), positive cells were counted, and fluorescence images obtained (Olympus Ix81, Biosystems, Munich, Germany).

### **3.2 Determination of receptor expression by flow cytometry (II)**

For flow cytometric analysis, cells ( $2 \times 10^5$ ) were incubated for 20 min at 4°C with the following primary antibodies at a 1:200 dilution: anti-CAR (clone RmcB; Upstate Cell Signaling Solutions/ Millipore, Lake Placid, NY), anti-HSPG (clone F58-10E4; Seikagaku, Falmouth, MA), anti- $\alpha_v\beta_3$  integrin (clone LM609; Chemicon International/Millipore, Temecula, CA), anti- $\alpha_v\beta_5$  integrin (clone P1F6; Chemicon International/Millipore), or anti-CD46 (clone E4.3; BD Biosciences, San Jose, CA). Cells were washed with fluorescence-activated cell-sorting (FACS) buffer (phosphate buffered saline, PBS containing 2% serum) and incubated with a 1:200 dilution of phycoerythrin-labeled secondary antibody (goat anti-mouse polyclonal antibody; BD Biosciences) for 30 min at 4°C. Cells were washed with FACS buffer and analyzed by flow cytometry (FACSCalibur; BD Biosciences) to determine receptor expression levels. Unstained cells were used as a negative control.

### **3.3 Cytotoxicity assay (I, II)**

The 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfophenyl)-2H-tetrazolium, inner salt [“MTS”] cell killing assay was performed, and viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay; Promega), when at least one tested virus showed a cytotoxic effect in the two highest dilutions. In the crystal violet assay, cells were stained with crystal violet when the cytotoxic effect was as above.

### **3.4 Semiquantitative polymerase chain reaction (III)**

Transfected Hs173We cells were incubated with Proteinase K (Qiagen, Valencia, CA, USA) at 37°C for 30 min. Proteinase K was then inactivated by 20 min of incubation at

95°C. Amplification (30, 40, and 60 cycles, annealing at 50°C) of a 77-bp DNA fragment was carried out by using primers that were positioned at the left end of the adenovirus genome, between the left inverted terminal repeat and the packaging signal. Upstream: 5'-GCGACGGATGTGGCAAAAGT-3'(150-171bp) and downstream: 5'-CCTAAAACCGCGCGAAAA-3' (211-228bp).

### **3.5 Assessment of neutralizing antibody (NAb) titers (IV)**

A549 cells were seeded on 96-well plates and cultured overnight. Serum collected from the immunized ICR mice was incubated at 56°C for 60 min to inactivate the complement and then a 4-fold dilution series of serum was prepared in growth media without serum. Ad5(GL) (100 vp/cell) was mixed with diluted serum samples and incubated at room temperature on a rocker for 30 min, and the mix was added to cells in quadruplicates. Two hours later, the infection media was replaced by fresh growth media. After 48 h, luciferase expression was measured by FluorStar Optima luminometer (BMG LabTech, Offenburg, Germany) according to the manufacturer's instructions. NAb titer was determined as the lowest degree of dilution that blocked gene transfer by more than 50%.

### **3.6 Effect of NAb on gene transfer (IV)**

Human NSCLC and normal lung tissue samples were homogenized and infected with Ad5(GL), Ad5/3luc1, and Ad5lucRGD (1000 vp / cell). Viruses were preincubated with (0, 1:512, and 1:4 dilutions) inactivated serum from Ad5(GL)-immunized mice at room temperature for 30 min before infection of tissue explants. A luciferase assay was performed in quadruplicate 24 h later by the using Luciferase Assay System (Promega).

## **4 *In vivo* studies**

### **4.1 Mice and mice models used (I-IV)**

Female NMRI nude mice and immunocompetent ICR mice aged 3-4 weeks were obtained from Taconic (Ejby, Denmark) and quarantined for 2 weeks. All animal experiments were

approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

Subcutaneous models were created as follows:  $1 \times 10^6$  293 and  $5 \times 10^5$  A549 cells were injected into the flanks of mice. Tumors were allowed to develop for 30 days (293) or 14 days (A549) to reach 5 mm in diameter. An orthotopic model of locally advanced lung cancer was created by injecting  $2 \times 10^6$  cells (LNM35/EGFP) into the left lung of NMRI nude mice in a total volume of 200  $\mu$ l using a syringe with a 27G needle. The orthotopic breast cancer model used in these studies has been described in more detail by Ranki *et al.* (Ranki *et al.* 2007b). Briefly,  $2 \times 10^6$  M4A4-LM3 cells were injected into both the right and the left second lowest mammary fat pads. Tumors were allowed to develop until they reached a diameter of approximately 4 mm. Before injections, the mice were anesthetized with medetomidine (Domitor; Orion Pharma, Espoo, Finland) and ketamine (Ketalar; Pfizer, New York, NY) at 1:2.

#### **4.2 Biodistribution assay (I, IV)**

After tumors had developed (I) or 1 month after immunization (IV)  $3 \times 10^{10}$  vp were injected through the tail vein, and 48 h later, organs were harvested and frozen at  $-80^\circ\text{C}$ . Organs were homogenized in Cell Culture Lysis Buffer (Promega), and luciferase expression was measured by using FluoStar Optima luminometer.

#### **4.3 Therapeutic assay (I, II)**

Advanced intrapulmonary disease was allowed to develop for 5 days and mice were then divided randomly into 5 groups (n=12 mice per group) (I) and (n=6-10 mice per group) (II). Viruses were injected into the tail vein ( $2 \times 10^{10}$  vp/mouse). Mice were treated on days 5, 12, and 19 (I). Viruses injected along with MSC groups are as follows: either MSCs ( $7 \times 10^5$  cells/mouse, n=5), virus [ $1.4 \times 10^8$  vp of Ad5.pK7(GL),  $7 \times 10^7$  vp of Ad5.pK7- $\Delta$ 24, n=10], MSCs infected with nonreplicating adenovirus [Ad5.pK7(GL) at 200 vp/cell, Ad5pK7LacZ at 100 vp/cell,  $7 \times 10^5$  cells/mouse, n=6] or MSCs infected with oncolytic adenoviruses [Ad5.pK7(GL) at 200 vp/cell, Ad5.pK7- $\Delta$ 24 at 100 vp/cell,  $7 \times 10^5$  cells/mouse, n=10] 7, 11, and 15 days after cancer cell inoculation. In the breast cancer model, tumors were measured in two dimensions with calipers and tumor volumes were

calculated according to the following formula: length x width<sup>2</sup> x 0.5. The health of the mice was monitored daily, and mice were killed according to humane endpoint guidelines.

#### **4.4 *In vivo* transfection of plasmids and infection of viruses (III)**

Transfection was accomplished by using *in vivo* JetPei cationic polymer transfection reagent (Poly transfection, Illkirch, France) following the manufacturer's protocol. Twenty four hours after transfection,  $5 \times 10^9$  vp/tumor of Ad300wt in 100  $\mu$ l was injected intratumorally. Tumors were collected and luciferase expression was analyzed as described previously in the biodistribution assay.

#### **4.5 Noninvasive *in vivo* imaging (I-III)**

Imaging was done by using the IVIS 100 Imaging System (Xenogen, Alameda, CA). Fluorescence emission was quantitated by determination of regions of interest, which were normalized to reference regions in the same image, but without tumor. When luciferase was imaged, luciferin substrate (4.5 mg/mouse) was injected intraperitoneally before bioluminescence imaging.

#### **4.6 Induction of NAbs (IV)**

ICR mice were immunized with viruses subcutaneously. Injection of  $3 \times 10^8$  vp occurred either once on day 0 or thrice on days 0, 3, and 6. Control animals were mock-immunized with serum-free media. On day 27, blood was collected from the hindlimb artery and allowed to clot, and serum was then separated by centrifugation and stored at -20°C for further experiments.

#### **4.7 Effect of NAbs on gene transfer (IV)**

Orthotopic tumors of advanced lung cancer were allowed to develop for 9 days before mice were injected with either viruses or serum at 1:512 or 1:4. Serum was pooled from ICR mice immunized once or thrice with Ad5(GL). Tumors were harvested 48 h later and luciferase assay was performed by FluorStar Optima. The data are protein-normalized (to

account for possible differences in tumor size) and presented relative to gene transfer obtained with no NAb.

## 5 Statistics (I-IV)

Pairwise comparisons were performed with Student's *t* test, one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test, or ANOVA followed by Dunnett's pairwise multiple comparison *t*-test. Survival data were analyzed with a log-rank test using SPSS software (version 11.5; SPSS Inc., Chicago, IL) or PROC LIFETEST (SAS version 9.1; SAS Institute, Cary, NC), using a Weibull distribution. Analysis of whether MSCs could improve the efficacy of oncolytic virus was performed with  $\chi^2$  test (II). For comparison of tumor size, data were analyzed with a repeated measures growth model with PROC MIXED (SAS version 9.1). The effects of treatment group, time, and the interaction of treatment group and time were evaluated by *F*- tests (II). Analysis of TCID<sup>50</sup> data was performed with an unpaired *t*- test with Welch's correction (II). The results from Cox-2 induction *in vivo* (III) were analysed with Multtest, version 9.1 (SAS Institute Inc., Cary, NC, USA). For all analyses,  $p < 0.05$  was deemed significant.

## Results and discussion

### 1 Modified adenoviruses show enhanced gene transfer to NSCLC cell lines, MSCs, and primary lung cancer tissues *in vitro* (I, II)

One of the major concerns in adenoviral gene therapy is effective targeting since the primary receptor CAR exhibits variable expression on cancer cells (Bauerschmitz *et al.* 2002; Kanerva *et al.* 2002b). We hypothesized that by using modified adenoviruses, we could enhance gene transfer to NSCLC cell lines, bone marrow, or fat tissue-derived human mesenchymal stem cells and lung cancer tissue samples fresh from patients. Ten NSCLC cell lines (Figure 1 in Study I) were infected with Ad5luc1, Ad5/3luc1, Ad5lucRGD, Ad5(GL), Ad5.pK7(GL), Ad5.pK7.RGD(GL), Ad5LacZ, and Ad5F/K21 replication-deficient adenoviruses (Table 4). On the basis of our results,  $\alpha_v\beta$  integrins and HSPG targeted viruses enhanced transduction up to 270- and 100-fold respectively in NSCLC cell lines. To examine whether the increased gene transfer was due to enhanced infection of the cells, we stained Ad5luc1-, and Ad5lucRGD-infected cells with antihexon antibody, which confirmed the hypothesis. In addition, we investigated gene transfer in primary NSCLC samples fresh from patients without any passaging, since geno- and phenotypes of clinical tumor specimens better resemble human tumor tissue. The results of four adenocarcinoma and two squamous cell carcinoma samples were in line with those of the NSCLC cell lines. The HSPG targeted Ad5pK7(GL) virus had up to 200-fold better gene transfer compared to the unmodified control virus ( $p < 0.001$ ) (Figure 2 in Study I). Both unmodified and genetically engineered MSCs have been widely used for various therapeutic purposes preclinically, and a number of studies have suggested an immune-privileged nature for MSCs and their ability to avoid allogeneic rejection in human and animal models, broadening their potential for therapeutic use (Aggarwal and Pittenger 2005; Chamberlain *et al.* 2004; Le Blanc *et al.* 2004). Antitumor activity of retrovirus vector-transduced MSC, producing IL-12, has been tested in a mouse melanoma model (Elzaouk *et al.* 2006), and MSC, transduced by a lentivirus vector to express human

OPG (osteoprotegerin) has been evaluated for the treatment of osteolysis in a myeloma bone disease model (Rabin *et al.* 2007). Modified MSCs loaded with adenoviruses have been successfully used preclinically as treatment alternatives for glioma, melanoma, and ovarian and breast cancers (Komarova *et al.* 2006; Stoff-Khalili *et al.* 2007b; Stoff-Khalili *et al.* 2007b; Studeny *et al.* 2002; Studeny *et al.* 2004b; Sonabend *et al.* 2008).

Unfortunately, the infectivity of MSCs with serotype Ad5 viruses has been limited due to low expression of CAR (Conget & Minguell 2000; Mizuguchi *et al.* 2005; Studeny *et al.* 2002).

Based on our results,  $\alpha_v\beta$  integrin- or HSPG-targeted adenoviruses increased gene transfer 1100- and 11000-fold compared with the untargeted virus. To further confirm the data, we studied the expression levels of target receptors on MSCs by flow cytometry. Interestingly, the most expressed receptor was CD46, a proposed receptor of Ad3, although only slightly enhanced transduction of Ad3-targeted virus was seen (Figure 1 in Study II). These results are in accordance with data suggesting that CD46 is not the only receptor of Ad3 (Gaggar *et al.* 2003; Tuve *et al.* 2006b).

## **2 Biodistribution of capsid-modified adenoviruses (I)**

The orthotopic murine model of advanced NSCLC created in this study may resemble the actual disease to some degree since it is very aggressive. The disease spreads from the primary tumor (located in the left lung with this model), to the mediastinal lymph nodes and later to the peritoneum. Replication-deficient viruses Ad5luc1, Ad5/3luc1, Ad5lucRGD, Ad5(GL), Ad5. pK7(GL), and Ad5.pK7.RGD(GL) were used to examine biodistribution after systemic delivery (Figure 4 in Study I). Although systemic delivery of viruses might be useful for treatment of disseminated diseases, there are many hurdles that a therapeutic vector faces in the bloodstream, such as the innate and adaptive immune response, potential adsorption to erythrocytes and thrombocytes and clearance by specialized cells in the liver and spleen (Baker *et al.* 2007; Mohr *et al.* 2008).

In our study, no statistical differences were observed between viruses. Although Ad5lucRGD and Ad5. pK7(GL) viruses showed moderately high gene delivery to the

lungs, kidneys, peritoneum, and ovaries, transduction to the tumor was also seen with the same viruses. The route of administration clearly also affects biodistribution since when administered systemically, adenovirus is exposed to many factors in the blood, such as coagulation factors IX and X and complement component C4-binding protein, which might determine the biodistribution of the virus (Shayakhmetov *et al.* 2005).

### **3 Capsid-modified adenoviruses display improved cell killing efficacy in NSCLC *in vitro* and *in vivo* (I)**

Even though efficacy has been seen following local administration, nonreplicating adenoviral agents have not yet demonstrated systemic therapeutic efficacy in humans. To overcome this problem, oncolytic adenoviruses have been developed. An advantage gained by using replication-competent oncolytic viruses is the potential for local amplification within the tumor, following initial transduction of even a small number of cells. In theory, this oncolytic process continues as long as target cells for the virus persist. This process when rigorously controlled in the oncolytic adenovirus context could be of enormous therapeutic benefit. Oncolytic potency of replicating agents is mostly determined by the infectivity of the viruses (Douglas *et al.* 2001; Hemminki *et al.* 2001). Unsurprisingly, targeted oncolytic adenoviruses have shown impressive preclinical efficacy (Kanerva *et al.* 2003; Ranki *et al.* 2007; Suzuki *et al.* 2001).

We evaluated the potency of oncolytic adenoviruses harboring the same capsid modifications with a cell viability assay *in vitro* (Figure 3 in Study I). In contrast to previous results with replication-deficient viruses, Ad5/3- $\Delta$ 24 (Table 4) showed the best cell killing efficacy in all cell lines tested. The finding could be explained by the structure of RGD-4C- or pK7-containing virions, which may lead to less rapid packaging (in comparison to gene delivery), perhaps due to the “sticky” nature of these moieties.

*In vivo*, with the same panel of viruses in an orthotopic model of advanced lung cancer, the median survival times were 19, 22, 23, 26, 26, and 26 days for no virus, Ad5luc1, Ad5/3- $\Delta$ 24, Ad5- $\Delta$ 24E3, Ad5.pK7- $\Delta$ 24, and Ad5- $\Delta$ 24RGD, respectively (Figure 5a in Study I). The therapeutic efficacy of Ad5- $\Delta$ 24E3, Ad5.pK7- $\Delta$ 24, and Ad5- $\Delta$ 24RGD was



significantly enhanced compared with the untreated group ( $p < 0.01$ ). One mouse from the Ad5- $\Delta$ 24RGD-treated group survived until the end of the experiment (93 days). Ad5/3- $\Delta$ 24 virus did not enhance survival of the mice, which might be partly explained by the biodistribution data. This finding is in accordance with data obtained from an orthotopic breast cancer model (Ranki *et al.* 2006). The route of administration clearly has an effect on the infectivity and oncolytic potency of Ad5/3- $\Delta$ 24 since after intraperitoneal administration of Ad5/3- $\Delta$ 24 in an orthotopic ovarian cancer model, survival was enhanced dramatically (Kanerva *et al.* 2003).

#### **4 MSCs target lungs *in vivo* (II)**

To use MSCs as carriers for adenoviruses, they should support the replication of oncolytic adenoviruses. To study replication and subsequent cytolysis, we infected *in vitro* passaged MSCs with a panel of oncolytic adenoviruses containing a 24 bp deletion in the E1A region and cell viability was analyzed by MTS assay (Figure 2 in Study II). The oncolysis correlated with the gene transfer of the viruses with the same targeting moieties (Figure 1 in Study II). HSPG- and  $\alpha_v\beta$  integrin-targeted oncolytic viruses showed significantly higher cytolysis rates in both adipose tissue- and bone marrow-derived cells. Ad5/3- $\Delta$ 24 virus also enhanced cell killing significantly compared with the unmodified virus (All  $p < 0.001$ ). The data revealed that cytolysis correlates with gene transfer. Previous finding of Komarova *et al.* showed that MSCs enable the replication of 5/3 targeted adenovirus with intact E1 (Komarova *et al.* 2006). Our viruses have a 24 bp deletion in E1A, allowing them to replicate in cells with a defective p16/Rb pathway. However, *in vitro* passaged MSCs feature phosphorylation of the Rb protein, which would be expected to allow replication of  $\Delta$ 24-type viruses, explaining the replication seen here (Heise *et al.* 2000a). We found no differences between the ability of adipose tissue- and bone marrow-derived cells to support the replication and allow release of active virions.

Since MSCs have been shown to target tumors and act as a shield to pre-existing antibodies (Aggarwal & Pittenger 2005; Le Blanc *et al.* 2004; Mohr *et al.* 2008), these engineered stem cells might serve as therapeutic Trojan horses, therefore enhancing the

therapeutic efficacy *in vivo* compared with the virus alone. Recently, Gutova *et al.* provided evidence that urokinase plasminogen activator (uPA) and its receptor uPAR, involved in chemotaxis and cell guidance during normal development, are upregulated in invasive tumors and might augment neural and mesenchymal stem cell tropism (Gutova *et al.* 2008).

In this study, we tested whether bone marrow-derived MSC could support viral replication and release in the context of orthotopic lung tumors. Mice received a single dose of intravenous oncolytic adenovirus (Ad5.pK7- $\Delta$ 24) or MSCs infected either with replication-deficient or oncolytic adenovirus. Luciferase-expressing virus was included in each group, which allowed us to use the noninvasive bioimaging system to follow the biodistribution of the virus (Figures 3 and 4 in Study II). Mice were imaged 15 min, and 3, 7, and 10 days after treatment. Initially, the luciferase signal was detected from the lungs of all MSC-treated mice, but not in the virus-alone group because of a lack of sufficient time for luciferase production. On the third day, the oncolytic virus-loaded MSCs displayed a strong luciferase signal from the liver, suggesting that viruses had escaped from the MSCs and transduced the liver. A 60 time weaker luciferase signal was captured from the livers of mice treated with virus only. Replication-deficient virus with MSCs showed weak luciferase expression in the lungs. On the next imaging days, a weak luciferase signal was detected from the liver of both groups of MSC-treated mice, but not mice treated with virus alone (Figure 3 in Study II). In addition, the kinetic studies revealed that viral release from MSC carriers was relatively rapid because 24 h after MSC injection (i.e. 72 h after infection) some of the released virus was already detectable in the liver. Further, DiI-labeled MSCs were injected intravenously into tumor-free, lung tumor- or breast tumor-bearing mice to see more clearly whether the luciferase signal was from the lungs or from the tumor. A strong fluorescence signal was obtained symmetrically from both lungs, suggesting accumulation preferentially in the lungs instead of specificity for tumors. No MSCs were found in breast tumors. In conclusion, we were able to demonstrate that MSCs loaded with oncolytic viruses homed to lungs of mice bearing orthotopic tumors. In contrast to previous reports, no tumor tropism was seen (Komarova *et al.* 2006; Studeny *et al.* 2004b).

## **5 Oncolytic adenoviruses and MSCs loaded with oncolytic adenoviruses show therapeutic efficacy in an orthotopic murine model of advanced lung and breast cancers (II)**

Therapeutic efficacy of MSCs packed with oncolytic adenoviruses was investigated in orthotopic models of advanced lung and breast cancers (Figure 6 in Study II). Mice were treated with virus only (Ad5.pK7- $\Delta$ 24), MSC only, MSC + oncolytic adenovirus (Ad5.pK7- $\Delta$ 24), MSC + replication-deficient adenovirus (Ad5pK7LacZ) and median survival times were 23, 25, 27, and 28 days, respectively, for lung tumor-bearing mice, and survival was significantly enhanced ( $p=0.0031$ ) with MSC loaded with oncolytic adenovirus. In an advanced breast cancer model the MSCs with oncolytic adenoviruses inhibited tumor growth significantly ( $p<0.001$ ) compared with control mice. These results suggest that despite MSCs lacking a specific tumor-targeting capacity, they were able to release the virus for subsequent transduction of tumors, and enhanced therapeutic efficacy was seen.

## **6 *In vivo* monitoring of oncolytic adenoviruses (I, II)**

Imaging has become an indispensable tool in cancer research, clinical trials, and medical practice. Imaging systems can be grouped by the energy used to derive visual information (X-rays, positrons, photons, or sound waves) (Groot-Wassink *et al.* 2004), the spatial resolution attained (macroscopic, mesoscopic, or microscopic), or the type of information obtained (anatomical, physiological, cellular, or molecular). In the context of adenoviral gene therapy, longitudinal monitoring of tumor burden and viral spreading has advantages over survival alone.

In this study, fluorescence and bioluminescence imaging were used to follow up tumor burden and the spreading of adenoviruses *in vivo*. Overall, imaging data correlated well with data achieved from biodistribution and survival experiments. The fluorescence imaging used in Study I (Figure 6) even revealed individual variation in mice treated with oncolytic adenoviruses. Mice treated with Ad5- $\Delta$ 24RGD were grouped according to

response. Some mice had progressive disease, the disease stabilized in others, and others had dramatic responses. In addition, imaging allowed us to track MSCs loaded with the adenoviruses more precisely in mice (Figures 3-5 in this Study II)

## **7 Cox-2 and CMV promoter are induced following adenovirus infection, but the Cox-2 3'UTR element can retain specificity *in vitro* and *in vivo* (III)**

Achievement of oncolytic replication in tumor cells while avoiding nonspecific replication in normal tissues remains a major challenge in oncolytic gene therapy. In this regard, the elucidation of the critical pathways involved in the adenoviral infectious cycle has provided the basis for multiple levels of control to achieve the goal of selective replication. Tumor-specific promoters are at the foundation of transcriptional targeting, and Cox-2 promoter has been among the more popular candidates (Bauerschmitz *et al.* 2006; Casado *et al.* 2001; Hirschowitz *et al.* 2002; Liang *et al.* 2004; Nettelbeck *et al.* 2003; Yamamoto *et al.* 2001; Yamamoto *et al.* 2003; Kanerva *et al.* 2005).

However, the physiological function of Cox-2 as an enzyme expressed upon inflammation, infection and injury as well as in tumors is very well established (Kujubu *et al.* 1991; Smith 1992; Turini & DuBois 2002). The Ras/MAPK group, one of the common pathways to control the cell cycle, is mutated in most cancers. Cox-2 expression is both upregulated transcriptionally and downregulated posttranscriptionally by the RAS/p38/MAPK pathway (Lasa *et al.* 2000). Downregulation is in partly through AU-rich elements within the 3'-UTR, and their physiological function is to rapidly downregulate Cox-2 mediated transcripts, when the threat (e.g. infection) has passed. Naturally, cancer cells have learned to take advantage of this mechanism and Cox-2 is stabilized through defected Ras pathway in tumor cells (Cok & Morrison 2001; Dean *et al.* 1999; Dixon *et al.* 2000).

In this study, we hypothesized that Cox-2 promoter is induced by adenoviral infection, but the specificity could be regained by utilization of 3'UTR elements. First, nonmalignant 293 cells were transfected with pShuttleCox2Luc plasmid featuring Cox-2L promoter driving luciferase, and the cells were then infected with a wild-type Ad5, a selectively

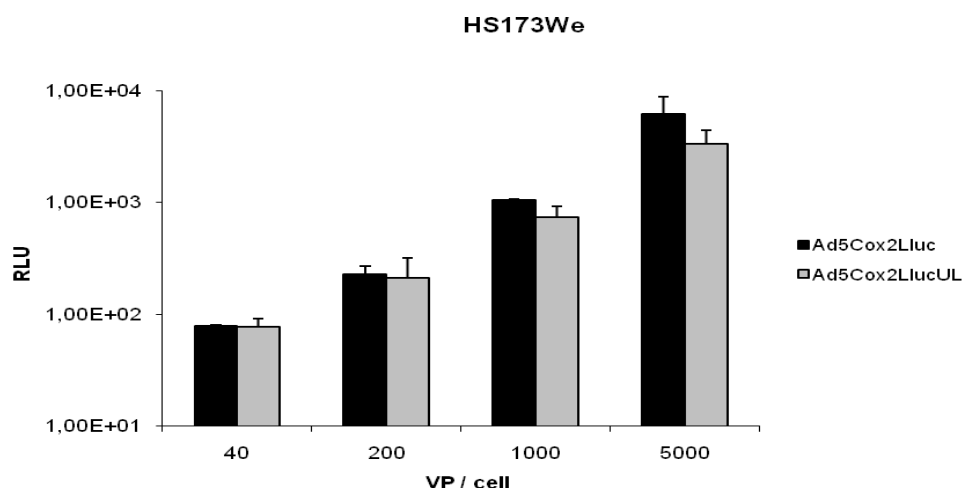
oncolytic adenovirus Ad5- $\Delta$ 24E3, or an E1-deleted adenovirus (Ad5LacZ) (Figure 2 A-C in Study III). Infection with a wild-type adenovirus or a conditionally replicating virus resulted in significant Cox-2 induction ( $p < 0.05$  and  $p < 0.01$ , respectively). Replication-deficient adenovirus also induced Cox-2 in these E1-containing cells, which allows replication of E1-deleted viruses ( $p < 0.05$ ). Further, we sought to investigate whether this Cox-2 induction is a cell type-specific phenomenon, and human fibroblasts were transfected and infected as previously, except that Ad5/3- $\Delta$ 24 virus was used instead of Ad300wt. Induction of the promoter seemed to be related to replication or E1 proteins since Cox-2 was induced by replication-competent adenoviruses but not by replication-deficient Ad5LacZ virus (Figure 3 in Study III). In addition, Ad5/3- $\Delta$ 24 virus, which replicates more efficiently in most cell types, also (Bauerschmitz *et al.* 2006; Kanerva *et al.* 2003). induced higher Cox-2 levels (Figures 3 and 4 in Study III).

When the same cell lines were transfected with a plasmid containing 3'UTR element after the luciferase gene (pShuttleCox2Luc+3'UTR) and infected with the same viruses, the 3'UTR element was able to abrogate induced Cox-2 expression in nonmalignant cells, but had little impact on malignant cells (Figures 2–4 in Study III). Interestingly, our control plasmids with CMV promoter with or without 3'UTR showed that CMV promoter is also induced by adenoviral infection to a similar degree as the Cox-2 promoter in all cell lines, and the UTR element also had a similar activity on the CMV promoter (Figures 2-4, panels D-F).

The induction of Cox-2 and CMV promoters was also studied *in vivo*. Subcutaneous 293 or A549 tumors were allowed to develop 30 and 14 days, respectively, and were then transfected with plasmids followed by intratumoral injection of wild-type adenovirus the next day. To investigate the induction, bioluminescence was imaged 15 min and 24 h after infection. Interestingly, there was a 4.3-fold induction in Cox-2 expression only 15 min after infection and this persisted to the next imaging. 3'UTR did not have time to affect expression during the first round of imaging, but 24 h later luciferase expressed by the Cox-2–3'UTR complex in infected cells decreased from 55 039 to 51 716, whereas in uninfected cells the luciferase continued to increase as expected (from 4339 to 14 519). The destabilization signal therefore appeared to work as predicted. However, there were variations typical of *in vivo* luciferase imaging and the complexities associated with *in vivo*

plasmid transfection followed by intratumoral infection, and therefore, no statistically significant differences between groups could be seen. After the second round of imaging, the tumors were excised and the amount of luciferase protein was analyzed. The results were in line with imaging data. Adenovirus infection induced expression of the Cox-2 and CMV promoters in both nonmalignant and malignant cells, and the 3'UTR element had an effect on the induction in 293 cells (Figures 5 and 6 in Study III).

In contrast to the promising data achieved from Cox-2 and 3'UTR expressing plasmids, the effect of the 3'UTR element was not powerful enough to downregulate the expression Cox-2 promoter driven luciferase in the context of replication deficient adenoviruses in human fibroblasts (Figure 5, unpublished data).



**Figure 5. Replication deficient adenovirus expressing 603bp long 3'UTR element has a minor impact on downregulation the expression of luciferase.** Human fibroblasts were infected with Ad5Cox2Luc and Ad5Cox2LucUL with, 40, 200, 1000 and 5000 vp/cell. Luciferase was imaged 24h after infection.

## 8 Switching the adenoviral fiber retains viral gene transfer in the presence of NAbs (IV)

The use of adenoviral vectors in gene therapy has been hampered by significant problems with attendant host immune responses that limit their safety and efficacy *in vivo* (Wilson 1996). Adenoviral vectors can effectively elicit the innate immune response immediately after infection, leading to the secretion of proinflammatory cytokines and chemokines in mice, humans, and nonhuman primates and that might damage healthy tissue (Raper *et al.* 2003; Schnell *et al.* 2001; Zhang *et al.* 2001). Problems particularly may arise after systemic delivery or upon readministration of adenoviruses. Recently, Zhu *et al.* described adenoviruses to elicit an innate immune response through the TLR9-MyD88 pathway in plasmacytoid DCs (pDCs) and a TLR-independent pathway in non-pDCs, leading to production of mainly type I IFNs (Zhu *et al.* 2007a).

We investigated whether the changes in the adenoviral fiber could allow the virus to escape from NAbs. To test the gene transfer of Ad5 in the presence of NAbs, immunocompetent mice were immunized with capsid-modified replication-deficient adenoviruses (see Table 6), and the inhibitory effect on Ad5(GL) gene transfer efficacy was analyzed. We used a single immunization to model a low level of NAb and a triple immunization to simulate a high NAb titer. Triple immunization with Ad5(GL) resulted in complete blocking of *in vitro* gene transfer by Ad5(GL). By contrast, after triple immunization with Ad5/3luc1 or Ad5pK7(GL), Ad5(GL) was not blocked. Anti-Ad5lucRGD NAbs marginally inhibited Ad5(GL) gene transfer (Table 2 in Study IV). These are consistent with previous results showing an ability of RGD or 5/3 modifications of the Ad5 fiber to avoid pre-existing anti-Ad5 NAb present in human serum or ascites (Hemminki *et al.* 2001; Kanerva *et al.* 2003). Immunized mice were injected intravenously with Ad5(GL), and organs were harvested for luciferase measurement (Table 6). A single immunization with Ad5(GL) reduced subsequent gene transfer with Ad5(GL) to the liver ( $p < 0.001$ ), heart ( $p < 0.001$ ), spleen ( $p < 0.05$ ), pancreas ( $p < 0.05$ ), and kidney ( $p < 0.01$ ), in contrast to a single immunization with the other viruses, which did not significantly inhibit gene transfer of Ad5(GL) in most organs. However, triple immunization did produce a

stronger antibody response, and NAb to capsid-modified adenoviruses started to reduce gene transfer of Ad5(GL). Liver transduction was decreased more than 1000-fold with Ad5(GL) compared with a single immunization with the same virus (Figures 2 and 3 in Study IV). Although a degree of cross-blocking was seen for all viruses, Ad5/3luc was affected the least, probably due to the complete knob-swap in the fiber, suggesting that anti-fiber knob NAb might be most relevant.

**Table 6. Induction of neutralizing antibodies and determination of their effect on gene transfer in immune-competent mice.**

Group	I	II	III	IV	V	VI	VII	VIII	IX	X
DAY										
0 (s.c.)	Ad5(GL)	Ad5(GL)	Ad5/3luc1	Ad5/3luc1	Ad5lucRG D	Ad5lucRG D	Ad5pK7( GL)	Ad5pK7( GL)	mock	mock
3 (s.c.)		Ad5(GL)		Ad5/3luc1		Ad5lucRG D		Ad5pK7( GL)		mock
6 (s.c.)		Ad5(GL)		Ad5/3luc1		Ad5lucRG D		Ad5pK7( GL)		mock
27	NAb	NAb	NAb	NAb	NAb	NAb	NAb	NAb	NAb	NAb
28 (i.v.)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)
29	organs & serum	organs & serum	organs	organs	organs	organs	organs	organs	organs	organs

Abbreviations: i.v., intravenous injection ( $3 \times 10^{10}$  vp); NAb, determination of neutralizing antibody titer from serum; s.c., subcutaneous immunization ( $3 \times 10^8$  vp); vp, viral particle

The effect on gene transfer in the presence NAb was also evaluated *in vivo*. Mice bearing orthotopic tumors of advanced lung cancer were injected intravenously with Ad5(GL), Ad5/3luc1, Ad5lucRGD, or Ad5pK7(GL) or with the same viruses preincubated with serum collected from Ad5(GL)-immunized mice. A low titer of anti-Ad5 NAb did not block intravenous Ad5(GL), but a high titer almost completely prevented gene transfer. Regardless of anti-Ad5 NAb titer, effective gene delivery was achieved with Ad5lucRGD, Ad5/3luc1, and Ad5pK7(GL) (Figure 4 in Study IV).



Two human squamous cell carcinomas, one adenocarcinoma, and one non-malignant lung tissue specimen were obtained fresh from patients and infected with viruses preincubated with serum collected from Ad5(GL)-immunized mice. Ad5(GL) was completely blocked by a high NAb titer, whereas Ad5/3luc1 and Ad5pK7(GL) continued to allow gene transfer. Both Ad5/3luc1 and Ad5pK7(GL) showed enhanced gene delivery compared with the Ad5(GL) in the presence of low or high NAb. Intriguingly, anti-Ad5 NAb blocked gene transfer by capsid-modified viruses to normal human lung explants much more than to tumor explants (Figures 5 and 6 in Study **IV**). This might be explained by the fiber knob-independent mechanisms proposed to be important in systemic biodistribution of mice and primates (Bayo-Puxan *et al.* 2006; Smith *et al.* 2003a; Smith *et al.* 2003b). In conclusion, our data suggest that relative escape from the NAbs is achieved after systemic delivery using capsid-modified adenoviruses.

## Summary and conclusions

The overall aim of this study was to obtain information on adenovirus tropism towards NSCLC cells and to enhance therapeutic outcome with oncolytic adenoviruses. To further improve the specificity, we sought to determine whether the Cox-2 promoter is induced by adenovirus infection and whether selectivity could be regained by 3'UTR elements. Finally, we tested whether switching the adenovirus fiber can retain gene delivery in the presence of NAbs.

HSPG- and  $\alpha_v\beta$  integrin-targeted viruses displayed enhanced gene transfer to NSCLC cells *in vitro* and *in vivo*. Respective oncolytic viruses showed effective cell killing and this translated into a therapeutic benefit in a highly aggressive orthotopic model of large-cell NSCLC. Combination therapies with radio- or chemotherapy and arming oncolytic viruses with immunostimulatory or cytotoxic transgenes could lead to an even better therapeutic outcome.

Most importantly, the data laid the foundation for clinical testing of these viruses in patients with NSCLC refractory to current treatments. The use of cells as oncolytic virus delivery vehicles offers a number of potential advantages. One lies in the tumor-homing capacity of certain cells. A second advantage is that carrier cells can be engineered to express a set of transgenes exceeding the cloning capacity of the viral vector considered, thereby acting as local virus factories allowing high amplification of the inoculated virus. Finally, oncolytic viruses harbored by carrier cells remain at least partly protected from inactivation by the host until released at their destination.

MSCs possess multilineage properties making them useful for a number of potential therapeutic applications, and they have suggested targeting many different cancers. Infectivity of MSCs could be enhanced by using capsid-modified adenoviruses. In addition, MSCs were able to support the replication and viral release of oncolytic adenoviruses harboring a 24-bp deletion in the E1A region both *in vitro* and *in vivo*. Further, MSCs loaded with oncolytic adenoviruses prolonged the survival of mice with

advanced lung cancer and showed enhanced antitumor efficacy with breast cancer tumors after systemic delivery. These data suggest that MSCs could improve the bioavailability of adenoviruses in the context of advanced and aggressive disease.

Viruses are known to induce Cox-2 (not previously known for adenovirus), but the mechanisms behind this phenomenon are poorly understood. It is unclear whether Cox-2 induction occurs due to a protective antiviral response initiated by human cells, or whether viruses can also actively promote Cox-2 induction for their own purposes. Our results suggest that both Cox-2 and CMV promoters are induced by adenoviral infection, but selectivity could be rescued by the 3'UTR element of the Cox-2. It would be interesting to study the activity of these elements in the context of oncolytic adenoviruses, and further, to determine whether other tumor specific promoters could be controlled by these elements. Another approach to enhance the efficacy of adenoviral gene therapy is to reduce the effect of Nabs. Our data proposed that minor changes in adenovirus fiber can allow the virus to escape from pre-existing Nabs, and therefore, more effective systemic readministration could be achieved. It might be even more beneficial to use viruses from different serotypes, or viruses pseudotyped with the entire fiber (instead of just the knob) or hexon might yield further advances. It would also be interesting to study MSCs in the context of NAbs.

## Acknowledgements

This work was done in the Cancer Gene Therapy Group, which is part of the Molecular Cancer Biology Program and Transplantation Laboratory and Haartman Institute and Finnish Institute for molecular Medicine in the University of Helsinki, and Helsinki University Central Hospital. The work was performed between May 2003 and May 2008. My sincere gratitude is owed to the large number of people who participated in this project.

I thank the former Head of the Transplantation Laboratory, Professor Pekka Häyry, the former coordinator of the Rational Drug Design Program, the current Head of Transplantation Laboratory, Professor Risto Renkonen, the former and current coordinators of the Molecular Cancer Biology Program, Professors Kari Alitalo, Marikki Laiho, and Jorma Keski-Oja, the Head of Haartman institute, Professor Seppo Meri, the Head of the Biomedicum and Faculty Research Programs, Professor Olli Jänne, for providing excellent research facilities in Biomedicum Helsinki.

My supervisor, Res. Professor Akseli Hemminki is thanked for introducing me to the fascinating world of gene therapy. I also thank Akseli for teaching me the basics of scientific thinking and for his many intriguing ideas, and a never-ending positive attitude. Most of all, I am grateful to him for being such a kind and understanding group leader.

I am also indebted to my second supervisor, Docent Anna Kanerva, for teaching me a very precise way of conducting research. I am especially thankful for all of the time she spent in discussions and troubleshooting with me.

Docent Anna-Liisa Levonen and Docent Maija Lappalainen, the official reviewers of this thesis, for critical review and constructive comments that greatly improved this manuscript.

I thank Carol Ann Pelli for linguistic revision of this thesis.

The members of Cancer Gene Therapy Group (CGTG) at the University of Helsinki: Akseli, Anna, Sari, Camilla, Laura, Vincenzo, Minna, Elina, Aila, Eerika, Kikka, Iulia, João, Sergio, Petri, Otto, Kilian, Marta, Ilkka, Anniina, Maria, and Marko are warmly thanked for making the lab such a fun place to work. I am especially grateful to the “old team”, Lotta, Mari, and Tuuli for being such good and understanding friends.

I thank former members of CGTG, Kirsi Saukkonen, Tanja Hakkarainen, Minna Eriksson, and Gerd Bauerschmitz for helping me with numerous problems during the thesis and also for friendship.

My collaborator at the University of Helsinki, Dr, Ari Ristimäki, is thanked for sharing his vast knowledge and plasmids.

My collaborators at Universities of Tampere and Oulu, Drs, Susanna Miettinen, Timo Ylikomi, Riitta Suuronen, and Petri Lehenkari are acknowledged for proving the mesenchymal stem cells and for sharing their superb knowledge.

My collaborator at UAB, Dr, Renee Desmond, is thanked for excellent statistical analyses.

I thank my mother Terttu for supporting me throughout my life, Petteri's parents Margareta and Erkki for taking such good care of Meri-Vuokko and enabling me to write this thesis. Finally, with all my heart I thank my dearest daughter Meri-Vuokko and my beloved partner Petteri for loving and believing me and for making my life complete.

Financial support provided by: Helsinki University Central Hospital Research Funds, University of Helsinki Internal Funds, the Foundation of Rauha-and Jalmari Ahokas, Biomedicum Helsinki Foundation, the Finnish Cancer Society and the Research and Science Foundation of Farnos, Foundation of Väinö ja Laina Kivi, Research Foundation of Virology and Maud Kuistila Memorial Foundation is gratefully acknowledged.

Helsinki, September 2008

Merja Särkioja

## REFERENCES

- Aggarwal, S. and Pittenger, M. F. (2005).** Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **105**, 1815-1822.
- Ahmed, A., Thompson, J., Emiliusen, L., Murphy, S. Beauchamp, R. D., Suzuki, K., Alemany, R., Harrington, K. and Vile, R. G. (2003).** A conditionally replicating adenovirus targeted to tumor cells through activated RAS/P-MAPK-selective mRNA stabilization. *Nat.Biotechnol.* **21**, 771-777.
- Alemany, R., Suzuki, K. and Curiel, D. T. (2000).** Blood clearance rates of adenovirus type 5 in mice. *J.Gen.Virol.* **81**, 2605-2609.
- Arstila P. (2004).** Adeno-ja Korona virukset. *Duodecim julk.* mbi00199, Mikrobiologia ja infektiosairaudet.
- Bainbridge, J. W., Smith, A. J., Barker, S. S., Robbie, S., Henderson, R., Balaggan, K., Viswanathan, A., Holder, G. E., Stockman, A., Tyler, N., Petersen-Jones, S., Bhattacharya, S. S., Thrasher, A. J., Fitzke, F. W., Carter, B. J., Rubin, G. S., Moore, A. T. and Ali, R. R. (2008).** Effect of gene therapy on visual function in leber's congenital amaurosis. *N.Engl.J.Med.* .
- Baker, A. H. (2007a).** Adenovirus toxicity and tropism in vivo: Not as simple as A,B,C (or D,E,F). *Mol.Ther.* **15**, 2061-2062.
- Baker, A. H., Mcvey, J. H., Waddington, S. N., Di Paolo, N. C. and Shayakhmetov, D. M. (2007b).** The influence of blood on in vivo adenovirus bio-distribution and transduction. *Mol.Ther.* **15**, 1410-1416.
- Bangari, D. S. and Mittal, S. K. (2004).** Porcine adenoviral vectors evade preexisting humoral immunity to adenoviruses and efficiently infect both human and murine cells in culture. *Virus Res.* **105**, 127-136.
- Barajas M., Mazzolini G., Genove G., Bilbao R., Narvaiza I., Schmitz V., Sangro B., Melero I., Qian C. and Prieto J. (2001).** Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus codi€ng for interleukin 12. *Hepatology* **33**, 52–61.
- Barker, S. D., Dmitriev, I. P., Nettelbeck, D. M., Liu, B., Rivera, A. A., Alvarez, R. D., Curiel, D. T. and Hemminki, A. (2003a).** Combined transcriptional and transductional targeting improves the specificity and efficacy of adenoviral gene delivery to ovarian carcinoma. *Gene Ther.* **10**, 1198-1204.
- Barouch, D. H., Pau, M. G., Custers, J. H., Koudstaal, W., Kostense, S., Havenga, M. J., Truitt, D. M., Sumida, S. M., Kishko, M. G., Arthur, J. C., Koriath-Schmitz, B., Newberg, M. H., Gorgone, D. A., Lifton, M. A., Panicali, D. L., Nabel, G. J., Letvin, N. L. and Goudsmit, J.**

(2004). Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J.Immunol.* **172**, 6290-6297.

**Bauerschmitz, G. J., Barker, S. D. and Hemminki, A. (2002).** Adenoviral gene therapy for cancer: From vectors to targeted and replication competent agents (review). *Int.J.Oncol.* **21**, 1161-1174.

**Bauerschmitz, G. J., Guse, K., Kanerva, A., Menzel, A., Herrmann, I., Desmond, R. A., Yamamoto, M., Nettelbeck, D. M., Hakkarainen, T., Dall, P., Curiel, D. T. Hemminki, A. (2006).** Triple-targeted oncolytic adenoviruses featuring the Cox2 promoter, E1A transcomplementation, and serotype chimerism for enhanced selectivity for ovariaand n cancer cells. *Mol.Ther.* .

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

**Beer, S. J., Matthews, C. B., Stein, C. S., Ross, B. D., Hilfinger, J. M. and Davidson, B. L. (1998).** Poly (lactic-glycolic) acid copolymer encapsulation of recombinant adenovirus reduces immunogenicity in vivo. *Gene Ther.* **5**, 740-746.

**Belinsky, S. A., Nikula, K. J., Palmisano, W. A., Michels, R., Saccomanno, G., Gabrielson, E., Baylin, S. B. and Herman, J. G. (1998).** Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc.Natl.Acad.Sci.U.S.A.* **95**, 11891-11896.

**Bessis, N., GarciaCozar, F. J. and Boissier, M. C. (2004).** Immune responses to gene therapy vectors: Influence on vector function and effector mechanisms. *Gene Ther.* **11 Suppl 1**, S10-7.

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

**Brambilla, E., Moro, D., Gazzeri, S. and Brambilla, C. (1999).** Alterations of expression of rb, p16(INK4A) and cyclin D1 in non-small cell lung carcinoma and their clinical significance. *J.Pathol.* **188**, 351-360.

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

**Brandtner, E. M., Kodajova, P., Hlavaty, J., Jandl, G., Tabotta, W., Salmons, B., Gunzburg, W. H. and Hohenadl, C. (2008).** Reconstituting retroviral (ReCon) vectors facilitating delivery of cytotoxic genes in cancer gene therapy approaches. *J.Gene Med.* **10**, 113-122.

**Bruder, J. T. and Kovesdi, I. (1997).** Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. *J.Virol.* **71**, 398-404.

- Brunetti-Pierri, N., Palmer, D. J., Beaudet, A. L., Carey, K. D., Finegold, M. and Ng, P. (2004).** Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum. Gene Ther.* **15**, 35-46.
- Casado, E., Gomez-Navarro, J., Yamamoto, M., Adachi, Y., Coolidge, C. J., Arafat, W. O., Barker, S. D., Wang, M. H., Mahasreshti, P. J., Hemminki, A., Gonzalez-Baron, M., Barnes, M. N., Pustilnik, T. B., Siegal, G. P., Alvarez, R. D. and Curiel, D. T. (2001).** Strategies to accomplish targeted expression of transgenes in ovarian cancer for molecular therapeutic applications. *Clin. Cancer Res.* **7**, 2496-2504.
- Cascallo, M., Capella, G., Mazo, A. and Alemany, R. (2003).** Ras-dependent oncolysis with an adenovirus VAI mutant. *Cancer Res.* **63**, 5544-5550.
- Cascallo, M., Gros, A., Bayo, N., Serrano, T., Capella, G. and Alemany, R. (2006).** Deletion of VAI and VAI RNA genes in the design of oncolytic adenoviruses. *Hum. Gene Ther.* **17**, 929-940.
- Chamberlain, J. R., Schwarze, U., Wang, P. R., Hirata, R. K., Hankenson, K. D., Pace, J. M., Underwood, R. A., Song, K. M., Sussman, M., Byers, P. H. and Russell, D. W. (2004).** Gene targeting in stem cells from individuals with osteogenesis imperfecta. *Science* **303**, 1198-1201.
- Chana, J. S., Grover, R., Tulley, P., Lohrer, H., Sanders, R., Grobbelaar, A. O. and Wilson, G. D. (2002).** The c-myc oncogene: use of a biological prognostic marker as a potential target for gene therapy in melanoma. *Br. J. Plast. Surg.* **55**, 623-627.
- Chen, Q. R., Zhang, L., Gasper, W. and Mixson, A. J. (2001).** Targeting tumor angiogenesis with gene therapy. *Mol. Genet. Metab.* **74**, 120-127.
- Cheng, J., Sauthoff, H., Huang, Y., Kutler, D. I., Bajwa, S., Rom, W. N. and Hay, J. G. (2007).** Human matrix metalloproteinase-8 gene delivery increases the oncolytic activity of a replicating adenovirus. *Mol. Ther.* **15**, 1982-1990.
- Chillon, M., Lee, J. H., Fasbender, A. and Welsh, M. J. (1998).** Adenovirus complexed with polyethylene glycol and cationic lipid is shielded from neutralizing antibodies in vitro. *Gene Ther.* **5**, 995-1002.
- Chiocca, E. A., Abbed, K. M., Tatter, S., Louis, D. N., Hochberg, F. H., Barker, F., Kracher, J., Grossman, S. A., Fisher, J. D., Carson, K., Rosenblum, M., Mikkelsen, T., Olson, J., Markert, J., Rosenfeld, S., Nabors, L. B., Brem, S., Phuphanich, S., Freeman, S., Kaplan, R. and Zwiebel, J. (2004).** A phase I open-label, dose-escalation, multi-institutional trial of injection with an E1B-attenuated adenovirus, ONYX-015, into the peritumoral region of recurrent malignant gliomas, in the adjuvant setting. *Mol. Ther.* **10**, 958-966.
- Cichon, G., Boeckh-Herwig, S., Schmidt, H. H., Wehnes, E., Muller, T., Pring-Akerblom, P. and Burger, R. (2001).** Complement activation by recombinant adenoviruses. *Gene Ther.* **8**, 1794-1800.



**Cohen, C. J., Shieh, J. T., Pickles, R. J., Okegawa, T., Hsieh, J. T. and Bergelson, J. M. (2001).** The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc.Natl.Acad.Sci.U.S.A.* **98**, 15191-15196.

**Cok, S. J. and Morrison, A. R. (2001).** The 3'-untranslated region of murine cyclooxygenase-2 contains multiple regulatory elements that alter message stability and translational efficiency. *J.Biol.Chem.* **276**, 23179-23185.

**Cole, C., Qiao, J., Kottke, T., Diaz, R. M., Ahmed, A., Sanchez-Perez, L., Brunn, G., Thompson, J., Chester, J. and Vile, R. G. (2005).** Tumor-targeted, systemic delivery of therapeutic viral vectors using hitchhiking on antigen-specific T cells. *Nat.Med.* **11**, 1073-1081.

**Conget, P. A. & Minguell, J. J. (2000).** Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. *Exp.Hematol.* **28**, 382-390.

**Cripe, T. P., Dunphy, E. J., Holub, A. D., Saini, A., Vasi, N. H., Mahller, Y. Y., Collins, M. H., Snyder, J. D., Krasnykh, V., Curiel, D. T., Wickham, T. J., DeGregori, J., Bergelson, J. M. and Currier, M. A. (2001).** Fiber knob modifications overcome low, heterogeneous expression of the coxsackievirus-adenovirus receptor that limits adenovirus gene transfer and oncolysis for human rhabdomyosarcoma cells. *Cancer Res.* **61**, 2953-2960.

**Croyle, M. A., Chirmule, N., Zhang, Y. and Wilson, J. M. (2002).** PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. *Hum.Gene Ther.* **13**, 1887-1900.

**Croyle, M. A., Le, H. T., Linse, K. D., Cerullo, V., Toietta, G., Beaudet, A. and Pastore, L. (2005).** PEGylated helper-dependent adenoviral vectors: Highly efficient vectors with an enhanced safety profile. *Gene Ther.* **12**, 579-587.

**Davidoff, A. M., Stevenson, S. C., McClelland, A., Shochat, S. J. and Vanin, E. F. (1999).** Enhanced neuroblastoma transduction for an improved antitumor vaccine. *J.Surg.Res.* **83**, 95-99.

**De Jong, J. C., Wermenbol, A. G., Verweij-Uijterwaal, M. W., Slaterus, K. W., Wertheim-Van Dillen, P., Van Doornum, G. J., Khoo, S. H. and Hierholzer, J. C. (1999).** Adenoviruses from human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. *J.Clin.Microbiol.* **37**, 3940-3945.

**Dean, J. L., Brook, M., Clark, A. R. and Saklatvala, J. (1999).** p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *J.Biol.Chem.* **274**, 264-269.

**DeWeese, T. L., van der Poel, H., Li, S., Mikhak, B., Drew, R., Goemann, M., Hamper, U., DeJong, R., Detorie, N., Rodriguez, R., Haulk, T., DeMarzo, A. M., Piantadosi, S., Yu, D. C., Chen, Y., Henderson, D. R., Carducci, M. A., Nelson, W. G. and Simons, J. W. (2001).** A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* **61**, 7464-7472.

- Dixon, D. A., Kaplan, C. D., McIntyre, T. M., Zimmerman, G. A. and Prescott, S. M. (2000).** Post-transcriptional control of cyclooxygenase-2 gene expression. the role of the 3'-untranslated region. *J.Biol.Chem.* **275**, 11750-11757.
- Dmitriev, I., Kashentseva, E., Rogers, B. E., Krasnykh, V. and Curiel, D. T. (2000).** Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. *J.Virol.* **74**, 6875-6884.
- Dmitriev, I., Krasnykh, V., Miller, C. R., Wang, M., Kashentseva, E., Mikheeva, G., Belousova, N. and Curiel, D. T. (1998).** An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J.Virol.* **72**, 9706-9713.
- Dmitriev, I. P., Kashentseva, E. A. and Curiel, D. T. (2002).** Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. *J.Virol.* **76**, 6893-6899.
- Douglas, J. T., Kim, M., Sumerel, L. A., Carey, D. E. and Curiel, D. T. (2001).** Efficient oncolysis by a replicating adenovirus (ad) in vivo is critically dependent on tumor expression of primary ad receptors. *Cancer Res.* **61**, 813-817.
- Douglas, J. T., Rogers, B. E., Rosenfeld, M. E., Michael, S. I., Feng, M. and Curiel, D. T. (1996).** Targeted gene delivery by tropism-modified adenoviral vectors. *Nat.Biotechnol.* **14**, 1574-1578.
- Duggan, B. J., Maxwell, P., Kelly, J. D., Canning, P., Anderson, N. H., Keane, P. F., Johnston S. R. and Williamson, K.E. (2001).** The effect of antisense Bcl-2 oligonucleotides on Bcl-2 protein expression and apoptosis in human bladder transitional cell carcinoma, *J. Urol.* **166**, 1098-1105.
- Duque, P. M., Alonso, C., Sanchez-Prieto, R., Quintanilla, M., Ramon, S. and Ramon y Cajal, S. (1998).** Antitumoral effect of E1B defective adenoviruses in human malignant cells. *Gene Ther.* **5**, 286-287.
- Dvorak, H. F. (1986).** Tumors: Wounds that do not heal. similarities between tumor stroma generation and wound healing. *N.Engl.J.Med.* **315**, 1650-1659.
- Elzaouk, L., Moelling, K. and Pavlovic, J. (2006).** Anti-tumor activity of mesenchymal stem cells producing IL-12 in a mouse melanoma model. *Exp.Dermatol.* **15**, 865-874.
- Everts, M., Kim-Park, S. A., Preuss, M. A., Passineau, M. J., Glasgow, J. N., Pereboev, A. V., Mahasreshti, P. J., Grizzle, W. E., Reynolds, P. N. and Curiel, D. T. (2005).** Selective induction of tumor-associated antigens in murine pulmonary vasculature using double-targeted adenoviral vectors. *Gene Ther.* **12**, 1042-1048.
- Falgout, B. and Ketner, G. (1988).** Characterization of adenovirus particles made by deletion mutants lacking the fiber gene. *J.Virol.* **62**, 622-625.

- Farina, S. F., Gao, G. P., Xiang, Z. Q., Rux, J. J., Burnett, R. M., Alvira, M. R., Marsh, J., Ertl, H. C. and Wilson, J. M. (2001).** Replication-defective vector based on a chimpanzee adenovirus. *J.Virol.* **75**, 11603-11613.
- Freytag, S. O., Rogulski, K. R., Paielli, D. L., Gilbert, J. D. and Kim, J. H. (1998).** A novel three-pronged approach to kill cancer cells selectively: Concomitant viral, double suicide gene, and radiotherapy. *Hum.Gene Ther.* **9**, 1323-1333.
- Freytag, S. O., Stricker, H., Peabody, J., Pegg, J., Paielli, D., Movsas, B., Barton, K. N., Brown, S. L., Lu, M. and Kim, J. H. (2007).** Five-year follow-up of trial of replication-competent adenovirus-mediated suicide gene therapy for treatment of prostate cancer. *Mol.Ther.* **15**, 636-642.
- Freytag, S. O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D. G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M. and Kim, J. H. (2003a).** Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate-to-high-risk prostate cancer. *Cancer Res.* **63**, 7497-7506.
- Fritz, V. and Jorgensen, C. (2008).** Mesenchymal stem cells: An emerging tool for cancer targeting and therapy. *Curr.Stem Cell.Res.Ther.* **3**, 32-42.
- Fueyo, J., Gomez-Manzano, C., Alemany, R., Lee, P. S., McDonnell, T. J., Mitlianga, P., Shi, Y. X., Levin, V. A., Yung, W. K. and Kyritsis, A. P. (2000).** A mutant oncolytic adenovirus targeting the rb pathway produces anti-glioma effect in vivo. *Oncogene* **19**, 2-12.
- Fukazawa, T., Maeda, Y., Sladek, F. M. and Owen-Schaub, L. B. (2004).** Development of a cancer-targeted tissue-specific promoter system. *Cancer Res.* **64**, 363-369.
- Gaggar, A., Shayakhmetov, D. M. and Lieber, A. (2003).** CD46 is a cellular receptor for group B adenoviruses. *Nat.Med.* **9**, 1408-1412.
- Gahery-Segard, H., Farace, F., Godfrin, D., Gaston, J., Lengagne, R., Tursz, T., Boulanger, P. and Guillet, J. G. (1998).** Immune response to recombinant capsid proteins of adenovirus in humans: Antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. *J.Virol.* **72**, 2388-2397.
- Galanis, E., Okuno, S. H., Nascimento, A. G., Lewis, B. D., Lee, R. A., Oliveira, A. M., Sloan, J. A., Atherton, P., Edmonson, J. H., Erlichman, C., Randlev, B., Wang, Q., Freeman, S. and Rubin, J. (2005).** Phase I-II trial of ONYX-015 in combination with MAP chemotherapy in patients with advanced sarcomas. *Gene Ther.* **12**, 437-445.
- Gall, J., Kass-Eisler, A., Leinwand, L. and Falck-Pedersen, E. (1996).** Adenovirus type 5 and 7 capsid chimera: Fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J.Virol.* **70**, 2116-2123.
- Ganly, I., Kirn, D., Eckhardt, G., Rodriguez, G. I., Soutar, D. S., Otto, R., Robertson, A. G., Park, O., Gulley, M. L., Heise, C., Von Hoff, D. D. and Kaye, S. B. (2000).** A phase I study of

onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin.Cancer Res.* **6**, 798-806.

**Glasgow, J. N., Bauerschmitz, G. J., Curiel, D. T. and Hemminki, A. (2004).** Transductional and transcriptional targeting of adenovirus for clinical applications. *Curr.Gene Ther.* **4**, 1-14.

**Goldman, C. K., Rogers, B. E., Douglas, J. T., Sosnowski, B. A., Ying, W., Siegal, G. P., Baird, A., Campaign, J. A. and Curiel, D. T. (1997).** Targeted gene delivery to kaposi's sarcoma cells via the fibroblast growth factor receptor. *Cancer Res.* **57**, 1447-1451.

**Griffiths, L., Binley, K., Iqbal, S., Kan, O., Maxwell, P., Ratcliffe, P., Lewis, C., Harris, A., Kingsman, S. and Naylor, S. (2000).** The macrophage - a novel system to deliver gene therapy to pathological hypoxia. *Gene Ther.* **7**, 255-262.

**Grimm, C.F., Ortmann, D., Mohr, L., Michalak, S., Krohne, T.U., Meckel, S., Eisele, S., Encke, J., Blum H.E. and Geissler M. (2000).** Mouse alpha-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. *Gastroenterology* **119**, 1104-1112.

**Groot-Wassink, T., Aboagye, E. O., Wang, Y., Lemoine, N. R., Reader, A. J. and Vassaux, G. (2004).** Quantitative imaging of Na/I symporter transgene expression using positron emission tomography in the living animal. *Mol.Ther.* **9**, 436-442.

**Guse, K., Dias, J. D., Bauerschmitz, G. J., Hakkarainen, T., Aavik, E., Ranki, T., Pisto, T., Sarkioja, M., Desmond, R. A., Kanerva, A. and Hemminki, A. (2007a).** Luciferase imaging for evaluation of oncolytic adenovirus replication in vivo. *Gene Ther.* **14**, 902-911.

**Guse, K., Ranki, T., Ala-Opas, M., Bono, P., Sarkioja, M., Rajecski, M., Kanerva, A., Hakkarainen, T. and Hemminki, A. (2007b).** Treatment of metastatic renal cancer with capsid-modified oncolytic adenoviruses. *Mol.Cancer.Ther.* **6**, 2728-2736.

**Gutova, M., Najbauer, J., Frank, R. T., Kendall, S. E., Gevorgyan, A., Metz, M. Z., Guevorkian, M., Edmiston, M., Zhao, D., Glackin, C. A., Kim, S. U. and Aboody, K. S. (2008).** uPA and uPAR mediate human stem cell tropism to malignant solid tumors. *Stem Cells* .

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

**Haisma, H. J., Pinedo, H. M., Rijswijk, A., der Meulen-Muileman, I., Sosnowski, B. A., Ying, W., Beusechem, V. W., Tillman, B. W., Gerritsen, W. R. and Curiel, D. T. (1999).** Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen EpCAM. *Gene Ther.* **6**, 1469-1474.

**Hakkarainen, T., Hemminki, A., Curiel, D. T. and Wahlfors, J. (2006).** A conditionally replicative adenovirus that codes for a TK-GFP fusion protein (Ad5Delta24TK-GFP) for evaluation

of the potency of oncolytic virotherapy combined with molecular chemotherapy. *Int.J.Mol.Med.* **18**, 751-759.

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

**Hanke, M., Serwe, F., Dombrowski, T., Sauerbruch and Caselmann, W. H. (2002).** DNA vaccination with AFP-encoding plasmid DNA prevents growth of subcutaneous AFP-expressing tumors and does not interfere with liver regeneration in mice. *Cancer Gene Ther.* **9**, 346-355.

**Hashimoto, M., Boyer, J. L., Hackett, N. R., Wilson, J. M. and Crystal, R. G. (2005).** Induction of protective immunity to anthrax lethal toxin with a nonhuman primate adenovirus-based vaccine in the presence of preexisting anti-human adenovirus immunity. *Infect.Immun.* **73**, 6885-6891.

**Hashimoto, Y., Watanabe, Y., Shirakiya, Y., Uno, F., Kagawa, S., Kawamura, H., Nagai, K., Tanaka, N., Kumon, H., Urata, Y. and Fujiwara, T. (2008).** Establishment of biological and pharmacokinetic assays of telomerase-specific replication-selective adenovirus. *Cancer.Sci.* **99**, 385-390.

**Haviv, Y. S., van Houdt, W. J., Lu, B., Curiel, D. T. and Zhu, Z. B. (2004).** Transcriptional targeting in renal cancer cell lines via the human CXCR4 promoter. *Mol.Cancer.Ther.* **3**, 687-691.

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

**Hedley, S. J., Auf der Maur, A., Hohn, S., Escher, D., Barberis, A., Glasgow, J. N., Douglas, J. T., Korokhov, N. and Curiel, D. T. (2006).** An adenovirus vector with a chimeric fiber incorporating stabilized single chain antibody achieves targeted gene delivery. *Gene Ther.* **13**, 88-94.

**Hedman, M., Hartikainen, J., Syvanne, M., Stjernvall, J., Hedman, A., Kivela, A., Vanninen, E., Mussalo, H., Kauppila, E., Simula, S., Narvanen, O., Rantala, A., Peuhkurinen, K., Nieminen, M. S., Laakso, M. and Yla-Herttuala, S. (2003).** Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: Phase II results of the kuopio angiogenesis trial (KAT). *Circulation* **107**, 2677-2683.

**Heise, C., Hermiston, T., Johnson, L., Brooks, G., Sampson-Johannes, A., Williams, A., Hawkins, L. and Kirn, D. (2000a).** An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat.Med.* **6**, 1134-1139.

**Hemminki, A., Belousova, N., Zinn, K. R., Liu, B., Wang, M., Chaudhuri, T. R., Rogers, B. E., Buchsbaum, D. J., Siegal, G. P., Barnes, M. N., Gomez-Navarro, J., Curiel, D. T. and Alvarez, R. D. (2001).** An adenovirus with enhanced infectivity mediates molecular chemotherapy of ovarian cancer cells and allows imaging of gene expression. *Mol.Ther.* **4**, 223-231.

**Hemminki, A., Kanerva, A., Kremer, E. J., Bauerschmitz, G. J., Smith, B. F., Liu, B., Wang, M., Desmond, R. A., Keriel, A., Barnett, B., Baker, H. J., Siegal, G. P. and Curiel, D. T. (2003).** A canine conditionally replicating adenovirus for evaluating oncolytic virotherapy in a syngeneic animal model. *Mol. Ther.* **7**, 163-173.

**Hemminki, A., Zinn, K. R., Liu, B., Chaudhuri, T. R., Desmond, R. A., Rogers, B. E., Barnes, M. N., Alvarez, R. D. and Curiel, D. T. (2002).** In vivo molecular chemotherapy and noninvasive imaging with an infectivity-enhanced adenovirus. *J.Natl.Cancer Inst.* **94**, 741-749.

**Henning, P., Andersson, K. M., Frykholm, K., Ali, A., Magnusson, M. K., Nygren, P. A., Granio, O., Hong, S. S., Boulanger, P. and Lindholm, L. (2005).** Tumor cell targeted gene delivery by adenovirus 5 vectors carrying knobless fibers with antibody-binding domains. *Gene Ther.* **12**, 211-224.

**Heo D.S. (2002).** Progress and limitations in cancer gene therapy. *Genet. Med.* **4** (6 Suppl.), pp. 52–55.

**Hermiston, T. W. and Kuhn, I. (2002).** Armed therapeutic viruses: Strategies and challenges to arming oncolytic viruses with therapeutic genes. *Cancer Gene Ther.* **9**, 1022-1035.

**Hirschowitz, E., Hidalgo, G. and Doherty, D. (2002).** Induction of cyclo-oxygenase-2 in non-small cell lung cancer cells by infection with DeltaE1, DeltaE3 recombinant adenovirus vectors. *Gene Ther.* **9**, 81-84.

**Hodges, B. L., Taylor, K. M., Chu, Q., Scull, S. E., Serriello, R. G., Anderson, S. C., Wang, F. and Scheule, R. K. (2005).** Local delivery of a viral vector mitigates neutralization by antiviral antibodies and results in efficient transduction of rabbit liver. *Mol. Ther.* **12**, 1043-1051.

**Hofmann, C., Loser, P., Cichon, G., Arnold, W., Both, G. W. and Strauss, M. (1999).** Ovine adenovirus vectors overcome preexisting humoral immunity against human adenoviruses in vivo. *J.Virol.* **73**, 6930-6936.

**Holm, P. S., Bergmann, S., Jurchott, K., Lage, H., Brand, K., Ladhoff, A., Mantwill, K., Curiel, D. T., Dobbelstein, M., Dietel, M., Gansbacher, B. and Royer, H. D. (2002).** YB-1 relocates to the nucleus in adenovirus-infected cells and facilitates viral replication by inducing E2 gene expression through the E2 late promoter. *J.Biol.Chem.* **277**, 10427-10434.

**Hu, C.R. Shew, M.B. Bally and Madden, T.D. (2001).** Programmable fusogenic vesicles for intracellular delivery of antisense oligodeoxynucleotides: enhanced cellular uptake and biological effects. *Biochim. Biophys. Acta* **1514**, 1–13.

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

**Jakubczak, J. L., Ryan, P., Gorziglia, M., Clarke, L., Hawkins, L. K., Hay, C., Huang, Y., Kaloss, M., Marinov, A., Phipps, S., Pinkstaff, A., Shirley, P., Skripchenko, Y., Stewart, D.,**

**Forry-Schaudies, S. and Hallenbeck, P. L. (2003).** An oncolytic adenovirus selective for retinoblastoma tumor suppressor protein pathway-defective tumors: Dependence on E1A, the E2F-1 promoter, and viral replication for selectivity and efficacy. *Cancer Res.* **63**, 1490-1499.

**Jevremovic, D., Gulati, R., Hennig, I., Diaz, R. M., Cole, C., Kleppe, L., Cosset, F. L., Simari, R. D. and Vile, R. G. (2004).** Use of blood outgrowth endothelial cells as virus-producing vectors for gene delivery to tumors. *Am.J.Physiol.Heart Circ.Physiol.* **287**, H494-500.

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

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

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

**Kanerva, A., Zinn, K. R., Peng, K. W., Ranki, T., Kangasniemi, L., Chaudhuri, T. R., Desmond, R. A., Wang, M., Takayama, K., Hakkarainen, T., Alfthan, H., Stenman, U. H., Curiel, D. T. and Hemminki, A. (2005).** Noninvasive dual modality in vivo monitoring of the persistence and potency of a tumor targeted conditionally replicating adenovirus. *Gene Ther.* **12**, 87-94.

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

**Kass-Eisler, A., Leinwand, L., Gall, J., Bloom, B. and Falck-Pedersen, E. (1996).** Circumventing the immune response to adenovirus-mediated gene therapy. *Gene Ther.* **3**, 154-162.

**Kelly, F. J., Miller, C. R., Buchsbaum, D. J., Gomez-Navarro, J., Barnes, M. N., Alvarez, R. D. and Curiel, D. T. (2000).** Selectivity of TAG-72-targeted adenovirus gene transfer to primary ovarian carcinoma cells versus autologous mesothelial cells in vitro. *Clin.Cancer Res.* **6**, 4323-4333.

**Khuri, F. R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I. F., Romel, L., Gore, M., Ironside, J., MacDougall, R. H., Heise, C., Randlev, B., Gillenwater, A. M., Brusio, P., Kaye, S. B., Hong, W. K. and Kirn, D. H. (2000a).** a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat.Med.* **6**, 879-885.

- Kikuchi, E., Menendez, S., Ozu, C., Ohori, M., Cordon-Cardo, C., Logg, C. R., Kasahara, N. and Bochner, B. H. (2007a).** Highly efficient gene delivery for bladder cancers by intravesically administered replication-competent retroviral vectors. *Clin.Cancer Res.* **13**, 4511-4518.
- Kirn, D. H., Wang, Y., Le Boeuf, F., Bell, J. and Thorne, S. H. (2007).** Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. *PLoS Med.* **4**, e353.
- Koc, O.N., Davis, B.M., Reese, J.S., Friebert, S. E. and Gerson, S. L. (1999).** Transfer of drug-resistance genes into hematopoietic progenitors. In: E.C. Lattime and S.L. Gerson, Editors, *Gene Therapy of Cancer*, Academic Press, San Diego 177-200.
- Komarova, S., Kawakami, Y., Stoff-Khalili, M. A., Curiel, D. T. and Pereboeva, L. (2006).** Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol.Cancer.Ther.* **5**, 755-766.
- Konopka, J. B., Watanabe, S. M., Singer, J. W., Collins, S. J. and Witte, O. N. (1985).** Cell lines and clinical isolates derived from Ph1-positive chronic myelogenous leukemia patients express c-abl proteins with a common structural alteration. *Proc.Natl.Acad.Sci.U.S.A.* **82**, 1810-1814.
- Korokhov, N., Mikheeva, G., Krendelshchikov, A., Belousova, N., Simonenko, V., Krendelshchikova, V., Pereboev, A., Kotov, A., Kotova, O., Triozzi, P. L., Aldrich, W. A., Douglas, J. T., Lo, K. M., Banerjee, P. T., Gillies, S. D., Curiel, D. T. and Krasnykh, V. (2003).** Targeting of adenovirus via genetic modification of the viral capsid combined with a protein bridge. *J.Virol.* **77**, 12931-12940.
- Krasnykh, V., Belousova, N., Korokhov, N., Mikheeva, G. and Curiel, D. T. (2001).** Genetic targeting of an adenovirus vector via replacement of the fiber protein with the phage T4 fibrin. *J.Virol.* **75**, 4176-4183.
- Krasnykh, V. N., Mikheeva, G. V., Douglas, J. T. and Curiel, D. T. (1996).** Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J.Virol.* **70**, 6839-6846.
- Kruys, V., Marinx, O., Shaw, G., Deschamps, J. and Huez, G. (1989).** Translational blockade imposed by cytokine-derived UA-rich sequences. *Science* **245**, 852-855.
- Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W. and Herschman, H. R. (1991).** TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J.Biol.Chem.* **266**, 12866-12872.
- Lambright, E. S., Amin, K., Wiewrodt, R., Force, S. D., Lanuti, M., Propert, K. J., Litzky, L., Kaiser, L. R. and Albelda, S. M. (2001).** Inclusion of the herpes simplex thymidine kinase gene in a replicating adenovirus does not augment antitumor efficacy. *Gene Ther.* **8**, 946-953.
- Lamfers, M. L., Grill, J., Dirven, C. M., Van Beusechem, V. W., Georger, B., Van Den Berg, J., Alemany, R., Fueyo, J., Curiel, D. T., Vassal, G., Pinedo, H. M., Vandertop, W. P. and Gerritsen, W. R. (2002).** Potential of the conditionally replicative adenovirus Ad5-Delta24RGD in



the treatment of malignant gliomas and its enhanced effect with radiotherapy. *Cancer Res.* **62**, 5736-5742.

**Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J. and Clark, A. R. (2000).** Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. *Mol.Cell.Biol.* **20**, 4265-4274.

**Le Blanc, K., Rasmusson, I., Sundberg, B., Gotherstrom, C., Hassan, M., Uzunel, M. and Ringden, O. (2004).** Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* **363**, 1439-1441.

**Le, L. P., Everts, M., Dmitriev, I. P., Davydova, J. G., Yamamoto, M. and Curiel, D. T. (2004).** Fluorescently labeled adenovirus with pIX-EGFP for vector detection. *Mol.Imaging* **3**, 105-116.

**Leskela, H. V., Risteli, J., Niskanen, S., Koivunen, J., Ivaska, K. K. and Lehenkari, P. (2003).** Osteoblast recruitment from stem cells does not decrease by age at late adulthood. *Biochem.Biophys.Res.Commun.* **311**, 1008-1013.

**Li, H. J., Everts, M., Pereboeva, L., Komarova, S., Idan, A., Curiel, D. T. and Herschman, H. R. (2007).** Adenovirus tumor targeting and hepatic untargeting by a coxsackie/adenovirus receptor ectodomain anti-carcinoembryonic antigen bispecific adapter. *Cancer Res.* **67**, 5354-5361.

**Li, J., Lad, S., Yang, G., Luo, Y., Iacobelli-Martinez, M., Primus, F. J., Reisfeld, R. A. and Li, E. (2006).** Adenovirus fiber shaft contains a trimerization element that supports peptide fusion for targeted gene delivery. *J.Virol.* **80**, 12324-12331.

**Li, N., Zhou, J., Weng, D., Zhang, C., Li, L., Wang, B., Song, Y., He, Q., Lin, D., Chen, D., Chen, G., Gao, Q., Wang, S., Xu, G., Meng, L., Lu, Y. and Ma, D. (2007).** Adjuvant adenovirus-mediated delivery of herpes simplex virus thymidine kinase administration improves outcome of liver transplantation in patients with advanced hepatocellular carcinoma. *Clin.Cancer Res.* **13**, 5847-5854.

**Liang, Q., Yamamoto, M., Curiel, D. T. and Herschman, H. R. (2004).** Noninvasive imaging of transcriptionally restricted transgene expression following intratumoral injection of an adenovirus in which the COX-2 promoter drives a reporter gene. *Mol.Imaging Biol.* **6**, 395-404.

**Licht, T. and Peschel, C. (2002).** Restoration of transgene expression in hematopoietic cells with drug-selectable marker genes. *Curr. Gene Ther.* **2**, 227-34.

**Lieber, A., He, C. Y., Meuse, L., Schowalter, D., Kirillova, I., Winther, B. and Kay, M. A. (1997).** The role of kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J.Virol.* **71**, 8798-8807.

**Lin, T., Huang, X., Gu, J., Zhang, L., Roth, J. A., Xiong, M., Curley, S. A., Yu, Y., Hunt, K. K. and Fang, B. (2002).** Long-term tumor-free survival from treatment with the GFP-TRAIL fusion gene expressed from the hTERT promoter in breast cancer cells. *Oncogene* **21**, 8020-8028.

- Liszewski, M. K., Post, T. W. and Atkinson, J. P. (1991).** Membrane cofactor protein (MCP or CD46): Newest member of the regulators of complement activation gene cluster. *Annu.Rev.Immunol.* **9**, 431-455.
- Magnusson, M. K., Hong, S. S., Boulanger, P. and Lindholm, L. (2001).** Genetic retargeting of adenovirus: Novel strategy employing "deknobbing" of the fiber. *J.Virol.* **75**, 7280-7289.
- Mastrangeli, A., Harvey, B. G., Yao, J., Wolff, G., Kovesdi, I., Crystal, R. G. and Falck-Pedersen, E. (1996).** "Sero-switch" adenovirus-mediated in vivo gene transfer: Circumvention of anti-adenovirus humoral immune defenses against repeat adenovirus vector administration by changing the adenovirus serotype. *Hum.Gene Ther.* **7**, 79-87.
- Miller, C. R., Buchsbaum, D. J., Reynolds, P. N., Douglas, J. T., Gillespie, G. Y., Mayo, M. S., Raben, D. and Curiel, D. T. (1998a).** Differential susceptibility of primary and established human glioma cells to adenovirus infection: Targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res.* **58**, 5738-5748.
- Mittal, S. K., Prevec, L., Graham, F. L. and Babiuk, L. A. (1995).** Development of a bovine adenovirus type 3-based expression vector. *J.Gen.Virol.* **76 ( Pt 1)**, 93-102.
- Mizuguchi, H. and Hayakawa, T. (2002).** Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. *Gene* **285**, 69-77.
- Mizuguchi, H., Koizumi, N., Hosono, T., Utoguchi, N., Watanabe, Y., Kay, M. A. and Hayakawa, T. (2001).** A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther.* **8**, 730-735.
- Mizuguchi, H., Sasaki, T., Kawabata, K., Sakurai, F. and Hayakawa, T. (2005).** Fiber-modified adenovirus vectors mediate efficient gene transfer into undifferentiated and adipogenic-differentiated human mesenchymal stem cells. *Biochem.Biophys.Res.Commun.* **332**, 1101-1106.
- Mohr, A., Lyons, M., Deedigan, L., Harte, T., Shaw, G., Howard, L., Barry, F., O'Brien, T. and Zwacka, R. (2008).** Mesenchymal stem cells expressing TRAIL lead to tumour growth inhibition in an experimental lung cancer model. *J.Cell.Mol.Med.* .
- Molinier-Frenkel, V., Prevost-Blondel, A., Hong, S. S., Lengagne, R., Boudaly, S., Magnusson, M. K., Boulanger, P. and Guillet, J. G. (2003).** The maturation of murine dendritic cells induced by human adenovirus is mediated by the fiber knob domain. *J.Biol.Chem.* **278**, 37175-37182.
- Morris, J. C. and Wildner, O. (2000).** Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-tk. *Mol.Ther.* **1**, 56-62.
- Morsy, M. A., Gu, M., Motzel, S., Zhao, J., Lin, J., Su, Q., Allen, H., Franlin, L., Parks, R. J., Graham, F. L., Kochanek, S., Bett, A. J. and Caskey, C. T. (1998).** An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. *Proc.Natl.Acad.Sci.U.S.A.* **95**, 7866-7871.

- Mueller, C. and Flotte, T. R. (2008).** Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Ther.*
- Mulvihill, S., Warren, R., Venook, A., Adler, A., Randlev, B., Heise, C. and Kirn, D. (2001).** Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: A phase I trial. *Gene Ther.* **8**, 308-315.
- Muruve, D. A., Barnes, M. J., Stillman, I. E. and Libermann, T. A. (1999).** Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo. *Hum. Gene Ther.* **10**, 965-976.
- Nakamura, K., Ito, Y., Kawano, Y., Kurozumi, K., Kobune, M., Tsuda, H., Bizen, A., Honmou, O., Niitsu, Y. and Hamada, H. (2004).** Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther.* **11**, 1155-1164.
- Nanda, A., Lynch, D. M., Goudsmit, J., Lemckert, A. A., Ewald, B. A., Sumida, S. M., Truitt, D. M., Abbink, P., Kishko, M. G., Gorgone, D. A., Lifton, M. A., Shen, L., Carville, A., Mansfield, K. G., Havenga, M. J. and Barouch, D. H. (2005).** Immunogenicity of recombinant fiber-chimeric adenovirus serotype 35 vector-based vaccines in mice and rhesus monkeys. *J. Virol.* **79**, 14161-14168.
- Nanda, D., Vogels, R., Havenga, M., Avezaat, C. J., Bout, A. and Smitt, P. S. (2001).** Treatment of malignant gliomas with a replicating adenoviral vector expressing herpes simplex virus-thymidine kinase. *Cancer Res.* **61**, 8743-8750.
- Nemunaitis, J., Cunningham, C., Buchanan, A., Blackburn, A., Edelman, G., Maples, P., Netto, G., Tong, A., Randlev, B., Olson, S. and Kirn, D. (2001).** Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: Safety, feasibility and biological activity. *Gene Ther.* **8**, 746-759.
- Nemunaitis, J., Ganly, I., Khuri, F., Arseneau, J., Kuhn, J., McCarty, T., Landers, S., Maples, P., Romel, L., Randlev, B., Reid, T., Kaye, S. and Kirn, D. (2000).** Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: A phase II trial. *Cancer Res.* **60**, 6359-6366.
- Nemunaitis, J., Khuri, F., Ganly, I., Arseneau, J., Posner, M., Vokes, E., Kuhn, J., McCarty, T., Landers, S., Blackburn, A., Romel, L., Randlev, B., Kaye, S. and Kirn, D. (2001).** Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J. Clin. Oncol.* **19**, 289-298.
- Nemunaitis, J., Senzer, N., Sarmiento, S., Zhang, Y. A., Arzaga, R., Sands, B., Maples, P. and Tong, A. W. (2007).** A phase I trial of intravenous infusion of ONYX-015 and enbrel in solid tumor patients. *Cancer Gene Ther.* **14**, 885-893.
- Nettelbeck, D. M., Rivera, A. A., Davydova, J., Dieckmann, D., Yamamoto, M. and Curiel, D. T. (2003).** Cyclooxygenase-2 promoter for tumour-specific targeting of adenoviral vectors to melanoma. *Melanoma Res.* **13**, 287-292.

- Nicklin, S. A., Von Seggern, D. J., Work, L. M., Pek, D. C., Dominiczak, A. F., Nemerow, G. R. and Baker, A. H. (2001).** Ablating adenovirus type 5 fiber-CAR binding and HI loop insertion of the SIGYPLP peptide generate an endothelial cell-selective adenovirus. *Mol. Ther.* **4**, 534-542.
- Nicklin, S. A., Wu, E., Nemerow, G. R. and Baker, A. H. (2005).** The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol. Ther.* **12**, 384-393.
- Okegawa, T., Li, Y., Pong, R. C., Bergelson, J. M., Zhou, J. and Hsieh, J. T. (2000).** The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. *Cancer Res.* **60**, 5031-5036.
- Okegawa, T., Pong, R. C., Li, Y., Bergelson, J. M., Sagalowsky, A. I. and Hsieh, J. T. (2001).** The mechanism of the growth-inhibitory effect of coxsackie and adenovirus receptor (CAR) on human bladder cancer: A functional analysis of car protein structure. *Cancer Res.* **61**, 6592-6600.
- Ong, H. T., Hasegawa, K., Dietz, A. B., Russell, S. J. and Peng, K. W. (2007).** Evaluation of T cells as carriers for systemic measles virotherapy in the presence of antiviral antibodies. *Gene Ther.* **14**, 324-333.
- Osaki, T., Tanio, Y., Tachibana, I., Hosoe, S., Kumagai, T., Kawase, I., Oikawa, S. and Kishimoto, T. (1994).** Gene therapy for carcinoembryonic antigen-producing human lung cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene. *Cancer Res.* **54**, 5258-5261.
- O'Shea, C. C., Soria, C., Bagus, B. and McCormick, F. (2005).** Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. *Cancer Cell.* **8**, 61-74.
- Oshikiri, T., Miyamoto, M., Hiraoka, K., Shichinohe, T., Kawarada, Y., Kato, K., Suzuoki, M., Nakakubo, Y., Kondo, S., Dosaka-Akita, H., Kasahara, N. and Katoh, H. (2006).** Transcriptional targeting of adenovirus vectors with the squamous cell carcinoma-specific antigen-2 promoter for selective apoptosis induction in lung cancer. *Cancer Gene Ther.* **13**, 856-863.
- Pellinen, R., Hakkarainen, T., Wahlfors, T., Tulimaki, K., Ketola, A., Tenhunen, A., Salonen, T. and Wahlfors, J. (2004).** Cancer cells as targets for lentivirus-mediated gene transfer and gene therapy. *Int.J.Oncol.* **25**, 1753-1762.
- Prockop, D. J. (1997).** Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71-74.
- Qian Z. M., Li H, Sun H, and Ho K. (2002).** Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacol Rev.* **54**, 561-87.
- Rabin, N., Kyriakou, C., Coulton, L., Gallagher, O. M., Buckle, C., Benjamin, R., Singh, N., Glassford, J., Otsuki, T., Nathwani, A. C., Croucher, P. I. and Yong, K. L. (2007).** A new xenograft model of myeloma bone disease demonstrating the efficacy of human mesenchymal stem cells expressing osteoprotegerin by lentiviral gene transfer. *Leukemia* **21**, 2181-2191.

- Rahman, A., Tsai, V., Goudreau, A., Shinoda, J. Y., Wen, S. F., Ramachandra, M., Ralston, R., Maneval, D., LaFace, D. and Shabram, P. (2001).** Specific depletion of human anti-adenovirus antibodies facilitates transduction in an in vivo model for systemic gene therapy. *Mol. Ther.* **3**, 768-778.
- Raki, M., Hakkarainen, T., Bauerschmitz, G. J., Sarkioja, M., Desmond, R. A., Kanerva, A. and Hemminki, A. (2007).** Utility of TK/GCV in the context of highly effective oncolysis mediated by a serotype 3 receptor targeted oncolytic adenovirus. *Gene Ther.* **14**, 1380-1388.
- Ramalingam, S. and Belani, C. (2008).** Systemic chemotherapy for advanced non-small cell lung cancer: Recent advances and future directions. *Oncologist* **13 Suppl 1**, 5-13.
- Ramirez, P. J., Vickers, S. M., Ono, H. A., Davydova, J., Takayama, K., Thompson, T. C., Curiel, D. T., Bland, K. I. and Yamamoto, M. (2008).** Optimization of conditionally replicative adenovirus for pancreatic cancer and its evaluation in an orthotopic murine xenograft model. *Am.J.Surg.* **195**, 481-490.
- Ranki, T., Kanerva, A., Ristimaki, A., Hakkarainen, T., Sarkioja, M., Kangasniemi, L., Raki, M., Laakkonen, P., Goodison, S. and Hemminki, A. (2007a).** A heparan sulfate-targeted conditionally replicative adenovirus, Ad5.pk7-Delta24, for the treatment of advanced breast cancer. *Gene Ther.* **14**, 58-67.
- Ranki, T., Sarkioja, M., Hakkarainen, T., von Smitten, K., Kanerva, A. and Hemminki, A. (2007b).** Systemic efficacy of oncolytic adenoviruses in imagable orthotopic models of hormone refractory metastatic breast cancer. *Int.J.Cancer* **121**, 165-174.
- Raper, S. E., Chirmule, N., Lee, F. S., Wivel, N. A., Bagg, A., Gao, G. P., Wilson, J. M. and Batshaw, M. L. (2003).** Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol.Genet.Metab.* **80**, 148-158.
- Raykov, Z. and Rommelaere, J. (2008).** Potential of tumour cells for delivering oncolytic viruses. *Gene Ther.* **15**, 704-710.
- Reid, T., Galanis, E., Abbruzzese, J., Sze, D., Andrews, J., Romel, L., Hatfield, M., Rubin, J. and Kirn, D. (2001).** Intra-arterial administration of a replication-selective adenovirus (dl1520) in patients with colorectal carcinoma metastatic to the liver: A phase I trial. *Gene Ther.* **8**, 1618-1626.
- Reynolds, P. N., Nicklin, S. A., Kaliberova, L., Boatman, B. G., Grizzle, W. E., Balyasnikova, I. V., Baker, A. H., Danilov, S. M. and Curiel, D. T. (2001).** Combined transductional and transcriptional targeting improves the specificity of transgene expression in vivo. *Nat.Biotechnol.* **19**, 838-842.
- Reynolds, P. N., Zinn, K. R., Gavrilyuk, V. D., Balyasnikova, I. V., Rogers, B. E., Buchsbaum, D. J., Wang, M. H., Miletich, D. J., Grizzle, W. E., Douglas, J. T., Danilov, S. M. and Curiel, D. T. (2000).** A targetable, injectable adenoviral vector for selective gene delivery to pulmonary endothelium in vivo. *Mol. Ther.* **2**, 562-578.

- Rocconi, R. P., Zhu, Z. B., Stoff-Khalili, M., Rivera, A. A., Lu, B., Wang, M., Alvarez, R. D., Curiel, D. T. and Makhija, S. K. (2007).** Treatment of ovarian cancer with a novel dual targeted conditionally replicative adenovirus (CRAd). *Gynecol.Oncol.* **105**, 113-121.
- Roelvink, P. W., Lizonova, A., Lee, J. G., Li, Y., Bergelson, J. M., Finberg, R. W., Brough, D. E., Kovesdi, I. and Wickham, T. J. (1998).** The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J.Virol.* **72**, 7909-7915.
- Rogulski, K. R., Wing, M. S., Paielli, D. L., Gilbert, J. D., Kim, J. H. and Freytag, S. O. (2000).** Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. *Hum.Gene Ther.* **11**, 67-76.
- Russell, W. C. (2000).** Update on adenovirus and its vectors. *J.Gen.Virol.* **81**, 2573-2604.
- 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**, 1083-1092.
- Sanz, L., Qiao, J., Vile, R. G. and Alvarez-Vallina, L. (2005).** Antibody engineering, virus retargeting and cellular immunotherapy: One ring to rule them all? *Curr.Gene Ther.* **5**, 63-70.
- Sarkar, D., Su, Z. Z., Vozhilla, N., Park, E. S., Randolph, A., Valerie, K. and Fisher, P. B. (2005).** Targeted virus replication plus immunotherapy eradicates primary and distant pancreatic tumors in nude mice. *Cancer Res.* **65**, 9056-9063.
- Sarkioja, M., Hakkarainen, T., Eriksson, M., Ristimaki, A., Desmond, R. A., Kanerva, A. and Hemminki, A. (2008).** The cyclo-oxygenase 2 promoter is induced in nontarget cells following adenovirus infection, but an AU-rich 3'-untranslated region destabilization element can increase specificity. *J.Gene Med.* .
- Saukkonen, K. and Hemminki, A. (2004).** Tissue-specific promoters for cancer gene therapy. *Expert Opin.Biol.Ther.* **4**, 683-696.
- Schiedner, G., Hertel, S., Johnston, M., Dries, V., van Rooijen, N. and Kochanek, S. (2003).** Selective depletion or blockade of kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol.Ther.* **7**, 35-43.
- Schnell, M. A., Zhang, Y., Tazelaar, J., Gao, G. P., Yu, Q. C., Qian, R., Chen, S. J., Varnavski, A. N., LeClair, C., Raper, S. E. and Wilson, J. M. (2001).** Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol.Ther.* **3**, 708-722.
- Schoggins, J. W., Nociari, M., Philpott, N. and Falck-Pedersen, E. (2005).** Influence of fiber detargeting on adenovirus-mediated innate and adaptive immune activation. *J.Virol.* **79**, 11627-11637.

- Segerman, A., Mei, Y. F. and Wadell, G. (2000).** Adenovirus types 11p and 35p show high binding efficiencies for committed hematopoietic cell lines and are infective to these cell lines. *J.Virol.* **74**, 1457-1467.
- Seidman, M. A., Hogan, S. M., Wendland, R. L., Worgall, S., Crystal, R. G. and Leopold, P. L. (2001).** Variation in adenovirus receptor expression and adenovirus vector-mediated transgene expression at defined stages of the cell cycle. *Mol.Ther.* **4**, 13-21.
- Shayakhmetov, D. M., Gaggar, A., Ni, S., Li, Z. Y. and Lieber, A. (2005).** Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J.Virol.* **79**, 7478-7491.
- Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G. and Lieber, A. (2000).** Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. *J.Virol.* **74**, 2567-2583.
- Shi F., Rakhmilevich, AL., Heise, CP., Oshikawa, K., Sondel, PM., Yang, NS. and Mahvi, DM. (2002).** Intratumoral injection of interleukin-12 plasmid DNA, either naked or in complex with cationic lipid, results in similar tumor regression in a murine model. *Mol. Cancer Ther.* **1**. 949-957.
- Shirakawa, T., Terao, S., Hinata, N., Tanaka, K., Takenaka, A., Hara, I., Sugimura, K., Matsuo, M., Hamada, K., Fuji, K., Okegawa, T., Higashihara, E., Gardner, T. A., Kao, C., Chung, L. W., Kamidono, S., Fujisawa, M. and Gotoh, A. (2007).** Long-term outcome of phase I/II clinical trial of ad-OC-TK/VAL gene therapy for hormone-refractory metastatic prostate cancer. *Hum.Gene Ther.* **18**, 1225-1232.
- Short, J. J., Pereboev, A. V., Kawakami, Y., Vasu, C., Holterman, M. J. and Curiel, D. T. (2004).** Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. *Virology* **322**, 349-359.
- Sirena, D., Lilienfeld, B., Eisenhut, M., Kalin, S., Boucke, K., Beerli, R. R., Vogt, L., Ruedl, C., Bachmann, M. F., Greber, U. F. and Hemmi, S. (2004).** The human membrane cofactor CD46 is a receptor for species B adenovirus serotype 3. *J.Virol.* **78**, 4454-4462.
- Small, E. J., Carducci, M. A., Burke, J. M., Rodriguez, R., Fong, L., van Ummersen, L., Yu, D. C., Aimi, J., Ando, D., Working, P., Kirn, D. and Wilding, G. (2006).** A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol.Ther.* **14**, 107-117.
- Smith, T. A., Idamakanti, N., Marshall-Neff, J., Rollence, M. L., Wright, P., Kaloss, M., King, L., Mech, C., Dinges, L., Iverson, W. O., Sherer, A. D., Markovits, J. E., Lyons, R. M., Kaleko, M. and Stevenson, S. C. (2003a).** Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. *Hum.Gene Ther.* **14**, 1595-1604.
- Smith, T. A., Idamakanti, N., Rollence, M. L., Marshall-Neff, J., Kim, J., Mulgrew, K., Nemerow, G. R., Kaleko, M. and Stevenson, S. C. (2003b).** Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. *Hum.Gene Ther.* **14**, 777-787.

- Smith, T. A., White, B. D., Gardner, J. M., Kaleko, M. and McClelland, A. (1996).** Transient immunosuppression permits successful repetitive intravenous administration of an adenovirus vector. *Gene Ther.* **3**, 496-502.
- Smith, W. L. (1992).** Prostanoid biosynthesis and mechanisms of action. *Am.J.Physiol.* **263**, F181-91.
- Sonabend, A. M., Ulasov, I. V., Tyler, M. A., Rivera, A. A., Mathis, J. M. and Lesniak, M. S. (2008).** Mesenchymal stem cells effectively deliver an oncolytic adenovirus to intracranial glioma. *Stem Cells* **26**, 831-841.
- Spano, J. P., Andre, F., Morat, L., Sabatier, L., Besse, B., Combadiere, C., Deterre, P., Martin, A., Azorin, J., Valeyre, D., Khayat, D., Le Chevalier, T. and Soria, J. C. (2004).** Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: Pattern of expression and correlation with outcome. *Ann.Oncol.* **15**, 613-617.
- Stecher, H., Shayakhmetov, D. M., Stamatoyannopoulos, G. and Lieber, A. (2001).** A capsid-modified adenovirus vector devoid of all viral genes: Assessment of transduction and toxicity in human hematopoietic cells. *Mol.Ther.* **4**, 36-44.
- Steel, J. C., Cavanagh, H. M., Burton, M. A. and Kalle, W. H. (2004).** Microsphere-liposome complexes protect adenoviral vectors from neutralising antibody without losses in transfection efficiency, in-vitro. *J.Pharm.Pharmacol.* **56**, 1371-1378.
- Stewart, P. L., Fuller, S. D. and Burnett, R. M. (1993).** Difference imaging of adenovirus: Bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J.* **12**, 2589-2599.
- Stoff-Khalili, M. A., Rivera, A. A., Mathis, J. M., Banerjee, N. S., Moon, A. S., Hess, A., Rocconi, R. P., Numnum, T. M., Everts, M., Chow, L. T., Douglas, J. T., Siegal, G. P., Zhu, Z. B., Bender, H. G., Dall, P., Stoff, A., Pereboeva, L. and Curiel, D. T. (2007a).** Mesenchymal stem cells as a vehicle for targeted delivery of CRAbs to lung metastases of breast carcinoma. *Breast Cancer Res.Treat.* **105**, 157-167.
- Stone, D., Ni, S., Li, Z. Y., Gaggar, A., DiPaolo, N., Feng, Q., Sandig, V. and Lieber, A. (2005).** Development and assessment of human adenovirus type 11 as a gene transfer vector. *J.Virol.* **79**, 5090-5104.
- Studeny, M., Marini, F. C., Champlin, R. E., Zompetta, C., Fidler, I. J. and Andreeff, M. (2002).** Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res.* **62**, 3603-3608.
- Studeny, M., Marini, F. C., Dembinski, J. L., Zompetta, C., Cabreira-Hansen, M., Bekele, B. N., Champlin, R. E. and Andreeff, M. (2004).** Mesenchymal stem cells: Potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J.Natl.Cancer Inst.* **96**, 1593-1603.
- Sudimack J, and Lee R. J. (2000).** Targeted drug delivery via the folate receptor. *Adv*



**Svensson, L., Hjalmarsson, A. and Everitt, E. (1999).** TCID50 determination by an immuno dot blot assay as exemplified in a study of storage conditions of infectious pancreatic necrosis virus. *J. Virol. Methods* **8**, 17-24.

**Swisher, S. G., Roth, J. A., Komaki, R., Gu, J., Lee, J. J., Hicks, M., Ro, J. Y., Hong, W. K., Merritt, J. A., Ahrar, K., Atkinson, N. E., Correa, A. M., Dolormente, M., Dreiling, L., El-Naggar, A. K., Fossella, F., Francisco, R., Glisson, B., Grammer, S., Herbst, R., Huaranga, A., Kemp, B., Khuri, F. R., Kurie, J. M., Liao, Z., McDonnell, T. J., Morice, R., Morello, F., Munden, R., Papadimitrakopoulou, V., Pisters, K. M., Putnam J. B. Jr., Sarabiam, A. J., Shelton, T., Stevens, C., Shin, D. M., Smythe, W. R., Vaporciyan, A. A., Walsh, G. L. and Yin, M. (2003).** Induction of p53-regulated genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral p53 (INGN 201) and radiation therapy. *Clin. Cancer Res.* **9**, 93–101.

**Suzuki, K., Fueyo, J., Krasnykh, V., Reynolds, P. N., Curiel, D. T. and Alemany, R. (2001).** A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency. *Clin. Cancer Res.* **7**, 120-126.

**Tanaka, H., Fujii, Y., Hirabayashi, H., Miyoshi, S., Sakaguchi, M., Yoon, H. E. and Matsuda, H. (1998).** Disruption of the RB pathway and cell-proliferative activity in non-small-cell lung cancers. *Int.J.Cancer* **79**, 111-115.

**Thanarajasingam, U., Sanz, L., Diaz, R., Qiao, J., Sanchez-Perez, L., Kottke, T., Thompson, J., Chester, J. and Vile, R. G. (2007).** Delivery of CCL21 to metastatic disease improves the efficacy of adoptive T-cell therapy. *Cancer Res.* **67**, 300-308.

**Tillman, B. W., de Gruijl, T. D., Luykx-de Bakker, S. A., Scheper, R. J., Pinedo, H. M., Curiel, T. J., Gerritsen, W. R. and Curiel, D. T. (1999).** Maturation of dendritic cells accompanies high-efficiency gene transfer by a CD40-targeted adenoviral vector. *J.Immunol.* **162**, 6378-6383.

**Tokunaga, T., Tsuchida, T., Kijima, H., Okamoto, K., Oshika, Y., Sawa, N., Ohnishi, Y., Yamazaki, H., Miura, S., Ueyama, Y. and Nakamura, M. (2000).** Ribozyme-mediated inactivation of mutant K-ras oncogene in a colon cancer cell line. *Br. J. Cancer* **8**, 833–839

**Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. and Croce, C. M. (1985).** The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* **229**, 1390-1393.

**Tsukuda, K., Wiewrodt, R., Molnar-Kimber, K., Jovanovic, V. P. and Amin, K. M. (2002).** An E2F-responsive replication-selective adenovirus targeted to the defective cell cycle in cancer cells: Potent antitumoral efficacy but no toxicity to normal cell. *Cancer Res.* **62**, 3438-3447.

**Turini, M. E. and DuBois, R. N. (2002).** Cyclooxygenase-2: A therapeutic target. *Annu.Rev.Med.* **53**, 35-57.

**Tuve, S., Wang, H., Ware, C., Liu, Y., Gaggar, A., Bernt, K., Shayakhmetov, D., Li, Z., Strauss, R., Stone, D. and Lieber, A. (2006a).** A new group B adenovirus receptor is expressed at high levels on human stem and tumor cells. *J.Virol.* **80**, 12109-12120.

**Tyler, M. A., Sonabend, A. M., Ulasov, I. V. and Lesniak, M. S. (2008).** Vector therapies for malignant glioma: Shifting the clinical paradigm. *Expert Opin.Drug Deliv.* **5**, 445-458.

**Vasey, P. A., Shulman, L. N., Campos, S., Davis, J., Gore, M., Johnston, S., Kirn, D. H., O'Neill, V., Siddiqui, N., Seiden, M. V. and Kaye, S. B. (2002).** Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J.Clin.Oncol.* **20**, 1562-1569.

**Vigne, E., Mahfouz, I., Dedieu, J. F., Brie, A., Perricaudet, M. and Yeh, P. (1999).** RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J.Virol.* **73**, 5156-5161.

**Vogels, R., Zuijdgceest, D., van Rijnsoever, R., Hartkoorn, E., Damen, I., de Bethune, M. P., Kostense, S., Penders, G., Helmus, N., Koudstaal, W., Cecchini, M., Wetterwald, A., Sprangers, M., Lemckert, A., Ophorst, O., Koel, B., van Meerendonk, M., Quax, P., Panitti, L., Grimbergen, J., Bout, A., Goudsmit, J. and Havenga, M. (2003).** Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: Efficient human cell infection and bypass of preexisting adenovirus immunity. *J.Virol.* **77**, 8263-8271.

**Vollmer C. M., Eilber, F. C., Jr., Butterfield, L. H., Ribas, A., Dissette, V. B., Koh, A., Montejo, J. D., Lee, M. C., Andrews, K.J., McBride, W. H., Glaspy J. G. and Economou, J.S. (1999).** Alpha-fetoprotein-specific genetic immunotherapy for hepatocellular carcinoma. *Cancer Res.* **59**, 3064–3067.

**Volpers, C., Thirion, C., Biermann, V., Hussmann, S., Kewes, H., Dunant, P., von der Mark, H., Herrmann, A., Kochanek, S. and Lochmuller, H. (2003).** Antibody-mediated targeting of an adenovirus vector modified to contain a synthetic immunoglobulin g-binding domain in the capsid. *J.Virol.* **77**, 2093-2104.

**Von Seggern, D. J., Chiu, C. Y., Fleck, S. K., Stewart, P. L. and Nemerow, G. R. (1999).** A helper-independent adenovirus vector with E1, E3, and fiber deleted: Structure and infectivity of fiberless particles. *J.Virol.* **73**, 1601-1608.

**Vulink, A., Radford, K. J., Melief, C. and Hart D. (2008).** Dendritic Cells in Cancer Immunotherapy. *Ad. Cancer Res.* **99**, 363-407.

**Waddington, S. N., McVey, J. H., Bhella D., Parker, A. L., Barker, K., Atoda, H., Pink, R., Buckley, S. M, Greig, J. A., Denby, L., Custers, J., Morita, T., Francischetti, I. M., Monteiro, R. Q., Barouch, D. H. , van Rooijen, N., Napoli, C., Havenga, M. J., Nicklin, S.A. and Baker A. H. (2008).** Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell* **8**, 397-409.

**Wesseling, J. G., Yamamoto, M., Adachi, Y., Bosma, P. J., van Wijland, M., Blackwell, J. L., Li, H., Reynolds, P. N., Dmitriev, I., Vickers, S. M., Huibregtse, K. and Curriel, D. T. (2001).**

Midkine and cyclooxygenase-2 promoters are promising for adenoviral vector gene delivery of pancreatic carcinoma. *Cancer Gene Ther.* **8**, 990-996.

**Westra, W. H., Slebos, R. J., Offerhaus, G. J., Goodman, S. N., Evers, S. G., Kensler, T. W., Askin, F. B., Rodenhuis, S. and Hruban, R. H. (1993).** K-ras oncogene activation in lung adenocarcinomas from former smokers. evidence that K-ras mutations are an early and irreversible event in the development of adenocarcinoma of the lung. *Cancer* **72**, 432-438.

**Wheeler, C. J., Felgner, P. L., Tsai, Y. J., Marshall, J., Sukhu, L., Doh, S. G., Hartikka, J., Nietupski, J., Manthorpe, M., Nichols, M., Plewe, M., Liang, X., Norman, J., Smith, A. and Cheng, S. H. (1996).** A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. *Proc Natl Acad Sci USA.* **93**: 11454-9.

**Wickham, T. J., Carrion, M. E. and Kovesdi, I. (1995).** Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. *Gene Ther.* **2**, 750-756.

**Wickham, T. J., Tzeng, E., Shears, L. L., 2nd, Roelvink, P. W., Li, Y., Lee, G. M., Brough, D. E., Lizonova, A. and Kovesdi, I. (1997).** Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J. Virol.* **71**, 8221-8229.

**Wildner, O. and Morris, J. C. (2000).** The role of the E1B 55 kDa gene product in oncolytic adenoviral vectors expressing herpes simplex virus-tk: Assessment of antitumor efficacy and toxicity. *Cancer Res.* **60**, 4167-4174.

**Wildner, O., Morris, J. C., Vahanian, N. N., Ford, H., Jr, Ramsey, W. J. and Blaese, R. M. (1999).** Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. *Gene Ther.* **6**, 57-62.

**Wilson, J. M. (1996).** Adenoviruses as gene-delivery vehicles. *N.Engl.J.Med.* **334**, 1185-1187.

**Witlox, A. M., Van Beusechem, V. W., Molenaar, B., Bras, H., Schaap, G. R., Alemany, R., Curiel, D. T., Pinedo, H. M., Wuisman, P. I. and Gerritsen, W. R. (2004).** Conditionally replicative adenovirus with tropism expanded towards integrins inhibits osteosarcoma tumor growth in vitro and in vivo. *Clin. Cancer Res.* **10**, 61-67.

**Wu, D. H., Liu, L. and Chen, L. H. (2005).** Antitumor effects and radiosensitization of cytosine deaminase and thymidine kinase fusion suicide gene on colorectal carcinoma cells. *World J. Gastroenterol.* **11**, 3051-3055.

**Wu, H., Seki, T., Dmitriev, I., Uil, T., Kashentseva, E., Han, T. and Curiel, D. T. (2002).** Double modification of adenovirus fiber with RGD and polylysine motifs improves coxsackievirus-adenovirus receptor-independent gene transfer efficiency. *Hum. Gene Ther.* **13**, 1647-1653.

**Xia, Z. J., Chang, J. H., Zhang, L., Jiang, W. Q., Guan, Z. Z., Liu, J. W., Zhang, Y., Hu, X. H., Wu, G. H., Wang, H. Q., Chen, Z. C., Chen, J. C., Zhou, Q. H., Lu, J. W., Fan, Q. X., Huang, J. J. and Zheng, X. (2004a).** Phase III randomized clinical trial of intratumoral injection of E1B

gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus. *Ai Zheng* **23**, 1666-1670.

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

**Yamamoto, M., Davydova, J., Wang, M., Siegal, G. P., Krasnykh, V., Vickers, S. M. and Curiel, D. T. (2003).** Infectivity enhanced, cyclooxygenase-2 promoter-based conditionally replicative adenovirus for pancreatic cancer. *Gastroenterology* **125**, 1203-1218.

**Yotnda, P., Savoldo, B., Charlet-Berguerand, N., Rooney, C. and Brenner, M. (2004).** Targeted delivery of adenoviral vectors by cytotoxic T cells. *Blood* **104**, 2272-2280.

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

**Zhu, J., Huang, X. and Yang, Y. (2007a).** Innate immune response to adenoviral vectors is mediated by both toll-like receptor-dependent and -independent pathways. *J. Virol.* **81**, 3170-3180.

**Zhu, Z. B., Rivera, A. A., Makhija, S. K., Lu, B., Wang, M., Izumi, M., Cerfolio, R. J., Stoff-Khalili, M. A., Zhou, F., Takayama, K., Siegal, G. P. and Curiel, D. T. (2007b).** Targeting lung cancer using an infectivity enhanced CXCR4-CRAd. *Lung Cancer* **55**, 145-156.