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IMPROVING ONCOLYTIC ADENOVIRAL THERAPIES FOR GASTROINTESTINAL CANCERS AND TUMOR INITIATING CELLS

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ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Faculty of Medicine of the University of Helsinki, in Haartman Institute Lecture Hall 2, Haartmaninkatu 3, Helsinki, on the 29th of January 2010, at 12 noon.

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ISBN 978-952-92-6738-5 (paperback)
ISBN 978-952-10-6004-5 (pdf)
http://ethesis.helsinki.fi

Helsinki 2010
Yliopistopaino

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PART A

i. List of original publications

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

I. Kangasniemi L, Kiviluoto T, Kanerva A, Raki M, Ranki T, Särkioja M, Wu H, Marini F, Hockerstedt K, Isoniemi H, Alfthan H, Stenman UH, Curiel DT, Hemminki A. Adenoviral gene therapy for gastric cancer with tropism modified adenoviruses. Clin Cancer Res. 2006 May 15;12(10):3137-44.

II. Bauerschmitz GJ*, Ranki T*, Kangasniemi L*, Ribacka C, Eriksson M, Porten M, Ristimäki A, Virkkunen P, Tarkkanen M, Hakkarainen T, Kanerva A, Rein D, Pesonen S, Hemminki A. Tissue specific promoter controlled oncolytic adenoviruses for killing of $CD44^{+}CD24^{-/low}$ breast cancer cells. Cancer Res 2008 Jul 15;68(14):5533-9. *equal contribution.

III. Kangasniemi L, Koskinen M, Jokinen M, Toriseva M, Ala-Aho R, Kähäri V-M, Jalonen H, Ylä-Herttuala S, Moilanen H, Stenman U-H, Diaconu I, Kanerva A, Pesonen S, Hakkarainen T, Hemminki A. Extended release of adenovirus from silica implants *in vitro* and *in vivo.* Gene Ther. 2009 Jan;16(1):103-10.

IV. Kangasniemi L, Pisto T, Koskinen M, Jokinen M, Kiviluoto T, Cerullo V, Jalonen H, Kangasniemi A, Koski A, Rajecki M, Escutenaire S, Kanerva A, Pesonen S, Hemminki A. Capsid modified oncolytic adenoviruses for treatment of orthotopic pancreatic cancer as single agents, with gemcitabine, or in silica implants Submitted.

ii. Abbreviations

5-FC	5-fluorocytosine
5-FU	5-fluorouracil
Ad	adenovirus
Ad3	adenovirus of serotype 3
Ad5	adenovirus of serotype 5
AFP	alpha-phetoprotein
APC	adenoidal-pharyngeal-conjuctivis virus
C4BP	C4-binding protein
CAR	coxsackie-adenovirus receptor
CD	cytosine deaminase
CE	carboxylesterase
CEA	carcinoembryonic antigen
CIC	cancer-initiating cell
CIK	cytokine-induced killer
COX-2	cyclooxygenase-2
CPT-11	irinotecan
CSC	cancer stem cell
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EGF	epidermal growth factor
FIX	coagulation factor IX
FVII	coagulation factor VII
FX	coagulation factor X
GCV	ganciclovir
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCC	hepatocellular carcinoma
HSPG	heparan sulfate proteoglycan
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
hTERT	human telomerase reverse transcriptase
IFN	interferon
ITR	inverted terminal repeat
LDL	low-density lipoprotein
mAb	monoclonal antibody
MHC	major histocomplatibility complex
MSCs	mesenchymal stem cells
NK	natural killer (cell)
OTC	ornithine transcarbamylase
PEG	polyethylene glycol
PKR	protein kinase R
pRb	retinoblastoma protein
PSA	prostate-specific antigen
RGD	arginine-glycine-aspartic acid
SPB	surfactant protein B
TLR	toll-like receptor
TK	thymidine kinase
TNF-α	tumor necrosis factor
Treg	regulatory T cell
TSP	tissue spesific promoter
VEGF	anti-vascular endothelial growth factor

iii. Abstract

Although the treatment of most cancers has improved steadily, only few metastatic solid tumors can be cured. Despite frequent responses, refractory clones often emerge and the disease becomes refractory to available treatment modalities. Although chemotherapeutic agents and radiation therapy target various cellular structures and pathways, the majority of them kill cancer cells through induction of apoptosis and selectivity is based mostly on more rapid replication of tumor cells in comparison to normal cells. As malignant cells are characterized by an ability to adapt to the environment, apoptosis-resistant clones frequently develop following standard treatment. Furthermore, resistance factors are shared between different treatment regimens and therefore loss of response typically occurs rapidly, and there is a tendency for cross-resistance between agents. Therefore, new agents with novel mechanisms of action and lacking cross-resistance to currently available approaches are desperately needed.

Oncolytic adenoviruses, featuring cancer-selective cell lysis and spread, constitute a particularly interesting drug platform towards the goals of tumor specificity and the implementation of potent multimodal treatment regimens, and have been engineered in a variety of ways with the aim of improving their selectivity and efficacy. Adenoviruses allow rational drug development by genetic incorporation of targeting mechanisms that can exert their function at different stages of the viral replication cycle. In this work, we demonstrate the applicability of capsid-modified, transcriptionally targeted oncolytic adenoviruses in targeting gastric, pancreatic and breast cancer.

A variety of capsid modified adenoviruses based on serotype 5 were tested *in vitro* in gastric and pancreatic cancer cells and fresh patient tissues for transduction specificity. Biodistribution analysis was done in an orthotopic gastric cancer model to confirm the targeting potential of capsid modified viruses *in vivo*. Then, the corresponding oncolytic viruses featuring the same capsid modifications were tested in their cell killing capacity. This confirmed that successful transductional targeting translated into enhanced oncolytic potential of the viruses. Capsid modified oncolytic viruses also prolonged the survival of tumor bearing orthotopic models of gastric and pancreatic cancer. Taken together, oncolytic adenoviral gene therapy could be a potent drug for gastric and pancreatic cancer, and its specificity, potency and safety can be modulated by means of capsid modification.

We also characterized a new intraperitoneal virus delivery method in benefit for the persistence of gene delivery to intraperitoneal gastric and pancreatic cancer tumors. With a silica implant a steady and sustained virus release to the vicinity of the tumor improved the survival of the orthotopic tumor bearing mice. Furthermore, silica gel-based virus delivery lowered the toxicity mediating proimflammatory cytokine response and production of total and anti-adenovirus neutralizing antibodies (NAbs). On the other hand, silica shielded the virus against pre-existing NAbs, resulting in a more favorable biodistribution in the preimmunized mice. The virus in silica implant might therefore be of interest in treating intraperitoneally disseminated disease.

Cancer stem cells are thought to be resistant to conventional cancer drugs and might play an important role in breast cancer relapse and the formation of metastasis. Therefore, we examined if transcriptionally modified oncolytic adenoviruses are able to kill these cells. Complete eradication of putative breast cancer stem cells, suggested to reside in the $CD44^{+}CD24^{-/low}$ population, was seen *in vitro*. Furthermore, these viruses displayed significant antitumor activity in $CD44^{+}CD24^{-/low}$ -derived tumors in mice. These findings may have relevance for the elimination of cancer stem cells in humans.

In conclusion, the results presented here suggest that the genetically engineered oncolytic adenoviruses have potential in destroying cancer initiating cells and in treating gastric and pancreatic cancers.

PART B

REVIEW OF THE LITERATURE

1. Introduction

In 2002, estimated 11 million new cancer cases and 7 million cancer deaths were reported worldwide; nearly 25 million people living with cancer (Parkin, Bray et al. 2005). World population growth and ageing imply a progressive increase in cancer burden – 15 million new cases and 10 million new deaths are expected in 2020 (Parkin 2001). Past decades have increased the knowledge of molecular background dramatically. Cancer has been revealed to be a disease involving dynamic changes in the genome. The foundation has been set in the discovery of mutations leading to inactivation of tumor suppressor genes and activation of oncogenes. Both classes of genes have been identified (Bishop 1996). Better understanding of the disease and emerging modern technologies have led to the development of more sensitive diagnostic methods and new therapies. Cancer has, however, remained mostly incurable especially in the advanced stages when metastatic. There are more than 100 distinct types of cancer, and subtypes can be found within organs. Tumor cells are disrupted in distinct regulatory circuits, which may vary from cell to cell even within a tumor. Some of these circuits operate on cell-autonomous basis, some are coupled with signals that cells receive from within a tissue (Hanahan and Weinberg 2000). Due to the complexity of the disease, the great breakthrough in cancer treatment is still to come.

Gene therapy is an exciting relatively novel approach for treating cancers resistant to currently available modalities. Treatment approaches are based on taking advantage of molecular differences between normal and tumor cells (Hemminki 2002). Suitable gene transfer vector is chosen based on the characteristics of the disease. Efficient vector for cancer treatment necessitates efficient transduction and gene expression in target cells, whereas sustained gene expression is subsidiary (Bauerschmitz, Barker et al. 2002). Adenoviruses are convenient as gene delivery vectors for cancer treatment, in which context they have been widely studied. Oncolytic adenoviruses utilize a straight forward means of action: Instead of

correcting a mutated gene in cancer cell, the whole tumor cell is killed following viral replication. Unfortunately, although the results in several oncolytic adenovirus cancer therapy trials have been encouraging in terms of safety, efficacy as a single agent is limited for several reasons. After intravenous administration, blood clearance and liver sequestration dramatically decrease the amount of virus available in the circulation. Tumor microenvironment further restricts viral spread. Several approaches have been taken to address these issues, with studies well underway to address specificity, delivery, and potency of adenoviruses.

2. Oncolytic viruses

Many viruses are known to be cancer-selective and oncolytic by nature. For instance adenoviruses infect quiescent cells and induce them into the S phase of the cell cycle so that viral replication can proceed (Van Dyke 1994). After the first round of replication, cancer cells are lysed and virus progeny are released to infect the neighbouring cancer cells. In theory, the rounds of infection and replication would continue until the whole tumor mass is eradicated. Normal cells are spared and thus toxicity is limited. Herpes simplex virus type 1 (HSV-1) has natural tropism for neuronal tissue, which makes it suitable for treating brain tumors. HSV-1 can be directed to replicate selectively in dividing cells by mutating crucial virulence genes, and has been studied widely as an oncolytic agent (Varghese and Rabkin 2002). Vaccinia represents another well characterized oncolytic agent, and has a long history as a smallpox vaccine. Genome size allows the insertion of large transgenes for virotherapeutic purposes, and conditionally replicating deletion mutants specific for cancer have been developed (Thorne, Hwang et al. 2005). Porcine Seneca Valley virus is a newly discovered native picornavirus. *In vitro* and *in vivo* studies have proposed this virus possess potential for the treatment of metastatic neuroendocrine cancers (Reddy, Burroughs et al. 2007). Other recently studied viruses with oncolytic activity include Poxvirus family member Myxoma (Wang, Barrett et al. 2006), vesicular stomatitis virus (Barber 2004) and Semliki Forest virus (SFV) (Smyth, Fleeton et al. 2005).

The exact virus-tumor interactions leading to natural oncolytic potential are not well understood. It is known that most tumors are defective in interferon/protein kinase R (PKR) signalling, because of the anti-tumoral effects of interferon. Lack of interferon also renders tumor cells more susceptible to viruses. This may be one explanation which underlies the

natural tumor selectivity of some viruses (Balachandran and Barber 2007). Oncolytic activity of the Newcastle disease virus, for example, is possibly due to cancer-specific defects in the interferon signalling pathway (Sinkovics and Horvath 2000).

2.1 Adenoviruses

Adenoviruses infect many post-mitotic cell types and have a wide host-range. Since they deliver their genome to the nucleus and can replicate with high efficiency, they are good candidates for the expression and delivery of therapeutic genes (Russell 2000). Adenoviruses are currently divided into three genera with further subdivision into species A to F. The division of human serotypes, based mainly on immunological criteria, has historically been the basis of classification (Lukashok and Horwitz 1998).

Capsid is nonenveloped and icosahedral consisting of three major proteins (**figure 1**): Hexon, penton base and a knobbed fiber, along with a number of other minor proteins, VI, VIII, IX, IIIa and IVa2 (Stewart, Fuller et al. 1993).

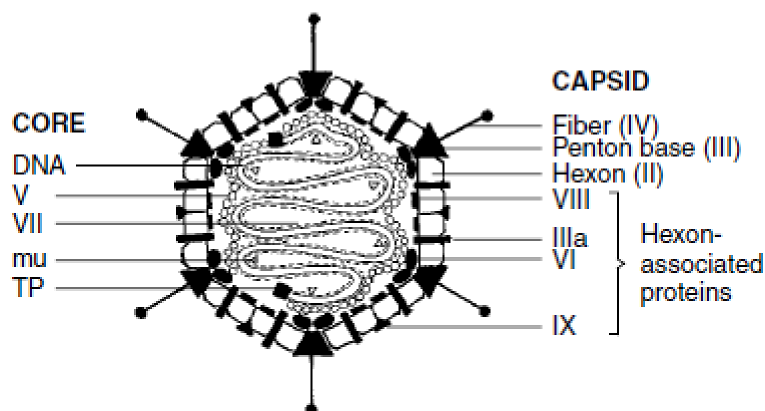


Figure 1. Structure of the adenovirus particle. The principal components are the homotrimeric hexons on the faces and edges of the capsid, together with the pentons consisting of penton bases and extended fibers on the apices. Other capsid proteins (IIIa, VI, VIII, IX) are also called 'minor components'. There are six other structural components in the core, of which the five associated with the genome are shown. The remaining component not shown is the 23K virion protease which plays pivotal role in the assembly of the virion; adapted from: (Volpers & Kochanek 2004).

The adenovirus genome consists of 36 kb double-stranded DNA. Genome is divided into E1A, E1B, E2A, E2B, E3 and E4 regions, which regulate the gene expression (**figure 2**). Transcripts are encoded via alternative splicing of each transcription unit to generate multiple products from each region (Berget, Moore et al. 1977; Berk and Sharp 1978). The infectious

cycle can be clearly defined into early and late phases (before and after viral DNA replication, respectively).

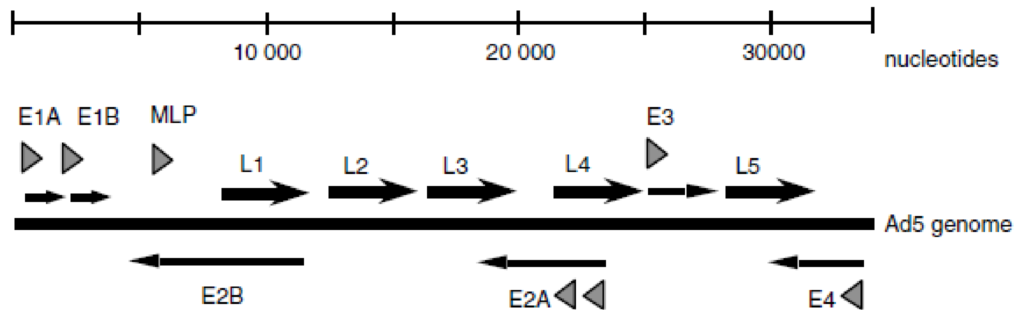


Figure 2. Genomic organization of Ad5. The first Ad gene to be expressed is the immediate early E1A gene encoding a transactivator for the transcription of the early genes E1B, E2A, E2B, E3 and E4, as well as protein functions involved in cellular transformation, together with an E1B protein. Promoters are depicted by arrowheads; early(E) and late (L) mRNAs are depicted by thin and heavy arrows, respectively. The adenovirus major late promoter (MLP) is active during both the early and late phases of infection; adapted from: (Volpers & Kochanek 2004).

The early phase covers the entry of the virus into the host cell and the passage of the virus genome to the nucleus, followed by the selective transcription and translation of early genes (**figure 3**). The binding of virus to target cell involves high-affinity binding via the knob portion of the fiber to receptor, primary receptor being coxsackie-adenovirus receptor (CAR) (Bergelson, Cunningham et al. 1997). The exceptions are members of subgroup B, from which for example Ad3 binds to another, yet unidentified receptor (Stevenson, Rollence et al. 1995). The critical recognition mechanism for CAR binding is an arginine-glycine-aspartic acid (RGD) motif that is exposed on the penton base (Stewart, Chiu et al. 1997) and interacts with cellular $\alpha_v\beta$ integrins (Wickham, Mathias et al. 1993). In addition to integrins, heparin sulphates, major histocompatibility complex class I α2, vascular cell adhesion molecule 1 and scavenger receptors have been suggested as alternative or coreceptors for species C adenoviruses (Jonsson, Lenman et al. 2009). Adenoviruses can also use soluble components in the body fluids for indirect binding to the target cells. As an example, lactoferrin is secreted by, for instance, neutrophils into epithelial mucosa and tear fluid and interacts with fiber protein, thus mediating CAR-independent binding to and infection of epithelial cells (Johansson, Jonsson et al. 2007). Entry of the virus proceeds via clathrin coated pit mediated endocytosis (Wang, Huang et al. 1998). Virus capsid is further disrupted by the proteolysis of the structural protein VI (Greber, Webster et al. 1996). Partially disrupted virus is then

transported to the nuclear membrane and the genome is passaged through the nuclear pore into the nucleus, where the primary transcription events take place.

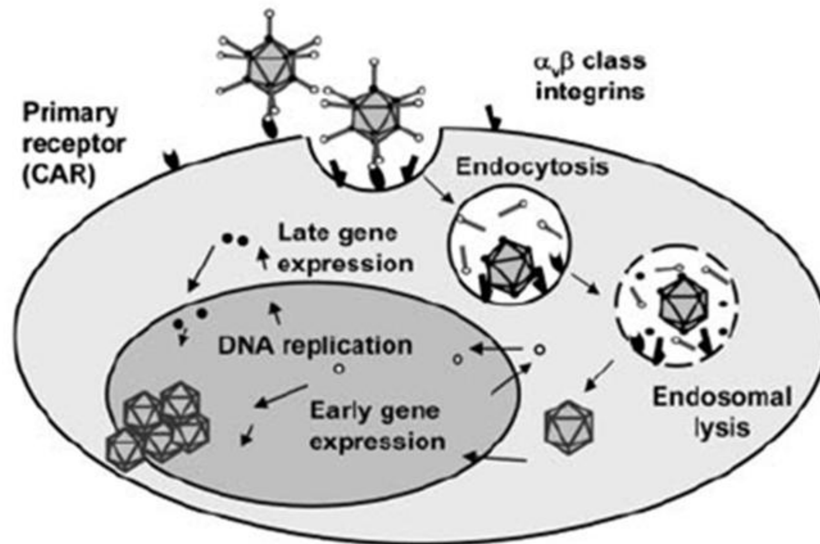


Figure 3. The adenoviral infection pathway. Cell entry is initiated by high-affinity binding of the fiber knob domain to its primary receptor, CAR. CAR-binding is followed by endocytosis, mediated by penton base RGD interaction with cellular $\alpha_v\beta$ integrins. After endosomal lysis, viral DNA is transported to the nucleus through a microtubule-mediated process, and viral genes and transgenes are expressed; adapted from: (Kanerva & Hemminki 2005).

Many of the early phase region (E1 through E4) products are necessary for downstream events in the early transcription cascade, and progression to late phase transcription. E1A both activates the transcription of further viral genes and manipulates the host cell physiology to make the environment hospitable for viral replication, with resultant transcription and translation of the late genes. One of the major functions of the E1B proteins is to counteract apoptosis (Rao, Debbas et al. 1992; Yew and Berk 1992). E2 gene products provide the machinery for the replication of virus DNA (Hay, Freeman et al. 1995), whereas E3 genes code for several proteins that suppress host immunodefence mechanisms (Russell 2000). Products of E4 gene function in concert with E1A and E1B to create a cellular environment permissive for efficient expression and processing of viral gene products (Goodrum and Ornelles 1999), leading to an assembly of the structural proteins in the nucleus, and the maturation of the infectious virus. The early phase in a permissive cell can take about 6±8 h (depending on the number of extraneous factors), while the late phase is normally much more rapid, yielding new virus in another 4±6 h.

3. Adenoviruses as gene transfer vehicles

Gene therapy for cancer has generated increasing interest for over two decades and experimental and clinical investigations are under way. In general, it comprises insertion of nucleic acids into cells of an individual to treat a disease. Therapeutic gene supplements a defective gene with a functional one, or encodes RNA or protein with therapeutic function. Whereas the first gene therapy trials were based on gene replacement for the treatment of monogenetic disorder, today only less than 10% of studies utilize this approach. Viral vectors are by far the most popular gene therapy approach, adenoviruses being the most commonly used viral vectors (25 % of clinical trials) (Edelstein, Abedi et al. 2007), while 75 % of adenoviral gene therapy trials are for the treatment of cancer. Based on the complex nature of cancer, gene therapy technologies to treat cancer are very heterogeneous, as demonstrated by a variety of used concepts such as immunomodulation, suicide gene therapy (i.e., transfer of the cDNA of a prodrug converting enzyme), replacing a faulty gene with a functional one, viral oncolysis, antiangiogenic and antiproteolytic gene therapy, or the delivery of drug resistance genes into hematopoietic precursor cells (Templeton 2009).

Adenoviruses possess many characteristics that make them a vector of choice for oncolytic virotherapy. They have a lytic replication cycle, their non-integrating genome stays episomal in the host cell, stable particles, an efficient gene transfer machinery and capability to infect both proliferating and nonproliferating cells. They can be produced in high titers and have for long been known to be oncolytic by nature (Huebner, Rowe et al. 1956). The vectors used for adenoviral gene therapy are derived from the subgroup C. Wild-type viruses from this subgroup cause mild upper respiratory tract infections, which resolves uneventfully in healthy individuals. Adenoviruses used in gene therapy are usually based on serotype 5 (Ad5).

Several regions of adenovirus genome can be deleted to accommodate up to 10 kb of foreign DNA (**figure 4**). Recombinant genomes with the size of 105% of 36 kb or less are efficiently incorporated into virus capsids resulting in stable viruses (Bett, Prevec et al. 1993). In many of the viruses used in trials, majority of the E1A and E1B regions are deleted to prevent virus replication. This also gives room for transgenes, such as therapeutic or suicide genes for enhanced oncolytic effect. In first generation adenoviruses, the E1A is replaced with a therapeutic transgene. Second generation adenoviruses typically have deletions either in E2 or E4, and deleted E1 and E3 regions, allowing to accommodate even larger or more transgenes. Helper dependent, also known as gutless, represent the third generation vectors. In

these vectors all viral genes except the inverted terminal repeats (ITRs) and the packaging signal have been deleted to further decrease immunogenicity and increase genetic payload (Shen 2006).

Figure 4. Different types of Ad5-derived vectors The first-generation vectors are based on the substitution of the E1 gene region by the transgene and are thus nonreplicating. The first-generation vectors can have an additional deletion in E3 which is dispensable for viral replication in cell culture. The second-generation Ad vectors are characterized by additional deletions. Third is shown a high-capacity vector, which is devoid of all coding viral genes, but contains only the ITRs and the packaging signal (Ψ), and can accommodate up to 36 kb of non-viral DNA. Fourth is shown one type of conditionally replicative Ad vector, characterized by a deletion in the gene encoding the 55 kDa E1B protein which normally binds to and inactivates p53, thereby activating the cell cycle. These vectors should productively infect and lyse p53-negative tumor cells, but not normal p53-positive cells; adapted from: (Volpers & Kochanek 2004).

3.1 Transductional targeting of adenoviruses

The capacity of an Ad5 vector to infect a given cell is dictated by the CAR- and integrin – expression levels of the cell. It has been shown that cells expressing both receptors below a certain threshold level are refractory to Ad infection (Freimuth 1996). A number of cell types such as endothelial, smooth muscle cells, differentiated airway epithelium cells, lymphocytes, fibroblasts and hematopoietic cells demonstrate either complete or partial resistance to Ad infection (Curiel and Douglas 2002). Adenovirus gene therapy vectors have also been reported to be incapable of transducting germ cells even with high doses (Gordon 2001). Importantly, many types of tumor cells express CAR at marginal or even undetectable levels and are thus Ad-refractory (Hemmi, Geertsen et al. 1998), leading to the development of several methods to improve poor infectivity due to low CAR expression (Pong, Lai et al. 2003). Histone deacetylase inhibitor trichostatin A is an upregulator of CAR expression, and has been used to improve adenoviral infectivity to low CAR cells (Kitazono, Goldsmith et al. 2001; Goldsmith, Kitazono et al. 2003). In combination with oncolytic adenovirus dl520 (ONYX-015) trichostatin had a significant effect on the replication and cytotoxicity (Bieler, Mantwill et al. 2006). Two distinct approaches have been employed to transductionally target

adenovirus vectors (**figure 5**): (1) targeting achieved via structural manipulation of the capsid by genetic means (Wickham, Tzeng et al. 1997), and (2) adapter molecule-based targeting. According to literature, there is some evidence suggesting that CAR may not be the primary Ad receptor *in vivo*. First, removal of the CAR-binding capacity of Ad vectors does not change their biodistribution in mice (Alemany and Curiel 2001). Secondly, mRNA expression of CAR correlates poorly with *in vivo* tropism of Ad vectors (Tomko, Xu et al. 1997). Third, CAR has been reported to have another relevant function in the fiber-CAR interaction, which is to facilitate viral escape from the site of infection (Walters, Freimuth et al. 2002).

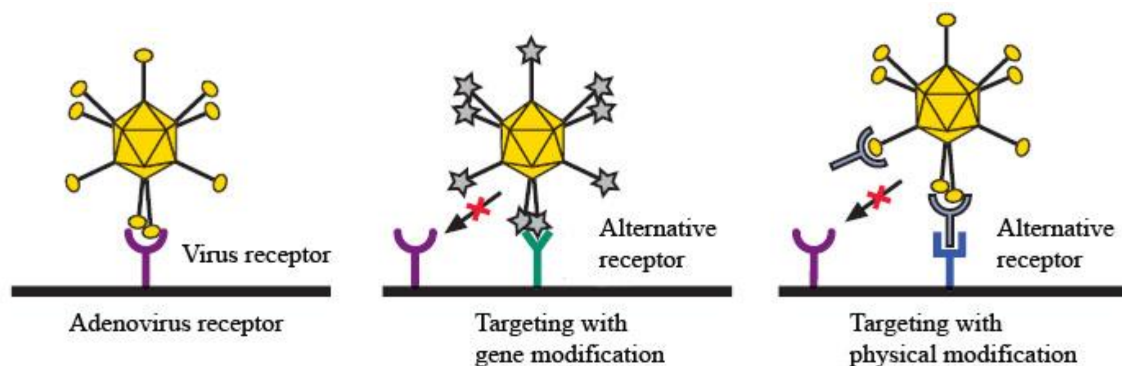


Figure 5. Adenovirus targeting. Virus can be genetically or physically modified to retarget binding from primary CAR receptor to alternative receptors expressed on target cells. In the middle, adenovirus knob is pseudotyped (changed to another serotype), or modified to display a peptide, resulting in altered receptor tropism. On the right, transductional targeting is achieved by utilizing bispesific adapter molecules that block interaction with CAR and redirect the virus to a novel receptor; adapted from: (Hakkarainen, Kanerva et al. 2005).

3.1.1 Transductional targeting through capsid modification

One approach to totally change transductional profile and to restrict broad natural tropism of an adenovirus is to genetically modify the capsid structure by ablating coxsackievirus-adenovirus receptor, α_v integrin, and heparan sulfate binding. This has been achieved through mutating FG loop in the fiber knob, deleting RGD motif of the penton base, and substituting the fiber shaft domain with that from serotype 35. Such triple-mutant adenovirus has been shown to display reduced *in vivo* tissue transduction and toxicity (Koizumi, Kawabata et al. 2006). This could offer a platform for subsequent retargeting of the the virus.

Adenovirus capsid can be retargeted to interact with other receptors than CAR (**figure 6**). For example, introducing an RGD-containing peptide in the HI loop of the fiber knob targets the virus to cells expressing $\alpha_v\beta$- integrins (Pasqualini, Koivunen et al. 1997; Wickham, Tzeng et al. 1997; Grill, Van Beusechem et al. 2001; Fueyo, Alemany et al. 2003). $\alpha_v\beta$- integrins are overly expressed in many cancers, as are heparan sulfates. They have been

targeted using adenoviruses with a COOH-terminal polylysine tail (Wu, Seki et al. 2002; Yotnda, Zompeta et al. 2004).

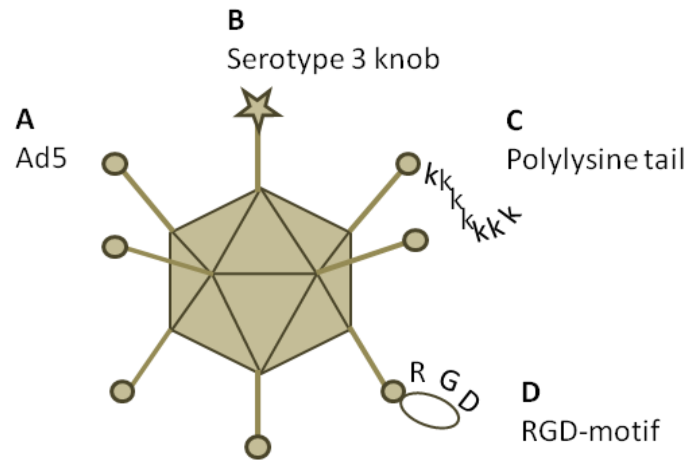


Figure 6. Adenovirus capsid modifications for transductional targeting. **A**, Ad5 “wild type” capsid with the default serotype 5 fiber binds to CAR receptor. **B**, 5/3 chimeric fiber targeted to serotype 3 yet unidentified receptor. **C**, polylysine motifs of different lengths in the C terminus of the knob bind to HSPG’s. **D**, arginine-glycine-aspartic acid (RGD)-motif in the HI-loop of the fiber targeted to $\alpha_v\beta$-integrins.

CAR deficiency has also been circumvented by serotype switching. Ad5/3 is chimeric serotype 5 adenovirus featuring serotype 3 (Ad3) knob, retargeting it to bind to Ad3 receptor (Kanerva, Mikheeva et al. 2002). Taking the "directed evolution" approach, viral diversity was increased by pooling an array of serotypes, then passaging the pools under conditions that invite recombination between serotypes. These highly diverse viral pools were then placed under stringent directed selection to generate and identify highly potent agents. ColoAd1, a complex Ad3/Ad11p chimeric virus, was the initial oncolytic virus derived by this methodology. This first described non-Ad5-based oncolytic Ad, is 2-3 logs more potent and selective than the parent serotypes or the clinically advanced oncolytic Ad, ONYX-015, *in vitro* (Kuhn, Harden et al. 2008). In addition to serotypes 3 and 11, also serotype 35 fiber has been used in replacement of Ad5 fiber for enhanced oncolysis. Ad5 vectors containing Ad35 fibers (Ad5/35) use CD46 as a receptor for infection of cells, which solves the problem with low CAR on cancer cells (Gaggar, Shayakhmetov et al. 2003). Adenovirus serotype 35 is also less prone to unspecific virus sequestration by blood components, including coagulation factor X (Liu, Wang et al. 2009; Wang, Li et al. 2009).

Initial attempts to reduce liver tropism were based on the hypothesis that CAR- and integrin-based interactions were required for liver transduction *in vivo*, and that fiber protein is one important structural determinant of liver tropism. For example, shortening of the native

fiber shaft domain of the Ad5 fiber (Vigne, Dedieu et al. 2003) or replacement of the Ad5 shaft with the short Ad3 shaft domain (Breidenbach, Rein et al. 2004) has been shown to attenuate liver uptake following intravenous delivery. Short-shafted Ads are unable to infect liver cells through CAR or through the KKTK shaft motif (Shayakhmetov, Li et al. 2004). Due to these features, they are not taken up by liver cells and are probably degraded within the sinusoids. In related work, the role of a putative heparan sulfate proteoglycan (HSPG)-binding motif, KKTK, in the third repeat of the native fiber shaft was examined. Replacement of this motif with an irrelevant peptide sequence reduced reporter gene expression in the liver by 90%. This was also the first indication of the importance of HSPG as an Ad receptor *in vivo* (Smith, Idamakanti et al. 2003). A recent report describes systemic delivery of $\alpha_v\beta$-targeted Ad to result in improved tumor uptake and reduced liver accumulation and hepatotoxicity in mice (Coughlan, Vallath et al. 2009). It remains uncertain, however, whether the above mentioned *in vivo* data has significance in human applications.

3.1.2 Adapter-based targeting

The majority of current adapter-based adenovirus targeting approaches incorporate the two mandates of delivery targeting, that of ablation of native CAR-dependent Ad tropism to restrict gene delivery exclusively to target cells, and formation of a novel tropism to previously identified cellular receptors. The formation of a molecular bridge between the adenovirus vector and the cell surface receptor constitutes the adapter-based concept of transductional targeting (**figure 5**). Bispecific adapter molecules include bi-specific antibodies (Korn, Nettelbeck et al. 2004), cell-selective ligands such as folate (Douglas, Rogers et al. 1996) and chemical conjugates (Reynolds, Zinn et al. 2000). Chemically conjugated bispesific moieties consisting of a Fab fragment and a natural ligand specific for cell surface receptor have an advantage that a variety of ligands, including vitamins, growth factors, antibodies, and peptides, can be chemically conjugated to the anti-knob Fab fragment to redirect Ad binding (Glasgow, Everts et al. 2006). However, the chemical conjugation results in a heterogeneous population of molecules. Moreover, the yield of appropriately conjugated bispesific molecules can be low (Curiel and Douglas 2002).

In recognition of the disadvantages associated with chemical conjugation strategies, bispesific targeting moieties have been generated in the form of recombinant fusion proteins (Korn, Nettelbeck et al. 2004). This permits the expression and purification of a homogenous population of retargeting molecules. The principle of bispesific proteins is that one site of the

protein is directed against Ad capsid protein, while a second site is specific for a cell surface molecule. This can be achieved by genetically fusing extracellular domain of CAR to a receptor-targeting moiety, yielding a truly targeted vector that blocks CAR binding: Once complexed with CAR-ligand fusion protein, an Ad vector will not be able to bind to its primary receptor. Utilizing this approach, a truncated, soluble form of CAR, sCAR, was fused to EGFR, and the soluble CAR-EGF fusion protein was expressed in insect cells using a baculovirus expression system. The bispesific fusion protein mediated EGFR-specific, CAR-independent Ad infection of target cells (Dmitriev, Kashentseva et al. 2000). Overall, adapter-based targeting studies provide compelling evidence that adenovirus tropism modification augments gene delivery to CAR-deficient cells *in vitro*. Adapter-targeted vectors have also performed well *in vivo*, although data so far are limited (Glasgow, Everts et al. 2006).

4. Polymers and vehicles in adenoviral gene delivery

As partial solution to adenovirus induced immune response and liver sequestration could be a disguise, such as carrier cells or coating agents. Adding anti-cancer drugs to implantable delivery matrix, such as silica-based sol-gel polymer, is one promising strategy for modifying biodistribution, reducing drug toxicity and thus improving the therapeutic efficacy of anti-tumor agents (Quintanar-Guerrero, Ganem-Quintanar et al. 2009). The manufacturing process is based on inorganic polymerization in conditions compatible with biologicals, allowing the association of mineral phases with organic materials. In the process, the sol (solvent) evolves towards the formation of a gel-like diphasic system (sol-gel polymerization). Drugs, proteins, cells and viruses can be immobilized into sol-gel polymers without loss of biological activity (Coradin, Boissiere et al. 2006). By changing the sol-gel synthesis parameters, drug concentration, size of the device and the dissolution rate can be adjusted according to purpose of use (Viitala, Jokinen et al. 2007). As examples of anti-cancer drugs, silica sol-gel implant has been studied as a delivery device for cisplatin (Czarnobaj and Lukasiak 2004) and doxorubicin (Prokopowicz 2007). The authors hypotize that higher local drug concentration, longer target exposure to the drug and spontaneous degradation of the implant might lead to reduced toxicity and improved delivery to the tumor site. The same hypotheses applied to our work, where we demonstrated that silica implants can be successfully used in intraperitoneal delivery of oncolytic adenoviruses *in vivo*, resulting in prolonged survival of intraperitoneal orthotopic tumor-bearing mice. Further, delivering the virus in silica had favorable effect on

anti-adenovirus immune response (**III-IV**). Another example of a polymer used for coating adenoviruses to disguise the virus from the immune system is polyethylene glycol (PEG). PEGylation has been reported to reduce blood clearance rate (Freytag, Stricker et al.). However, this procedure also reduces infectivity (Alemany, Suzuki et al. 2000).

Cytokine-induced killer (CIK) cells are known to home to and destroy tumors. CIK cells can be isolated from blood stream, infected with virus, and systemically readministered. Oncolytic vaccinia viruses and CIK cells have been shown to be synergistic in tumor cell killing in tumor-bearing mice (Thorne, Negrin et al. 2006). Mesenchymal stem cells (MSCs) have also been suggested to have inherent tumor tropism. Using MSCs as carriers, intravenously injected oncolytic adenoviruses were released into advanced orthotopic breast and lung tumors, and survival of the mice was increased. When the same dose of virus was injected intravenously without MSCs, liver transduction was prevalent (Hakkarainen, Sarkioja et al. 2007). Also intravenously injected tumor cells have been tested as carriers for tumor targeting *in vivo,* and were found to home to metastases (Garcia-Castro, Martinez-Palacio et al. 2005). The authors speculate this approach to be feasible also for targeting oncolytic viruses to tumors.

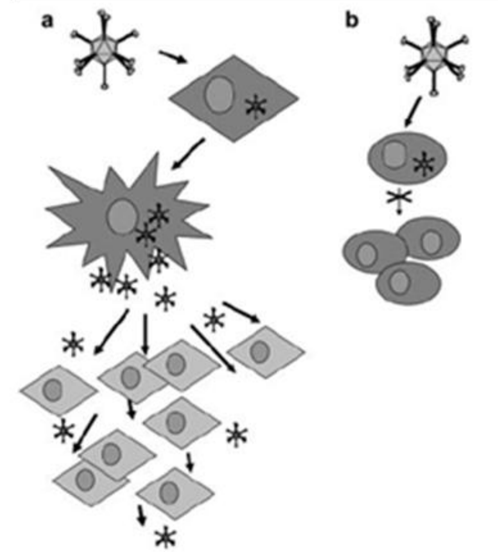
5. Transcriptional targeting of Ads

5.1 Type 1 oncolytic adenoviruses

Transductional targeting is not enough for achieving a potent, tumor-specific oncolytic virus. There is also need for controlled replication in cells. Transcomplementational approach for transcriptional targeting takes advantage of the fact that Ad infection and oncogenic transformation induce similar signalling cascades in eukaryotic cells: A number of the most critical early transcript functions of adenovirus (such as cell cycle deregulation and inhibition of apoptosis) are often complemented by the deregulated states associated with tumor cell differentiation (Yew and Berk 1992; Lukas, Muller et al. 1994; Han, Modha et al. 1998). These points within the adenoviral life cycle may be transcriptionally targeted to limit adenoviral replication preferentially to tumor cells. Partial deletion will impair replication potency in normal cells, whereas in malignant cells deletion will be transcomplemented by distinct cellular deregulated pathways, allowing productive replication (**figure 7**) (Kanerva

and Hemminki 2005). The loss-of function mutated adenoviruses that need transcomplemetation from cancer cells to replicate are called type I oncolytic adenoviruses.

Figure 7. Conditionally replicating adenoviruses are genetically modified to multiply only in cancer cells. **A,** infection of tumor cells results in replication, oncolysis (cell killing), and subsequent release of virus progeny. The new generation of viruses will then infect neighbouring cancer cells, leading to cycles of replication and lysis of malignant cells within the tumor. **B,** normal cells are spared due to lack of replication; adapted from: (Kanerva & Hemminki 2005).



One widely used transcomplementational approach is based on the knowledge of most of the advanced human tumors being deficient in retinoblastoma/p16 pathway (Sherr 1996; Hernando, Nahle et al. 2004). Δ24-mutated adenoviruses, such as Ad5-Δ24, have 24 bp deletion in constant region 2 of E1A, in which the pRb binding domain resides. Via this domain, wild-type E1A binds to and activates pRb, required for replication in normal cells. Δ24 is complemented by inactivation of pRb, enabling virus replication selectively in cancer cells (Fueyo, Gomez-Manzano et al. 2000; Heise, Hermiston et al. 2000).

The first and most studied oncolytic adenovirus dl1520 (ONYX-015) carries two mutations in the gene coding for the E1B-55 kDa protein. One purpose of this protein is binding and inactivation of cellular tumor suppressor p53, which is thought to respond to DNA damage by inducing cell cycle arrest or apoptosis. For this reason, tumors lacking p53 respond poorly to radiation or chemotherapy, and majority of human tumors are p53 mutated (Bischoff, Kirn et al. 1996). E1B-55 kDa mutated ONYX-015 can replicate in and lyse p53-deficient human tumor cells, but not cells with functional p53. Tumor cells that support ONYX-015 replication may do so by providing the function of E1B in late viral RNA export from the nucleus, allowing the virus to replicate selectively in tumor cells without normal p53 protein or with a deficient p53 pathway (O'Shea, Johnson et al. 2004). The same authors subsequently reported resistant tumor cell lines failing to provide the RNA export functions of E1B-55K necessary for ONYX-015 replication; viral 100K mRNA export being necessary for host protein shutoff. However, heat shock rescues late viral RNA export and renders refractory tumor cells permissive to ONYX-015. Thus, heat shock and late adenoviral RNAs

may converge upon a common mechanism for their export (O'Shea, Soria et al. 2005). Interestingly, this was hypotized to explain why patients with virus induced fever getting no antipyretic drugs showed enhanced response in H101 clinical trial (virus similar to ONYX-015) (Yu and Fang 2007). Of note, replication of ONYX-015 is severely impeded compared to wild type virus, probably resulting from a loss of E1B-55 kDa protein function for the late virus mRNA transcription (Harada and Berk 1999). Another shortage involves replication in some cultured cells lacking p53 mutations. Loss of E1B-55K leads to the induction, but not the activation, of p53 in ONYX-015-infected primary cells, and consequently replication in primary cells is not restricted (Goodrum and Ornelles 1998).

Adenovirus dl331 contains a 29-bp deletion in the coding region of VAI gene as a conditionally replicative oncolytic adenovirus for Ras-activated tumors. The selectivity of this virus stems from the inability of dl331 to block the activation of the double-stranded RNA-activated protein kinase (PKR) and, therefore, to prevent cellular anti-viral response. Oncogenic Ras also induces an inhibitor of PKR (Cascallo, Capella et al. 2003; Wang, Xue et al. 2005).

Replication-deficient E1A mutant adenovirus mutant dl520 contains an 11-bp deletion removing the 13S donor splicing junction and resulting in the loss of the E1A protein (Haley, Overhauser et al. 1984). This virus replicates efficiently and exhibits oncolytic potential in multidrug-resistant cells with nuclear localization of the human transcription factor YB-1, which binds to the adenoviral late E2 promoter resulting in E1A independent replication (Bieler, Mantwill et al. 2006).

5.2 Type II oncolytic adenoviruses

In oncolytic adenoviruses, the anti-tumour effect is caused by the replication of the virus *per se* and replication must be restricted to tumour cells to protect normal tissues from damage. Tissue-specific promoters (TSPs) represent a powerful tool for decreasing the toxicity of cancer gene therapy to normal tissues and have previously been utilised for specific mutation compensation or delivery of prodrug-converting enzymes (Hardcastle, Kurozumi et al. 2007). However, TSPs can also be tumor specific promoters used for controlling crucial viral replication regulators and consequent restriction of replication to tumor cells. This class of Ads are called type II oncolytic adenoviruses. Since the size of a candidate promoter construct is restricted by the packaging capacity of adenoviral virions for DNA, large or multiple promoter insertions may require deletions of viral sequences, such as the viral promoter to be

replaced. This strategy also deletes unwanted internal control mechanisms. Minimizing the promoter size is also advantageous for enabling insertion of therapeutic genes into viral genome for improved therapeutic efficacy. Other sequences, not essential for viral replication, can also be deleted. Viral genes that ensure optimal viral spread should be retained (Nettelbeck 2008). In the context of TSPs, tight promoter control gained specificity, rather than strong activity in the induced state, is critical (Hitt and Graham 1990).

E1A, being the master regulator of replication, offers the first choice to control Ad replication with TSPs. The first TSP driven oncolytic adenovirus was created from a serotype 5 adenovirus by placing human prostate-specific antigen (PSA) based promoter to drive E1A, thereby creating a prostate-specific virus, CN706 (Rodriguez, Schuur et al. 1997). In another study, melanoma specific oncolysis was achieved with melanoma differentiation marker tyrosinase enhancer/promoter controlling E1A. This was a Ad5-Δ24 based oncolytic virus (Nettelbeck, Rivera et al. 2002). Oncolytic adenovirus OV798 in turn utilized human carcinoembryonic antigen (CEA) promoter. In this virus, CEA-driven E1A tightly controls gene expression and viral replication in CEA-overexpressing colon cancer cells, which also translated into survival benefit in human colon tumor xenograft bearing mice (Li, Chen et al. 2003). E1A was placed under control of alpha-phetoprotein (AFP) promoter to create oncolytic adenovirus CV890 specific to hepatocellular carcinoma (HCC). In combination with doxorubicin, CV890 eliminated distant human liver tumors in HCC xenograft bearing mice (Li, Yu et al. 2001). Other examples of cancer specific promoter controlled replication include melanoma specific replication achieved with tyrosinase and hTERT promoters (Nettelbeck, Rivera et al. 2002; Peter, Graf et al. 2003), and cyclooxygenase-2 (Cox-2) promoter targeting for pancreatic cancer (Yamamoto, Davydova et al. 2003).

In addition to E1A gene, TSPs have been placed to control E1B, E2 and E4 (Nettelbeck 2008). By replacing the E4 promoter with the promoter for surfactant protein B (SPB), oncolytic adenovirus specific for alveolar and bronchial cancer cells was created. SPB promoter activity is restricted in the adult to type II alveolar epithelial cells and bronchial epithelial cells. In addition this virus had two E1A mutations, which made it replicate within and destroy only alveolar and bronchial cancer cells (Doronin, Kuppuswamy et al. 2001). Replication of oncolytic adenovirus VRX-009 is restricted to cells with a deregulated wnt signal transduction pathway by replacement of the wild-type Ad E4 promoter with a synthetic promoter consisting of five consensus binding sites for the T-cell factor transcription factor (Toth, Djeha et al. 2004). Cancer cell selective replication of ONYX-411 in turn resulted from Δ24 for pRb-selectivity, which was further enhanced by the replacement of the viral E1A and

E4 promoter regions with the human E2F1 gene promoter. The oncolytic activity of ONYX-411 is not limited to a particular tumor type. The combination of these attributes resulted in selective tumor cell killing both *in vitro* and *in vivo* (Johnson, Shen et al. 2002).

Adenoviral replication can also be tightly directed by controlling adenoviral E1A and E4 genes simultaneously with a single enhancer. This was achieved by creating a prostate cancer specific adenovirus with PSES enhancer controlling adenovirus E1A and E4 gene expression. This chimeric enhancer contains enhancer elements from prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) genes that are prevalently expressed in androgen-independent prostate cancers (Li, Zhang et al. 2005). Colon cancer targeted oncolytic adenovirus was developed to express the viral E1B and E2 genes from promoters controlled by the Tcf4 transcription factor. Tcf4 is constitutively activated in virtually all colon tumors by mutations in the adenomatous polyposis coli and beta-catenin genes, and is constitutively repressed in normal tissue (Brunori, Malerba et al. 2001). In another study, both E1 and E4 regions were controlled by a synthetic tyrosinase enhancer/promoter specific for melanocytes in melanoma-targeted oncolytic adenoviruses (Banerjee, Rivera et al. 2004).

6. Armed oncolytic adenoviruses

When replication-mediated oncolysis is the only means of tumor eradication, the efficacy of virotherapy can be limited. Virus induced inflammation in the tumor microenvironment also needs to be taken into account. Tumor microenvironment, being heterogeneous, hypoxic and compartmentalized, holds many obstacles for the efficient spreading of the virus. Although tumor hypoxia is known to limit group C adenovirus (serotype 5) replication, the expression level of CD46, a receptor some group B adenoviruses, is not altered in hypoxic conditions (Shen, Bauzon et al. 2006). Thus, hypoxic conditions might favour Ad5 vectors that are modified to favour receptors other than CAR, such as Ad5/3 fiber chimera binding to Ad3 receptor. Viruses can also be transductionally targeted to tumor matrix components, such as vascular endothelium. Fibroblast growth factor-2-retargeted adenovirus has been shown to selectively transduct primary glioblastoma multiforme endothelial cells (Gupta, Wang et al. 2006). It may in occasions be feasible to also transcriptionally target the virus to other than cancer cells in the tumor. Oncolytic adenoviruses have been transcriptionally targeted with high specificity to dividing endothelial cells within a tumor. This has been done by utilizing

regulatory elements shown to be highly overexpressed in angiogenic endothelial cells (Savontaus, Sauter et al. 2002).

Viral vectors can further be weaponed with therapeutic transgenes affecting tumor vasculature or matrix. Antiangiogenic treatments include soluble vascular endothelial growth factor (VEGF) receptors and antibodies that can be expressed by adenovirus. Such soluble VEGF receptor producing vector has been reported to result in pronounced tumor growth inhibition when injected intravenously or intratumorally into mice (Kuo, Farnebo et al. 2001; Thorne, Tam et al. 2006). Antiantiogenic soluble Flt-1 expression from oncolytic adenovirus has also been shown to reduce tumor growth and prolong the survival in mice (Zhang, Zou et al. 2005). Protease expressing viruses can be used for targeting tumor stroma. Oncolytic adenovirus encoding relaxin, a matrix-degrading protein, has been reported to enhance viral spread without causing significant toxicity (Kim, Lee et al. 2006). Tumor stromal matrix targeted human matrix metalloproteinase-8 gene delivery has been shown to increase oncolytic activity of replicating adenovirus (Cheng, Sauthoff et al. 2007). Degrading agents can also be coadministered with the virus. Coadministration of matrix-modifying metalloproteinases and bacterial collagenase have been shown to improve distribution and efficacy of oncolytic vectors (McKee, Grandi et al. 2006; Mok, Boucher et al. 2007)

Suicide gene therapy involves the tumor-targeted delivery of genes encoding enzymes that convert systemically delivered, innocuous prodrugs into toxic metabolites. This results in a high concentration of toxic product intratumorally, thereby avoiding the systemic toxicity often associated with conventional chemotherapy. Arming oncolytic viruses with genes encoding prodrug-converting enzymes yield enhanced anticancer efficacy by combining the effects of oncolytic replication and local prodrug activation. Examples of suicide gene delivery approaches include herpes simplex type 1 thymidine kinase (HSV-1 TK)/ganciclovir (GCV) and *Escherichia coli* cytosine deaminase (CD)/5-fluorocytosine (5-FC) therapy. HSV-1 TK phosphorylates GCV, converting it to a nucleotide analog that inhibits DNA synthesis, while CD converts 5-FC to its highly toxic metabolite, 5-FU. The combination of CD and HSV-1 TK gene delivery is an example of doublesuicide gene therapy. When both of the respective prodrugs (5-FC + GCV) were administered, therapeutic outcome was dramatically improved (Rogulski, Wing et al. 2000). Replication-competent adenovirus-mediated double suicide gene therapy has also shown promise in combination with radiation therapy in an orthotopic mouse prostate cancer model (Rogulski, Wing et al. 2000; Freytag, Paielli et al. 2002). Suicide gene therapy has also been utilized in the context of Δ24-type oncolytic adenovirus Ad5-Δ24.E3-sCE2, which utilizes prodrug converting enzyme

carboxylesterase/irinotecan (CE/CPT-11) system. Administrated CPT-11 is converted by CE into much more potent SN-38, which in this case augmented the cytotoxicity of the virus in colon cancer cells (Oosterhoff, Pinedo et al. 2005). ONYX-015-based replicating adenovirus has also been armed with CE/CPT-11 system. Here, cytotoxicity of CE-expressing virus was significantly enhanced in the presence of the prodrug, and combination treatment of CE-expressing virus and CPT-11 enhanced survival of tumor-bearing mice (Stubdal, Perin et al. 2003).

7. Immune response

Even though the results from clinical trials have been encouraging in terms of safety, there are downsides that need to be taken into account. Immune system has evolved to efficiently and rapidly recognize adenoviruses as pathogens in an innate and adaptive manner (Prestwich, Errington et al. 2009). Immune response is triggered by virus interaction with leukocytes, endothelial and epithelial cells. Tissue macrophages are derived from monocytes in the blood stream. Once making their way to the tissue, they develop into different phagocytic cell populations, which efficiently clear virus from the blood stream after systemic injection. These cells, along with activated dendritic cells (DCs) in the spleen, have an important role in provoking virus-induced inflammatory response (Muruve 2004).

The combined innate and adaptive response upon natural Ad-infection most commonly results in Ad-clearance and life-long immunity in the majority of hosts (Lenaerts, De Clercq et al. 2008). High immunogenicity of adenovirus remains difficult to classify either as an advantage or disadvantage. The immune system could decrease the efficacy of the vector, and it may prevent the spread to organs, which may on the other hand contribute to safety. In the case of cancer immunotherapy, virus-induced immune response is utilized to synergize with anti-tumor activity of the virus. Inflammatory response is optimal for antigen presentation and helps to reveal the hidden tumor antigens to dendritic cells (Prestwich, Harrington et al. 2008). Activation of tumor-antigen-specific T cells would thereby create a danger signal triggering not only anti-virus but also anti-tumor immunity (Tuve, Liu et al. 2009). The main concern with adenoviral gene therapy is the possibility of provoking a severe immune and inflammatory response, as was tragically exemplified in the case of a death of a patient with ornithine transcarbamylase (OTC) deficiency who participated in a Phase I study of gene therapy. The vector used for this trial was based on human adenovirus type 5, deleted

in E1 and E4. In this case, massive cytokine response and disseminated intravascular coagulation were reported (Raper, Chirmule et al. 2003). Despite this unfortunate case, it is noteworthy that 16 000 patients treated with adenoviral gene therapy have proven adenovirus to have a good safety profile compared to most conventional therapies.

7.1. Innate immune response

Innate immunity is the first line of defence against infections (**figure 8**). Unlike adaptive immunity, the innate response is mediated by the adenovirus particle and does not necessarily require viral transcription: Interaction of the viral capsid with the host cell is sufficient to activate the pathways leading to inflammatory responses (Muruve 2004). Mechanisms of innate immunity are either constitutively active or are activated very rapidly after infection (prior to the development of adaptive immune responses) and serve three very important functions. First, as the initial host response, innate defences limit or prevent infection by rapidly eliminating microbes (clearance). Second, the effector components of innate immunity interact and work together with components of adaptive immunity to synergistically augment microbial clearance. Third, innate immunity stimulates and can reprogram adaptive immune mechanisms to optimize clearance of specific types of microbes. The principal effector components of innate immunity involved in the clearance of microbes during *in vivo* infection include phagocytic and natural killer (NK) cells, cytokines, and complement (Zaiss, Machado et al. 2009).

The innate recognition process is initiated by pathogen recognition through a number of receptors in the intracellular and extracellular compartments (Girardin, Sansonetti et al. 2002; Muruve, Petrilli et al. 2008). The best studied family of receptors consists of the Toll-like receptors (TLRs). The recognition of pathogen-associated molecular patterns by innate receptors triggers the activation of inflammatory genes, which serves to control the infection locally and recruit effector leukocytes to the site of infection (Girardin, Sansonetti et al. 2002). The effector cells include granulocytes, natural killer (NK) cells, and monocytes/macrophages that perform cytolytic functions and secrete more cytokines/ chemokines to further amplify the immune response (Guidotti and Chisari 2001).

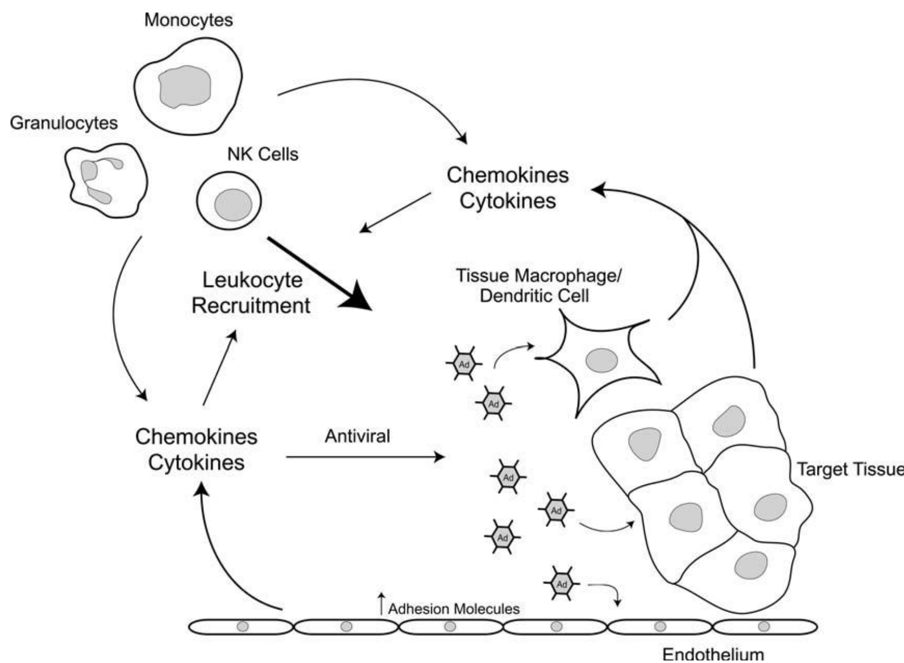


Figure 8. General overview of the innate response to adenovirus vectors. A number of different cell types are transduced and activated by Ad vectors, including endothelial cells. Ad vectors induce numerous inflammatory genes (chemokines and cytokines), which play a role in recruiting and activating innate effector cells to the site of infection. In addition, genes that are involved in leukocyte trafficking (such as adhesion molecules) are also expressed. Cytokine induction also occurs in innate effector cells such as dendritic cells and macrophages, further amplifying the response; adapted from: (Muruve 2004).

Innate immune response depends both on adenovirus species and the dose. Innate immune responses to the virus can also be a major hurdle for long-term gene expression and oncolytic potency. Within 24 h, the virus induced inflammatory response eliminates about 80 % of the adenoviral particles (Worgall, Wolff et al. 1997), a number which can be decreased by means of genetic modification.

7.2. Adaptive immunity

The hexon, being the major adenovirus capsid component, is a principal player in establishing the adaptive immune response – both humoral and cellular. The humoral and cellular immune response to recombinant adenoviral vectors, as described in several animal models, result in the extinction of transgene expression, severe local inflammation, and production of anti-adenovirus neutralizing antibodies (NAbs) that prevent readministration (Yang, Li et al. 1995). Hexon capsid can have at least nine hypervariable loops, and some of these appear to function as type specific neutralizing antigens and thus define the serotype (Russell 2009). The expression of adenoviral genes results in an immune response specifically directed

against the products of these genes. The adaptive response is far weaker with last-generation vectors, which are characterized by the deletion of all or part of the viral genes.

The adaptive response is thought to require the integration of both adenovirus specific memory CD4+ and cytotoxic CD8+ T cell responses. Adenovirus specific cytotoxic T lymphocytes (CTLs) are preferentially directed towards conserved epitopes within the virus capsid (mostly hexon protein) and kill infected cells (using multiple mechanisms that include perforin, Fas-L and TNFα). CTLs disrupt the adenovirus life cycle before progeny viruses are assembled (Leen, Christin et al. 2008).

Immune modulatory agents can be used in combination with oncolytic viruses to enhance viral spread, transgene expression and antitumoral efficacy. Cyclophosphamide has recently been successfully used in combination with oncolytic adenovirus in animal studies to suppress regulatory T cell (Treg) induction and decrease tumor infiltration by immune cells (Di Paolo, Tuve et al. 2006; Lamfers, Fulci et al. 2006). Clinical studies are needed to evaluate the effect in cancer patients who often have pre-existing immunosupression caused by the disease and chemotherapy.

7.3 Immunological obstacles to systemic administration

Even though some encouraging results have been obtained, the efficacy of systemically administrated oncolytic adenovirus has been somewhat limited in updated clinical trials (Reid, Warren et al. 2002). It seems likely that there are number of different mechanisms for virus neutralization, *e.g.* aggregation of virus may impede proper recognition at the cell surface, and there is also evidence that virus–antibody complexes can enter the cell and that inhibition occurs at a later stage (Varghese, Mikyas et al. 2004). Following systemic administration, virus uptake by tumor cells is hampered by systemic antiviral immune response, for example due to the complement and NAbs. As practically all adults have been exposed to the most widely used serotype 5 adenovirus (Ad5), the immune system is primed to rapidly produce NAbs on re-exposure. A direct correlation between NAb and block of readministration of vector has been established by passive transfer of serum from treated to naïve animals (Yang, Li et al. 1995). After genetic manipulation, the virus often becomes attenuated and thus even more prone to immune response before massive oncolysis takes place. A high NAb titer may not limit local injection, but it can compromise systemic delivery. In this context, transient removal of pre-existent antibodies by immunoapheresis prior to virus treatment has been suggested (Chen, Yu et al. 2000). In data obtained in immune-competent mice, changing of

the adenovirus fiber knob allowed the virus to overcome the neutralizing activity of the pre-existing NAbs (Sarkioja, Pesonen et al. 2008). This is supported by earlier observations where anti-adenovirus humoral immune defences against repeat adenovirus vector administration were circumvented by changing the adenovirus serotype (Mastrangeli, Harvey et al. 1996). Inducing immunological tolerance, or the use of polyethylene glycol (PEG), are other examples of how pre-existing antibodies can be partially overcome (Kass-Eisler, Leinwand et al. 1996; O'Riordan, Lachapelle et al. 1999). Transient immunosupression during initial administration of adenovirus can be used to prevent a rise in antibody titer, but it would not be expected to suppress the levels of pre-existing antibodies (Chen, Yu et al. 2000).

There are also studies suggesting that pre-existing immunity does not necessarily reduce the efficacy of an oncolytic virus. On the contrary, it has been reported that the oncolytic effect of modified HSV is enhanced in HSV-1 seropositive mice, possibly due to interferon (IFN) -γ mediated tumor cell killing (Zhu, Su et al. 2007). It was also recently shown that anti-tumor efficacy of intratumorally injected adenovirus in mice was increased by pre-immunisation against adenovirus despite the production of NAbs (Tuve, Liu et al. 2009).

After intravenous administration to mice, within minutes Ad vectors are predominantly sequestered by the liver (Shayakhmetov, Li et al. 2004) through hepatic macrophage (Kupffer cell) uptake (Tao, Gao et al. 2001) and hepatocyte transduction (Connelly 1999). Liver sequestration greatly reduces the ability of the virus to reach other tissues, and provokes toxic responses (Worgall, Wolff et al. 1997; Alemany, Suzuki et al. 2000). The clearance of adenovirus by Kupffer cells is mediated by scavenger receptors, natural antibodies, and complement (Xu, Tian et al. 2008).

Once opsonised by plasma proteins, such as coagulation factors, hepatocytes are infected in a CAR and integrin independent manner (Alemany and Curiel 2001). Coagulation factor IX (FIX) and complement component C4-binding protein (C4BP) can bind the Ad fiber knob domain and provide a bridge for virus uptake through hepatocellular HSPGs and low-density lipoprotein (LDL)-receptor-related protein. Kupffer cell sequestration of Ad particles is likewise heavily dependent on Ad association with FIX and C4BP (Shayakhmetov, Gaggar et al. 2005). Another coagulation factor, FX, was recently found to bind directly not to fiber, but to the central depression of the hexon. Binding affinity of FX to Ad is high compared to other blood factors, and results in efficient hepatocyte transduction (Kalyuzhniy, Di Paolo et al. 2008; Waddington, McVey et al. 2008). Also vitamin K-dependent coagulation factors have been reported to opsonise adenovirus and facilitate the transduction of hepatocytes (Parker, Waddington et al. 2006), and vitamin K dependent coagulation factor synthesis

inhibition in mice with warfarin has been reported to reduce transduction to liver, spleen and lung (Koski, Rajecki et al. 2009).

Systemic Ad administration is associated with thrombocytopenia, a phenomena shown to be dose-dependent, saturable and reversible. After systemic administration, Ad5 rapidly binds to circulating platelets, which causes their activation/aggregation and subsequent entrapment in liver sinusoids. Virus-platelet aggregates are taken up by the Kupffer cells and degraded. Ad sequestration in organs can be reduced by platelet depletion prior to vector injection (Stone, Liu et al. 2007). Depletion of the Kupffer cells by $GdCl_3$ has also been done *in vivo*, which resulted in increased viraemia (Alemany, Suzuki et al. 2000). Preinjecting polyinosinic acid poly(I), a ligand for scavenger receptor, has been used to reduce the Kupffer cell uptake and increase the circulating half-life of adenovirus *in vivo* (Ranki, Kanerva et al. 2007; Haisma, Kamps et al. 2008). In conclusion, considering the main immune response related problems, preventing anti-adenovirus NAbs or redirecting virus particles away from liver may make the virus more applicable for systemic use.

8. Cancer stem cells and oncolytic adenoviruses

Stem cells characteristics include asymmetric replication, capacity for self-renewal, pluripotency and the proliferative ability to drive continued expansion of the cell population. Cells with stem cell-like attributes have been isolated from haematological malignancies and from several solid tumor types. These cells are thought to be responsible for the initiation and growth of tumors, and have been commonly referred to as cancer stem cells (CSCs) or cancer-initiating cells (CICs). For tumors in which cancer stem cells play a role, at least three scenarios are possible (**Figure 9**). First, mutation of a normal stem cell or progenitor cell may create a cancer stem cell, which will then generate a primary tumor. Second, during treatment with chemotherapy, the majority of cells in a primary tumor may be destroyed, but if the cancer stem cells are not eradicated, the tumor may regrow and cause a relapse. Like their normal counterparts (Dean, Fojo et al. 2005; Rich 2007), putative cancer stem cells show remarkable resistance to radiation and chemotherapy (Reya, Morrison et al. 2001). Their capacity for surviving apparently curative treatment can result in tumor relapse. Third, cancer stem cells arising from a primary tumor may emigrate to distal sites and create metastatic lesions (Jordan, Guzman et al. 2006).

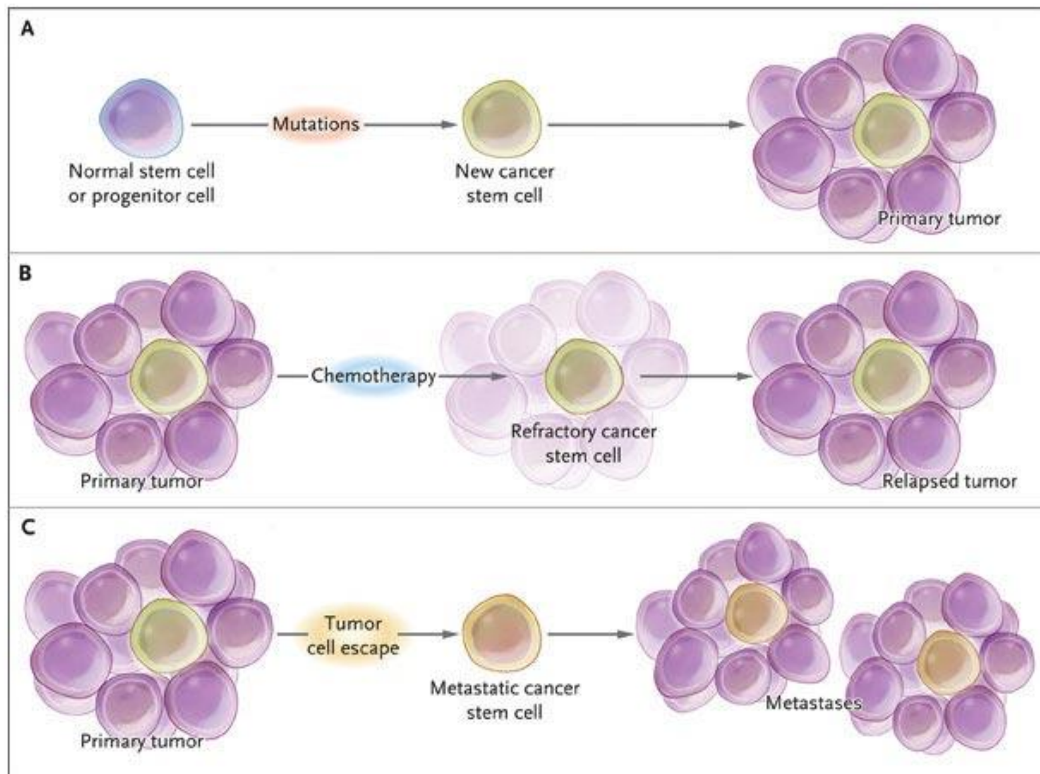


Figure 9. The role of cancer stem cells in tumor initiation, relapse and metastasis. **A**, mutated stem cell or progenitor cell may create a tumor initiating cancer stem cell. **B**, a relapse after seemingly successful chemotherapy may be caused by cancer stem cells refractory to the treatment. **C**, once detaching from the primary tumor, cancer stem cells may relocate to distant sites and initiate metastases; adapted from: (Jordan, Guzman et al. 2006)

Oncolytic viruses enter cells through infection and may therefore be resistant to defence mechanisms exhibited by the cancer stem cells, such as efflux pumps and defective apoptotic signalling (Coukos, Makrigiannakis et al. 2000). Oncolytic adenoviruses can be engineered to attack CSCs by utilizing linage specific cell surface markers, dysfunctional stem cell-signalling pathways, or upregulated oncogenic genes (Ribacka, Pesonen et al. 2008). Indeed, oncolytic viruses are the first approach shown to be effective against tumor-initiating cells (Ribacka and Hemminki 2008). However, mesenchymal stem cells are known to express CAR poorly, suggesting that lack of CAR may constitute an overall problem for infectivity of stem cells. Capsid-modified adenoviral vectors are able to overcome CAR deficiency for effective gene delivery to mesenchymal stem cells (Mizuguchi, Sasaki et al. 2005; Hakkarainen, Sarkioja et al. 2007), breast cancer CSCs (Eriksson, Guse et al. 2007) and brain tumor CSCs (Jiang, Gomez-Manzano et al. 2007). Another possible approach to transductionally target oncolytic adenovirus towards CSCs is to use single chain monoclonal

antibodies or other bispecific adapter molecules through which the virus can attach to a specific cell surface protein (Dmitriev, Kashentseva et al. 2000).

Fiber modified Δ24-mutated oncolytic adenoviruses have been shown to kill differentiated breast cancer cells *in vitro*, and to abrogate human breast cancer stem cell derived tumor growth *in vivo* (Eriksson, Guse et al. 2007; Bauerschmitz, Ranki et al. 2008). In addition, viruses may be engineered to express therapeutic transgenes that specifically target properties that CSCs rely upon for self-renewal and cell division.

9. Preclinical combination therapy

Because of the unique mode of tumor destruction, oncolytic virotherapy has the potential to augment the antineoplastic activity of chemotherapy and radiation therapy. Oncolytic adenoviruses do not have cross-resistance with existing treatments, and therefore tumors resistant to other therapies may be susceptible to adenoviral therapy (Kanerva, Zinn et al. 2003). Further, the side effects caused by the adenovirus treatments differ from the ones caused by chemotherapeutics or radiation therapy. Therefore, by combining conventional therapies with virus treatments, it is possible to reach additive or synergistic effect without increased side effects (Raki, Sarkioja et al. 2008).

First promising preclinical treatment studies combining oncolytic adenovirus with chemotherapy were done with ONYX-015 and cisplatin. Human xenograft bearing mice received ONYX-015 intratumorally or intravenously, with or without concurrent chemotherapy. Subsequent anti-tumor efficacy in the group receiving combination treatment was significantly greater than in the groups receiving either agent alone (Heise, Sampson-Johannes et al. 1997). In work done with lung cancer cell lines and primary cultures freshly made from lung cancer patients, ONYX-015 was reported to work synergistically also with paclitaxol and cisplatin (You, Yang et al. 2000). Another report showing antitumor synergy resulted from studying prostate cancer-specific adenovirus CV787 in combination with paclitaxel and docetaxel in xenograft bearing mice (Yu, Chen et al. 2001). In another study, recombinant adenovirus containing the thymidine kinase (TK) gene driven by the osteocalcin promoter was highly selective in blocking the growth of osteosarcoma in experimental models, when delivered concurrently with acyclovir (Cheon, Ko et al. 1997). Further evidence of the benefits of combination treatments was provided by studying the α-fetoprotein-specific

adenovirus CV890 in combination with doxorubicin in mice. Following the treatment, hepatocellular carcinoma liver xenografts were eliminated (Li, Yu et al. 2001).

Combining radiation therapy with oncolytic adenoviruses is an intriguing approach, since adenoviral E1A protein has been implicated in the potentiation of apoptosis induced by various external stimuli, including radiation. Ad5 E1A sensitizes cells to radiation-induced apoptosis by inhibiting NF-kappaB activity (Shao, Karunagaran et al. 1997). Preclinical studies combining the E1B-deleted, replication-competent ONYX-015 with radiation also led to enhanced oncolysis beyond that of either monotherapy both *in vitro* and *in vivo* (Rogulski, Freytag et al. 2000). Synergistic potential of combining radiation therapy with oncolytic adenovirus Ad5-Δ24RGD was revealed in glioma xenograft mouse model. Tumor regression occurred in all mice, resulting in long-term survival without evidence of tumor regrowth (Lamfers, Grill et al. 2002). Later, the experiment was repeated and even though combined treatment with Ad5-Δ24RGD and irradiation was reported to show enhanced antitumor activity in subcutaneous glioma xenografts, it did not work in an orthotopic glioma model (Lamfers, Idema et al. 2007). These discrepant results are a reminder of the importance of finding the suitable animal model when assessing the effects of combination therapies with oncolytic adenoviruses. Encouraging preclinical findings have also led to clinical trials, some of which will be discussed in the following chapter.

10. Clinical use of oncolytic viruses

Cancer has become the most treated disease with means of gene therapy (Edelstein, Abedi et al. 2004). This is in part due to the intrinsic oncolytic nature of some viruses. The first reports describing tumor regressions concomitant with naturally acquired virus infections dates to the mid-1800s (Sinkovics and Horvath 1993). Reported early cases were often patients with haematological malignancies, known to be associated with severely compromised immunological state. Remissions following natural virus infections were more often seen in young patients, and were short-lived and incomplete (Kelly and Russell 2007). The earliest clinical testing began properly in the mid-1900s. The clinical studies performed at that time were unsafe, as therapeutic material administered to patients often consisted of infectious body fluids or infected tissue harvested from patients with ongoing virus infections. In 1949, Hepatitis B virus was used in a clinical trial in which the patients suffering from Hodking's disease were treated with virus containing sera or tissue extracts. Some of the patients showed

improvement lasting at least one month, but also severe side effects were reported, leading to the death of one patient (Hoster, Zanes et al. 1949). Flaviviruses such as West Nile are spread by mosquitoes and exceedingly common in Egypt. A clinical trial with Egypt 101 (early passage West Nile) was enrolled in the early 50's for the treatment of advanced, unresponsive neoplasms. Responses were rare, and cases of encephalitis were reported concomitant with the treatment (Southam and Moore 1952). Also in the 50's, adenoidal-pharyngeal-conjuctivis virus (APC), now known to be an adenovirus, was used to treat cervical carcinoma, and localized tumor necrosis was frequently seen (Huebner, Rowe et al. 1956; Georgiades, Zielinski et al. 1959). In the early 70's, a large clinical trial was launched using non-attenuated mumps viruses to treat 18 types of tumors. Delivery methods varied from intravenous to oral administration utilizing virus containing bread or pieces of tampon. Rectal administration was also used. The results were among the best yet seen in oncolytic virus trials, with encouraging response rate and minimal toxicity (Asada 1974). In the 70's and 80's, regulatory barriers led to huge decrease in the number of reported clinical trials employing oncolytic viruses, and the need for diminished pathogenic potential had become evident.

Later on, recombinant technology enabled the modification of oncolytic viruses to become more selective for tumor cells, and thus safer to use. The new era with genetically engineered oncolytic viruses started in the early 1990's with the use of herpes simplex virus type one (HSV-1) in an experimental glioma model (Martuza, Malick et al. 1991). Five years later, E1B 55K gene-deleted ONYX-015 (Reid, Warren et al. 2002) established clinical proof-of-concept for oncolytic virotherapy in the first Phase I trial in which the virus was directly injected into head and neck tumors (Ganly, Kirn et al. 2000). ONYX-015 was well tolerated and showed localized efficacy in head and neck cancer trials as a single agent (Nemunaitis, Ganly et al. 2000). Only patients with advanced incurable cancers were initially enrolled. Once tentative safety was demonstrated, treatment of patients with premalignant conditions followed (Ries and Korn 2002). Finally, ONYX-015 became the first virus to undergo clinical trials combined with chemotherapy. Promising effects were obtained on localized head and neck tumors following direct injection combined with systemic cisplatin and 5-fluorouracil (5-FU) chemotherapy (Khuri, Nemunaitis et al. 2000). This was the first trial in history to demonstrate combined oncolytic virus and chemotherapy combination efficacy. A phase III clinical trial of head and neck carcinoma in combination with chemotherapy was halted in the US prematurely because of funding problems in 2003. After this, H101, oncolytic adenovirus similar to ONYX-015, was constructed in China. In addition to modified E1B-55KD, it has

partial E3 deletion of the Adenovirus Death Protein. In 2005, H101 was approved for treating advanced nasopharyngeal carcinoma in combination with chemotherapy (regimen of 5-FU + cisplatin), and became the first oncolytic virus product approved by a governmental agency for human use (Yu and Fang 2007). The efficacy of combining H101 with chemotherapeutics was comparable to the efficacy obtained previously in the phase II trial combining ONYX-015 with chemotherapeutics (Khuri, Nemunaitis et al. 2000). While ONYX-015 is well tolerated and shows activity when injected intratumorally, it is inefficient as a single agent. It has also failed targeting metastases when administrated systemically (Crompton and Kirn 2007).

The first published clinical trial reporting results of radiation therapy combined with an oncolytic virus, was a phase I trial using replicating adenovirus, Ad5-CD/TK, which has an E1B-deletion and carries a fusion gene expressing two prodrug-activating enzymes, *Escherichia coli* cytosine deaminase and HSV-TK. Virus in combination with a prodrug therapy (5-FC and valganciclovir) and radiation resulted in significant reduction in prostate-specific antigen (PSA) tumor marker level (Freytag, Stricker et al. 2003).

Transcriptionally controlled viruses with cancer specific TSP driven E1A have also been well tolerated in patients (DeWeese, van der Poel et al. 2001). The same applies to transcriptionally targeted oncolytic adenovirus expressing immunostimulatory cytokine GM-CSF. Phase I trial with CG0070 in recurrent bladder cancer was launched in 2005. Minimal toxicity was reported and the trial was expanded to a multiple-dose trial which is still ongoing. No clinical trials have yet been completed with targeted Ads, but we can expect some to be reported within the near future. A trial with Ad5-Δ24RGD was launched in 2004 for the treatment of ovarian cancer, and in 2007 for the treatment of recurrent glioblastoma.

A number of trials have revealed a proof of principle for oncolytic adenovirus replication in tumors. Importantly, the safety profile of these viruses has been encouraging. Side effects, mostly flu-like symptoms and transient hepatotoxicity, were tolerable even after systemic application of high virus titers (Reid, Warren et al. 2002; Ko, Hawkins et al. 2005). Although individual tumor responses were observed, the overall therapeutic efficacy of oncolytic adenovirus monotherapy needs to be improved. Clinical experiences to date suggest that short-term potential for this class of therapeutics lies in combination therapy regimens. Increasing potency of the vectors by means of arming, detargeting, retargeting, and coating of adenoviruses is necessary to improve the delivery of the agent to the treatment site.

AIMS OF THE STUDY

1. To evaluate different capsid modified adenoviruses in their transduction and oncolytic efficiency in gastric and pancreatic cancer cells, tissues and xenografts **(I, III-IV).**

2. Determine activity of oncolytic adenoviruses armed with tissue-specific promoters in $CD44^{+}CD24^{-/low}$ breast cancer cells from pleural effusions of breast cancer patients **(II)**.

3. To study release kinetics and stability of a silica embedded adenovirus *in vitro* and *in vivo*, and use of silica implants for intraperitoneal virus delivery in mice bearing orthotopic gastric or pancreatic cancer **(III-IV)**.

MATERIALS AND METHODS

1. Cell lines

All cell lines used in the studies are listed below (**table 1**). Cell lines were cultured as recommended by the supplier, and the culturing conditions are described in more detail in the studies **I-IV**.

Table 1. Human cell lines used in this study.

Cell line	Description	Source	Study
911	Transformed human embryonic retinoblasts	ATCC[1]	II
293	Transformed embryonic kidney cells	ATCC[1]	I-IV
A549	Lung adenocarcinoma cells	ATCC[1]	I-IV
KatoIII	Diffuse gastric cancer	ATCC[1]	I
Hs 766T	Lymph node methastasis of pancreatic cancer	ATCC[1]	IV
SW 1990	Spleen methastasis of pancreatic cancer	ATCC[1]	IV
Capan-2	Pancreatic adenocarsinoma	ATCC[1]	IV
HPAC	Pancreatic adenocarsinoma	ATCC[1]	IV
Panc-1	Pancreatic epithelial carcinoma	ATCC[1]	III-IV
MKN-1	Adenosquamous gastric cancer	Dr. Hiroshi Yokozaki[2]	I
MKN-7	Intestinal gastric cancer	Dr. Hiroshi Yokozaki[2]	I
MKN-28	Intestinal gastric cancer	Dr. Hiroshi Yokozaki[2]	I, III
MKN-74	Intestinal gastric cancer	Dr. Hiroshi Yokozaki[2]	I
MKN-45	Diffuse gastric cancer	Dr. Hiroshi Yokozaki[2]	I
JIMT-1	Breast cancer cell line, phenotypically of epithelial progenitor cell origin	Dr. J. Isola[3]	II

[1]Cell line purchased from Amercan Type Culture Collection (ATCC)
[2]Cell line is a gift from Dr. Hiroshi Yokozaki (Kobe University, Kobe, Japan)
[3]Cell line is a gift from Dr. Minna Tanner (University of Tampere and Tampere University Hospital, Tampere, Finland)

2. Fresh human cells and tissues

Informed consent from the patient and permission for studies on human tissue from the ethics committee were obtained prior to the experiments. Fresh liver samples were received from donor livers that were to be transplanted into recipients (Department of Surgery, Helsinki University Central Hospital). For slicing, infecting and culturing the liver slices, see study **I**. Fresh gastric (**I**) and pancreatic (**IV**) cancer samples were obtained from cancer patients undergoing surgery in Department of Surgery, Helsinki University Central Hospital. See the corresponding studies for details of processing, infecting and culturing of the tumor tissue.

Primary adult human skin fibroblasts (HSFs) (**III**) were established from a skin punch biopsy obtained from the arm of a healthy male volunteer (age 27).

3. Adenoviruses

Wild type adenovirus Ad300wt was purchased from ATCC (Manassas, VA, USA). All recombinant adenoviruses are listed in **table 2**. Ad5/3ala-Δ24, Ad5/3mdr-E1, Ad5/3mdr-Δ24 and Ad5/3-hTERT-Δgp were cloned as described in study **II**. Replicating and replication-deficient adenoviruses were amplified on A549 and 293 cells, respectively. Viruses were purified from cell extracts on double caesium chloride gradients. VP titer was measured at 260 nm, and the amount of infectious particles was determined by standard $TCID_{50}$ or plaque assay on 293 cells. The presence of gene insertions and deletions were confirmed with polymerase chain reaction (PCR) and sequencing.

Table 2. Recombinant adenoviruses used in this study. All viruses represent serotype 5.

Virus	**E1**	**Fiber**	**Used in**	**Reference**
Ad5luc1	Luciferase	Wild type	**I, IV**	(Kanerva, Mikheeva et al. 2002)
Ad5/3luc1	Luciferase	5/3 serotype chimerism	**I, III-IV**	(Kanerva, Mikheeva et al. 2002)
Ad5lucRGD	Luciferase	RGD motif in HI loop	**I, IV**	(Dmitriev, Krasnykh et al. 1998; Wu, Seki et al. 2002)
Ad5(GL)	GFP + luciferase	Wild type	**I, IV**	(Wu, Seki et al. 2002)
Ad5.pK7	GFP + luciferase	7 lysine residues at C-terminus	**I, IV**	(Wu, Seki et al. 2002)
Ad5RGD.pK7	GFP + luciferase	RGD motif in HI loop and 7 lysine residues at C-terminus	**I, IV**	(Wu, Seki et al. 2002)
Ad5LacZ	LacZ	Wild type	**I**	(Yotnda, Zompeta et al. 2004)
Ad5pK21LacZ	LacZ	21 lysine residues at C-terminus	**I**	(Yotnda, Zompeta et al. 2004)
RadlacZ	Replaced with CMV promoter driven lacZ	Wild type	**III**	(Wilkinson and Akrigg 1992)
RAd66	Deleted	Wild type	**III**	(Wilkinson and Akrigg 1992)
AdlacZ216	Deleted, replaced with β- galactosi-dase cDNA[3]	Wild type	**III**	(Laitinen, Mäkinen et al 1998)
Ad5/3-Δ24	Δ24[1]	5/3 serotype chimerism	**I-IV**	(Kanerva, Zinn et al. 2003)
Ad5-Δ24RGD	Δ24[1]	RGD motif in HI loop	**I, III-IV**	(Suzuki, Fueyo et al. 2001)

Ad5-Δ24E3[2]	Δ24[1]	Wild type	**I, IV**	(Suzuki, Fueyo et al. 2001)
Ad5/3ala-Δ24	α-Lactalbumin promoter, Δ24[1]	5/3 serotype chimerism	**II**	(Bauerschmitz, Ranki et al. 2008)
Ad5/3mdr-E1	Multidrug resistance protein promoter	5/3 serotype chimerism	**II**	(Bauerschmitz, Ranki et al. 2008)
Ad5/3mdr-Δ24	Multidrug resistance protein promoter, Δ24[1]	5/3 serotype chimerism	**II**	(Bauerschmitz, Ranki et al. 2008)
Ad5/3-hTERT-Δgp	Telomerase promoter, Δ24[1]	5/3 serotype chimerism	**II**	(Bauerschmitz, Ranki et al. 2008)
Ad5/3-Cox2L-Δ24	Cyclo-oxygenase 2 promoter, Δ24[1]	5/3 serotype chimerism	**II**	(Bauerschmitz, Guse et al. 2006)
Ad5/3-Δ24hCGβ[4]	Δ24[1]	5/3 serotype chimerism	**III**	(Rajecki, Kanerva et al. 2007)
Ad5/3-Δ24-TK-GFP	Δ24[1]	5/3 serotype chimerism	**III**	(Raki, Hakkarainen et al. 2007)

[1] 24 bp deletion in constant region 2 of E1A
[2] The virus has an intact E3 region, therefore it was originally named Ad5-Δ24E3
[3] Nuclear-targeted β-galactosidase cDNA under a β-actin promoter and a cytomegalovirus (CMV) enhancer
[4] Transgene for the beta-chain of human chorionic gonadotropin (hCGbeta) replaces the gp19K reading frame, but most of the E3region remains intact

4. *In vitro* studies

4.1 Isolating $CD44^{+}CD24^{-/low}$ cell populations (II)

Cells from pleural effusion samples and JIMT-1 cells were sorted with FITC-labeled anti-CD44 and phycoerythrin-labeled anti-CD24 antibodies as described in study **II**.

4.2 Gene expression analysis (II)

To predict the activity of different promoter constructs in $CD44^{+}CD24^{-/low}$ cells, the messenger RNA from sorted JIMT-1 cells was isolated. Expression levels of α-lactalbumin (ala), cyclo-oxygenase 2 (Cox-2), telomerase (hTERT) and multidrug resistance protein (mdr) were normalized to highly expressed house keeping gene β-actin. For the materials and RT-PCR conditions, see study **II**.

4.3 Release and infectivity of silica-embedded virus (III)

Silica gels were allowed to melt in buffer to study the degradation kinetics by measuring the Si content at different time points. Simultaneous release of adenovirus was measured with quantitative PCR and with $TCID_{50}$ to quantify the functionality of the released virus.

To study the effect of long term storage on functionality of the silicated virus, viability of the silica embedded β-galactosidase producing adenovirus was studied after incubating for

229 days at +4°C. To test the stability of silicated viruses at 37°C, β-galactosidase producing virus was embedded in silica and incubated in phosphate-buffered saline (PBS) for up to 32 days. In both cases, silica monoliths were then placed to cell cultures. After incubation cells were stained for β- or X-galactosidase to show the presence of functional viruses.

4.4 Marker gene transfer assays (I-IV)

To study the effects of the capsid modifications on gene transfer, cancer cells (**I-II**, **IV**) and CAR-positive nonmalignant 293 cells (**I, IV**), as well as gastric (**I**) and pancreatic cancer tissues (**IV**), were infected with replication deficient capsid modified viruses. In addition, one sample representing normal pancreatic tissue was analyzed (**IV**). Luciferase or β-gal assays were done to measure transgene activity. In tissue samples, protein concentrations were measured and included in the analysis as an internal control. In biodistribution analysis (**I, III**), mouse organs were snap-frozen at collection. The tissue samples were analyzed for transgene expression and protein content as with the clinical samples. For more detailed protocols, see 'Materials and Methods' from the respective studies.

4.5 Cytotoxicity assay (I-II, IV)

To compare the cell killing efficacy of oncolytic viruses, cell lines were infected with three different doses and followed for cytopathic effect. The most pronounced differences in viability of the cells infected with different viruses are seen when the most oncolytic viruses have killed most of the cells at 1 VP per cell. At that time point, the mitochondrial activity of the cells was measured with MTS assay.

4.6 Quantification of infectious particles in tissue samples (I, III)

To determine the amount of infectious particles in mouse organs, tissues were snap-frozen at collection. The tissue samples were homogenized and standard $TCID_{50}$ test was done. To study the adenoviral replication in human liver, human liver precision-cut slices were infected with oncolytic and control viruses. The number of infectious particles was determined by the $TCID_{50}$ at different time points.

5. *In vivo* studies

Three to four week old female mice were obtained from Taconic (Ejby, Denmark) and were quarantined for two weeks before starting the experiments. Animal experiments were

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

5.1 Biodistribution of capsid-modified viruses in orthotopic gastric cancer model (I).

To study the effects of capsid modifications on gene transfer *in vivo*, an orthotopic gastric cancer model was established. After eight days, capsid modified replication-deficient viruses, and the virus with the unmodified capsid, were injected intarperitoneally. Tumors and normal organs were collected 48 hours after the injection and luciferase or lacZ activity was measured from tissue homogenates as described above.

5.2 Silica biodegradation and release of functional virus *in vivo* (III).

Silica gel implants with LacZ-viruses were implanted subcutaneously into mice. Histological samples from tissue surrounding the implant were collected at selected time points and stained with X-gal to study the functionality of the virus released from the implants. Release of the functional virus from the silica was further assessed in intraperitoneal models. Orthotopic gastric cancer bearing mice received oncolytic GFP producing adenovirus as an intraperitoneal injection or in a silica implant. Tumors were collected at selected time points and stained with a GFP-specific antibody.

To study the effects of the silica implant on gene delivery efficacy and persistence of viral replication, orthotopic pancreatic cancer tumors were established. In this study, we utilized oncolytic adenovirus which secretes hCGβ that can be quantified from the blood. Mice received the virus either in a silica implant or as an injection. At selected time points, hCGβ was measured from the blood and selected organs were collected to analyze the amount of infectious particles with $TCID_{50}$.

5.3 Antibody formation against adenovirus delivered within or without silica (III, IV)

Silica implants containing adenovirus were implanted subcutaneously into C57Bl mice. Serum was collected at selected time points to measure anti-adenoviral antibodies with EIA (**III**). To study the effects of silica implant on the virus-induced NAb response, ICR mice were given Ad5/3-Δ24 as an injection or in a silica implant. Sera were collected 28 days after treatment, and NAbs were analyzed with gene transfer inhibition assay. Briefly, Ad5/3luc1

was mixed with different serum dilutions, and the effect on gene transfer was assayed on 293 cells. Reduction in luciferase activity reflects the amount of NAbs in the serum (**IV**).

5.4. Adenovirus induced pro-inflammatory cytokines (IV)

To study whether delivering the virus in silica implant affects innate toxicity, ICR mice were given Ad5/3-Δ24 either intraperitoneally or in silica implant. 6 hours post treatment, sera were collected to analyze early pro-inflammatory cytokines IL-6, IFN-γ and RANTES.

5.5 Survival experiments (I-IV)

An orthotopic gastric cancer model was established to study the effect of oncolytic virus capsid modifications on survival. MKN-28 cells were injected intraperitoneally into SCID mice. Ten days later, mice were treated with a panel of viruses as a single intraperitoneal injection, and the mice were followed for survival. In the same experiment, serum samples for hCGβ were collected from the tail vein 50 days after injecting the cells to see if the tumor burden would correlate with the upcoming survival results **(I)**.

To study the anti-tumor activity of tissue specific promoters in $CD44^{+}CD24^{-/low}$ cell originating tumors, sorted JIMT-1 cells were injected into the topmost mammary fat pads of nude mice. Intratumoral virus injections were performed thrice a week for a total of 5 weeks, and the tumor growth was followed throughout the experiment. In the same experiment, expression of CD44 and CD24 in tumors was measured by flow cytometry **(II)**.

To find out if silica embedded virus can prolong the survival of the orthotopic tumor bearing mice, silica implant was studied as intraperitoneal virus delivery device in studies **III** and **IV**. In study **III**, Orthotopic pancreatic cancer tumors were grown in SCID mice before treatment with Ad5/3-Δ24 in silica implants. Control mice were treated with silica implants without virus, and mice were followed for survival. In the study **IV**, we studied silica implant in the context of peritoneally disseminated pancreatic cancer. Mice were treated with a panel of capsid modified oncolytic adenoviruses or wild type Ad300wt and followed for survival. To represent the standard-of-care in the treatment of pancreatic cancer, a group receiving gemcitabine intraperitoneally was included and compared to a group receiving gemcitabine in combination with Ad5/3-Δ24. In addition, silica gel was used in delivery of Ad5/3-Δ24 and compared to the virus injected group.

6. Statistical analysis (I-IV)

Two-tailed Student's t test was used to compare the differences between viruses and their control groups. Survival was analyzed according to Kaplan-Meier with SPSS 11.5 for Windows (**I-IV**). In the *in vivo* survival experiment based on the subcutaneous tumor growth, a nonparametric change-point test was used to determine a systematic change in the pattern of observations as opposed to chance. Proc Mixed was used to examine the effects of group and time on tumor growth. Pair comparisons were performed so that each group was individually compared with all other groups in the experiment (**II**).

RESULTS AND DISCUSSION

1. Capsid modified adenoviruses show enhanced transduction to gastric and pancreatic cancer cells and tissues (I, IV)

One limiting factor in the clinical performance of the adenoviral (Ad) vectors may be attributed to their broad native tropism. Thus there is a need for the derivation of Ad agents that have the capacity for intrinsic, self-directed, specific localization to the disease-affected target tissue. Limiting factor for the most frequently used serotype 5 adenoviruses (Ad5) is dependence on the coxsackie- and adenovirus receptor (CAR), which is variably expressed in most advanced cancers (Bauerschmitz, Barker et al. 2002). Native Ad5 tropism can be modified to circumvent CAR deficiency in cancer cells. Transductional targeting of adenoviruses, e.g. by incorporating targeting moieties into the fiber knob region, aims at the enhanced transduction of the target cell (Glasgow, Everts et al. 2006). For instance, incorporation of Arg-Gly-Asp (RGD)-containing peptide in the HI loop of the fiber knob allows the virus to utilize $\alpha_v\beta$-class integrins for binding and internalization (Dmitriev, Krasnykh et al. 1998). These integrins are highly expressed in gastric and pancreatic cancers (Kawashima, Tsugawa et al. 2003; Grzesiak, Ho et al. 2007). Adenoviruses with a COOH-terminal polylysine tail are retargeted to bind to heparan sulfates (Wu, Seki et al. 2002; Yotnda, Zompeta et al. 2004). Substitution of the entire fiber knob was used in the construction of Ad5/3, an Ad5 vector that features a chimeric fiber with the adenovirus serotype 3 (Ad3) knob domain (Kanerva, Mikheeva et al. 2002). This virus binds to Ad3 receptor, which is yet not characterized.

In this work we compared these capsid modifications in terms of increasing the transduction efficacy to gastric and pancreatic cancer cells and tissues. Capsid modifications were found to increase gene transfer to the gastric and pancreatic cancer cell lines, whereas in CAR-positive nonmalignant 293 cells the effect was less than 7-fold (**figure 1 in study I and figure 1 in study IV**). In intestinal type gastric cancer cell lines, capsid modification with pK21 modification, with tail of 21 lysines in COOH-terminus, displayed the best gene transfer efficacy (up to 479-fold compared to the wild type capsid). In the diffuse-type cell lines, in

addition to pK21 modification, also pK7 (with 7 lysines in COOH terminus) resulted in over 70-fold enhancement in gene transfer efficacy, and these viruses also performed best in the adenosquamous gastric cancer cells. With RGD- and 5/3 modified viruses, the results remained less impressive, but they also enhanced gene transfer to some extent. Further, capsid modifications increased gene transfer to primary gastric cancer specimens (**figure 2 in study I**), in which 5/3 and RGD modified viruses were overall most efficient with up to 256- and 198-fold increase, respectively. As the cell lines are derived from different origins, it was not completely unexpected to see a different profile of gene transfer in comparison with the cell lines.

In pancreatic cancer cell lines, 5/3 modified virus was superior to others with as high as 95 000 fold increase in gene transfer. Impressive results were also obtained with RGD-modified virus, with 22 000-fold increase. Of the polylysine tail containing viruses, only pK7 was studied and was evaluated to be at best 67 more efficient than the unmodified capsid. These results correlated nicely with the ones obtained from the clinical pancreatic cancer samples (**figure 2 in study IV**), 5/3 modification increasing gene transfer in all cancer tissue samples up to 18 000-fold. The second best virus had RGD modification, which augmented gene transfer up to 7600-fold. Importantly, capsid modifications did not increase transduction to normal pancreatic tissue. Given that clinical cancer samples displayed inter-sample variation, it might be of interest to analyze the tumor before selecting a virus for the treatment.

To assess transductional efficacy in different organs, *in vivo* biodistribution analysis was performed in an orthotopic gastric cancer model resembling human metastatic disease (**figure 3 in study I**). 5/3 and pK7 modifications increased gene transfer to intraperitoneally disseminated tumors, but 5/3 modification also increased gene transfer to other organs. Since liver toxicity is a concern in the context of adenoviral gene therapy (Worgall, Wolff et al. 1997; Connelly 1999), it was important to discover that neither virus increased hepatic gene transfer.

We conclude that adenoviruses with capsid modifications transducer gastric and pancreatic cancer cells and tissues significantly better than viruses with wild type capsid. Since the rationale behind transductional targeting via genetic modifications is based on ubiquitous properties shared by most tumor cells, the abovementioned capsid modifications have proved powerful in other types of cancers as well. 5/3 virus for instance has been successfully used in transductional targeting in glioma (Zheng, Ulasov et al. 2007), ovarian (Kanerva, Zinn et al. 2003), cervical (Kanerva, Lavilla-Alonso et al. 2008), kidney (Guse,

Diaconu et al. 2009), renal (Guse, Ranki et al. 2007), breast (Stoff-Khalili, Stoff et al. 2005), prostate (Rajecki, Kanerva et al. 2007) and gallbladder cancer model (Tekant, Davydova et al. 2005). Next, we sought to find out whether the enhanced transductional efficacy would translate into enhanced anti-cancer activity of oncolytic viruses.

2. Improving oncolytic effect *in vitro* and *in vivo* with capsid modified adenoviruses (I, IV)

The efficacy of oncolytic adenoviruses is linked to the infection of target cells and subsequent productive replication. Most human tumors, including gastric and pancreatic cancers, are deficient in the retinoblastoma/p16 pathway (Fueyo, Gomez-Manzano et al. 2000; Heise, Hermiston et al. 2000; Hernando, Nahle et al. 2004). Δ24-mutated adenoviruses have a 24 bp deletion in constant region 2 of E1A, in which the pRb binding domain resides. Via this domain, wild-type E1A binds to and activates pRb, required for replication in normal cells. In cancer cells, Δ24 is complemented by the inactivation of pRb by p16/Rb pathway defects, enabling virus replication (Sherr 1996). We used oncolytic viruses with the Δ24 backbone to find out which capsid modification in combination with Δ24 would be the most potent in killing gastric and pancreatic cancer cells. We also wanted to study whether the same viruses would display enhanced anti-tumor activity in mice.

In cell killing assay on gastric (**figure 4 in study 1**) and pancreatic cancer cell lines (**figure 3 in study IV**), Ad5/3-Δ24 was overall the most potent. The same virus was the best in increasing the survival of mice bearing orthotopic gastric (**figure 5a in study 1**) and pancreatic cancer tumors (**figure 4a in study IV**). In gastric cancer model, also RGD-modified virus prolonged the survival significantly. In the same model, serum hCG-β concentration, a prognostic factor in patients with gastric cancer (Louhimo, Kokkola et al. 2004), was lower in mice treated with oncolytic viruses (**figure 5b in study 1**), possibly a further indication of true anti-tumor efficacy. As mentioned above, the same capsid modifications have proved useful in many cancer types, and as an example, clinical trials with Ad5-Δ24RGD are ongoing for the treatment of ovarian cancer and recurrent glioblastoma.

To study replication of oncolytic viruses in human liver, human liver explants were infected with unmodified, RGD, or 5/3-modified oncolytic viruses, and the wild type virus (**figure 6 in study 1**). No true replication was detected, since the amounts of infectious particles in the culture increased 4-fold at best over the whole period of 48h. This was an

important finding, since liver may be the main organ in the context of adenovirus associated toxicity (Worgall, Wolff et al. 1997; Connelly 1999). In conclusion, we saw that capsid modified oncolytic viruses improved antitumor efficacy in gastric and pancreatic cancer models *in vitro* and *in vivo*, but no replication was detected in human livers. Further preclinical studies will need to be done to evaluate safety, but after this is done, these viruses might be valuable for treating gastric or pancreatic cancers in humans.

3. Transductional and transcriptional targeting to $CD44^{+}CD24^{-/low}$ cells (II)

3.1 Tissue-specific promoters are active in $CD44^{+}CD24^{-/low}$ breast cancer cells

It has been proposed that human tumors contain stem cells that have a central role in tumor initiation and post-treatment relapse. Putative breast cancer stem cells may reside in the $CD44^{+}CD24^{-/low}$ population (Al-Hajj, Wicha et al. 2003; Hill and Perris 2007; Kelly, Dakic et al. 2007). As adenoviruses are not subject to the typical mechanisms of drug resistance such as drug efflux pumps and defective cell cycling (Coukos, Makrigiannakis et al. 2000), they might have potential in targeting cancer stem cells, known to be resistant to chemotherapy and radiation (Jordan, Guzman et al. 2006). We utilized capsid modified luciferase expressing viruses to investigate if gene transfer to $CD44^{+}CD24^{-/low}$ cells could be improved. 5/3 modification seemed to be the optimal capsid configuration as it allowed 10-fold higher gene transfer to $CD44^{+}CD24^{-/low}$ cells. Based on this and a previous report describing 5/3 modification to be efficient in targeting breast cancer initiating cells (Eriksson, Guse et al. 2007), for further experiments we chose to construct viruses with 5/3 chimeric fibers.

Oncolytic viruses can be transcriptionally targeted by utilizing tumor-specific promoters (TSP), which are active only in target cells. In normal cells adenovirus replication is prevented in the absence of E1A expression (Saukkonen and Hemminki 2004). To identify which TSPs would be feasible in targeting breast cancer initiating cells, we extracted $CD44^{+}CD^{24-/low}$ cells from pleural effusions of breast cancer patients. As there were no previous reports on which promoters might be useful in the context of cancer stem cells, we chose tumor-specific promoters previously used in adenoviral gene therapy approaches. α-lactalbumin (ala) (Li, Zhang et al. 2005), cyclo-oxygenase 2 (Cox-2) (Yamamoto, Alemany et al. 2001), telomerase (hTERT) (Horikawa, Cable et al. 1999), and multidrug resistance protein (mdr) (Walther, Wendt et al. 1997) promoters were introduced in luciferase producing 5/3 chimeric adenoviruses to compare promoter activities in $CD44^{+}CD24^{-/low}$ cells (**figure**

1a). Mdr showed the strongest activity in pleural effusion cells. Highly specific activity was also achieved with hTERT promoter. The activity of Cox-2 was near to highly active CMV promoter. Expression with ala is too low for the successful control of oncolytic adenoviruses. Taken together these results suggested that mdr, hTERT, and Cox-2 promoters are active in $CD44^{+}$ $^{+}CD24^{-/low}$ breast cancer cells, which encouraged us to find out whether oncolytic viruses featuring these tumor specific promoters would be efficient in killing breast cancer initiating cells.

3.2. Tissue-specific promoters are useful in killing $CD44^{+}CD24^{-/low}$ breast cancer cells *in vitro* and *in vivo*

Capsid modified Δ24 type oncolytic adenoviruses have previously been described to kill breast cancer initiating cells (Eriksson, Guse et al. 2007). As replication and toxicity of oncolytic viruses may be targeted via tumor specific promoters, we rationalized that such a virus could be targeted against breast cancer initiating cells. For transcriptional control, all our oncolytic viruses harbor the Δ24-bp deletion in E1A. Dual transcriptional control would be useful in the context of non-tumor cells in which the TSP is active. For example, normal tissue stem cells might express hTERT or mdr. Nevertheless, they would be expected to be intact in the Rb-p16 pathway. Thus in many cases, an Rb binding site deletion was included in the constructs (**figure 1c**).

When $CD44^{+}CD24^{-/low}$ cells from pleural effusions were infected, we found that all 5/3 modified viruses were more oncolytic than the wild-type Ad5 control (**figure 2**): In 2 out of 3 samples, Ad5/3-mdr-Δ24 was most oncolytic, with Ad5/3-hTERT-Δgp and Ad5/3-Cox2L-Δ24 closely following. Despite the use of a TSP, these viruses were sometimes more potent than Ad5/3-Δ24. Adding the Rb binding site deletion to Ad5/3-mdr-E1A to make Ad5/3-mdr-Δ24 did not reduce the activity of the virus but actually increased potency.

JIMT-1 is breast cancer derived cell line phenotypically of epithelial progenitor cell origin (Tanner, Kapanen et al. 2004). $CD44^{+}CD24^{-/low}$ sorted JIMT-1 cells were used for cell killing assay to look at the kinetics of cell killing between the different viruses (**Figure 3a**). Ad5/3-mdr-Δ24 and Ad5/3-hTERT-Δgp were the most effective TSP viruses and similar in the efficacy with the control virus Ad5/3-Δ24.

To assess antitumor efficacy *in vivo*, $CD44^{+}CD24^{-/low}$–derived tumors were established and injected with oncolytic viruses, all of which resulted in significantly smaller tumor size versus mock controls (**Figure 4a**). Ad5/3-Cox2L-Δ24 and Ad5/3-mdr-Δ24 showed the greatest antitumor efficacy and Ad5/3-mdr-Δ24 was superior when compared to control

virus Ad5/3-Δ24. Despite promising activity *in vitro*, Ad5/3-hTERT-Δgp was less effective *in vivo* than Ad5/3-Δ24.

In conclusion, oncolytic adenoviruses controlled by the TSPs were able to kill $CD44^{+}CD24^{-/low}$ cells. These findings may have relevance for the elimination of cancer stem cells in humans, reducing relapse rates and improving long-term outcome for patients with breast cancer. As drug-resistant tumor initiating cells have been reported to exist in a perivascular niche, intravenous administration of an oncolytic virus might be highly effective to reach this site (Calabrese, Poppleton et al. 2007). Capsid modified viruses capable for dual transcriptional control, such as Ad5/3-mdr-Δ24 and Ad5/3-Cox2L-Δ24, may be appealing for clinical testing. However, it will be important to ensure that these treatments do not adversely affect the function of normal tissue stem cells. Development of such innovative strategies to target cancer stem cell populations in human malignancies is likely to increase treatment success.

4. Quantification of putative stem cells in tumors originating from $CD44^{+}CD24^{-/low}$ cells (II)

Putative stem cells divide asymmetrically. At each division, one or both daughter cells retain the same biological properties as the parent cell. Since the other daughter cell can also become a progenitor cell, a mutated progenitor could divide to produce differentiated tumor cells (Jordan, Guzman et al. 2006). To study this hypothesis, $CD44^{+}/CD24^{-/low}$ tumors were established and treated with Ad5/3mdr-Δ24 (able to kill $CD44^{+}/CD24^{-/low}$ cells *in vitro*), or Ad5/3-ala-Δ24 (not able to kill $CD44^{+}/CD24^{-/low}$ cells *in vitro*), followed by fluorescence-activated cell sorting (FACS) (**figure 4c**). Interestingly, while Ad5/3mdr-Δ24 reduced tumor size (**figure 4b**), it also reduced the total number of $CD44^{+}/CD24^{-/low}$ cells, implying that the viruses also kill tumor-initiating cells, in accordance to what was seen *in vitro*. If viruses had only killed differentiated tumor cells, the proportion of $CD44^{+}CD24^{-/low}$ cells would have been higher, not lower, in virus versus mock-treated tumors. The proportion of $CD44^{+}/CD24^{-/low}$ cells in the tumor returned to the same level as before cell sorting, suggesting that asymmetrical cell division does occur.

5. Characterisation of silica implants for adenovirus preservation and release *in vitro* (III)

Since one major obstacle in the cancer gene therapy remains to be the level and persistence of gene delivery to sufficiently large areas of the tumor, it was of interest to characterize a new viral delivery method for intraperitoneally disseminated disease. Silica sol-gel polymers (the name referring to solvent to gel-transition during polymerization) have many characteristics that make it interesting for drug delivery, and they have been successfully used for spatially controlled, prolonged delivery of different drugs (Coradin, Boissiere et al. 2006). Since also large proteins have been moulded in silica implants (Viitala, Jokinen et al. 2007), we hypothesized that they might be applicable also in adenovirus gene therapy. First we studied the utility of silica gel implants for their capability to store and release functional virus. In theory, high water content might disturb structural homogeneity of the implant in long term storage. However, the water in which the implant is stored is rapidly saturated by SiO_2, stabilizing the implant from degradation (Jokinen, Koskinen et al. 2007). We therefore speculated, that silicated viruses might remain encapsulated and infective for long time.

Steady release kinetics of smaller molecules and proteins from silica sol-gels has previously been described (Coradin, Boissiere et al. 2006; Viitala, Jokinen et al. 2007). Our work was the first to report biochemical properties and release kinetics of the silica-virus matrix. Nearly linear virus release as a function of time was detected (**figure 1c**), encouraging us to further study whether comparable kinetics could be obtained *in vivo*. We also found the virus stay infective for weeks at +37°C (**figure 2a**) and months at +4°C (**table 1**), which may facilitate their storage and distribution. The current guideline in storing the virus requires a -80°C freezer, which is inconvenient for clinical use, for example. Retaining infectivity +37°C would be crucial in *in vivo* applications, where virus is embedded within the implant for long periods while slow degradation of the implant and concomitant virus release take place under physiological conditions.

6. *In vivo* release and antitumor efficacy of virus in silica implants (III-IV)

Since the *in vitro* results of virus preservation and release from the silica were encouraging, we wanted to study silica implants further for delivery of Ad to advanced orthotopic gastric and pancreatic cancer tumors i*n vivo*. The implant can with a minor surgical procedure be placed subcutaneously, or intraperitoneally. First we confirmed *in vivo* gene transfer with

replication deficient reporter gene expressing viruses. Subcutaneously implanted LacZ-virus resulted in reporter gene expression in subcutaneous space (**figure 2b in study III**). Similarly, luciferase gene expression was detected in selected organs after intraperitoneal delivery of luciferase expressing virus (**figure 3 in study III**). Next, capsid-modified oncolytic viruses were implanted into the very vicinity of an intraperitoneal tumor, and extended release of functional viruses was seen. The results were confirmed with immunohistochemical stainings of the tumor (**figure 2c in study III**). We also utilized hCGβ-expressing Ad5/3-Δ24hCGβ for indirect quantification of replication from the blood (**figure 4c in study II**), since replication of this virus is tightly linked to the secretion of hCGβ (Rajecki, Kanerva et al. 2007). For comparison, we collected tumors and livers at the same time points for direct quantification of infectious virus particles (**figures 4a and 4b in study II**). Indeed, hCGβ-levels in the virus injected mice peaked at the same time point when highest amounts of infectious virus were detected in the organs (2 days after treatment). In the silica treated mice, both hCGβ-levels and virus replication displayed protracted kinetics, hCGβ peaking at day 7 and viral loads peaking days later. hCGβ levels stayed elevated even when there was no detectable replicating virus in the liver (3 weeks after treatment). At this time, pronounced replication was still ongoing in the tumor according to pfu-quantification, and thus at this time it seems likely that the elevated hCGβ levels were resultant of replication in the tumor.

Treating pancreatic cancer bearing mice with virus implanted virus doubled the survival compared to the non-treated mice **(figure 4d in study III)**. Since only combining adenoviral cancer gene therapy with standard therapy has yielded positive efficacy data in randomized phase 3 trials (Yu and Fang 2007; Pan, Zhang et al. 2009), and since gemcitabine remains the global standard-of-care for advanced pancreatic cancer (Bernhard, Dietrich et al. 2008), we included gemcitabine group to study if combination treatment with gemcitabine and virus would bring synergy or additional effect. In mice that received both gemcitabine and Ad5/3-Δ24, median survival was longer than in the mice receiving gemcitabine only. There was a statistically non-significant trend for survival benefit in the combination treated mice when compared to mice receiving virus only. The modest survival benefit might be partially explained by the fact that gemcitabine reduces the rate of Ad5/3-Δ24 replication early after infection (Raki, Kanerva et al. 2005). However, it should not affect the total yield of the virus. There are also previously published studies implying that even when virus treatment or chemotherapy alone are effective, their combination is not, as in the case of doxorubicin and Ad5-Δ24RGD in primary osteosarcoma cells (Graat, Witlox et al. 2006). Still, we speculate that gemcitabine in combination with Ad5/3-Δ24 may be potent in treating pancreatic cancer,

but further work would need to be done to optimize the *in vivo* protocol. A previously described work utilizing the same drugs in ovarian cancer model implies that finding the optimal timing for delivery of the two drugs, either separately or at the same time, may proof critical (Raki, Kanerva et al. 2005).

Silica embedded virus was as good as the direct virus injection in prolonging the survival, and thus the overall lower level of gene transfer with the implant did not compromise the anti-tumor efficacy in survival experiments. Seeing superior survival benefit by the use of silica implant would have been unexpected because of the distinct virus release kinetics from the silica. Parameters such as dosing, timing and gel composition would require optimization to demonstrate true survival benefit of the silica implanted virus compared to virus injection. We still speculate that it might be obtainable for instance through lower virus doses and changing the gel composition to reach the optimal release kinetics. Still, our hypothesis that the release kinetics and possible shielding effect of the silica molecules surrounding the capsid would affect virus-induced immune response proved to be worth studying.

7. Silica implants and anti-adenovirus immune response (III-IV)

Since the release kinetics of the implanted virus are different from virus given as single intraperitoneal injection, we also wanted to study whether silica implant affects anti-viral immune response. Neutralizing antibody (NAb) response has been reported to limit readministration of the virus (Bierman, Crile et al. 1953; Chen, Yu et al. 2000; Tsai, Johnson et al. 2004). We hypothesized, that the use of silica implant might attenuate or postpone antibody formation.

When the virus was given in a subcutaneous implant, the development of anti-adenovirus antibodies was indeed slower than in the subcutaneously virus injected mice, in which the amount of antibodies was statistically significantly higher already a week before antibodies were even detected in the implant group **(figure 4e in study III)**. A similar pattern was seen in an intraperitoneal model: When the virus was delivered in an intraperitoneal implant, virus induced NAb response was low compared to the mice receiving intaperitoneal virus injection (**figure 6b in study IV**). NAbs were analyzed at the time point when the titers are expected to have reached their peak values (Sarkioja, Pesonen et al. 2008). In the virus implant group, the NAb titers were at least a magnitude lower than in the virus injected mice. Lowered antibody formation against the delivered virus might be useful for facilitating readministration. Although the tumor environment may be relatively immune privileged,

peritumoral and systemic viral dissemination might be more effective if the antibody induction is lower. Lower NAb titers may also be crucial in safe readministration of adenovirus, since in mice viral toxicity caused by vector challenge is reported to be greater in preimmunized animals (Vlachaki, Hernandez-Garcia et al. 2002; Varnavski, Calcedo et al. 2005). It was also of interest to find out whether intraperitoneal virus delivery would lead into a more favourable biodistribution when silica implant is used in preimmunized animals. In the presence of NAbs, the virus in silica implants resulted in more favourable pancreas to liver transduction profile **(figures 5a and 5c in study IV)**. Again, liver transduction was significantly lower in the mice receiving the virus in silica implant, which may reduce toxicity (Worgall, Wolff et al. 1997; Connelly 1999; Tao, Gao et al. 2001).

We hypothesized further that silica implant might also have an effect on virus-induced proimflammatory cytokine response critical for early viral toxicity (Raper, Chirmule et al. 2003). After receiving a large dose of virus intraperitoneally, IL-6 was found to be lower 6 h after the treatment in the silica implant group vs. the injected ones **(figure 6a in study IV)**, IFN-γ and RANTES displaying a similar pattern. These results suggest that co-administration of the virus with silica may partly prevent early viral toxicity, thus enabling administration of larger doses and/or more immunogenic viruses, if needed.

As a conclusion, the data received from the immunological studies suggests that silica implant might be a way to partially overcome the problems associated with high immunogenicity of the virus, further broadening the safety/efficacy window of intraperitoneally administered oncolytic adenoviruses.

SUMMARY AND CONCLUSIONS

The overall goal of this thesis was to improve the treatment options for incurable cancers using oncolytic adenoviruses, and capsid modified adenoviruses proved to be useful for transductional targeting to cancer cell lines and clinical tumor samples, as well as targeting for cancer initiating cells. More favorable *in vivo* biodistribution was also achieved with capsid modified adenoviruses. Furthermore, enhanced oncolytic potency was seen *in vitro*, which translated into improved anti-tumor effect *in vivo*. 5/3 chimerism emerged as the approach-of-choice in all studies, covering models of gastric, pancreatic and breast cancer. Importantly, the capsid modifications did not increase gene transfer to normal human pancreatic tissue, nor did the oncolytic capsid modified viruses replicate in normal human liver tissue *ex vivo*.

To gain strict transcriptional targeting to breast cancer initiating $CD44^{+}CD24^{-/low}$ cells, we inserted TSPs in the genome of capsid modified Δ24-based oncolytic viruses to control expression of E1A and subsequent replication. Cox-2, hTERT and mdr promoters proved useful in the *in vitro* studies, displaying even better oncolytic potential in $CD44^{+}CD24^{-/low}$ cells isolated from the pleural effusion samples than the highly active control virus without the TSP. Compared to the conventional oncolytic viruses, all of the viruses armed with TSPs were superior in eradicating tumors in mice. In conclusion, oncolytic adenoviruses controlled by the TSPs seem to be able to kill $CD44^{+}CD24^{-/low}$ cells.

The biochemical properties of silica sol-gel implant proved to be favorable for preserving the virus in different temperatures, and functional virus release correlated with the degrading of the silica-virus matrix. Utilizing orthotopic gastric and pancreatic cancer models, we found the silica implant to steadily release replication competent virus also *in vivo*, resulting in a lower level but more sustained replication in the tumor tissue. Intraperitoneal delivery also resulted in a more favorable biodistribution of the virus, with less virus accumulating in the liver. The survival benefit gained with the silica implant was comparable to the intraperitoneally injected virus. Silica gel-based virus delivery lowered toxicity mediating proimflammatory cytokine response, and production of total and anti-adenovirus neutralizing antibodies (NAbs). Further, silica shielded the virus against pre-existing NAbs, resulting in more favorable biodistribution in the preimmunized mice. In this thesis book we

describe adenovirus capsid modifications and TSPs that enhance safety, specificity and anti-tumor activity of oncolytic adenoviruses. Furthermore, new delivery methods, such as the studied silica implant, might further broaden the safety window and/or gene transfer efficacy of intraperitoneally administrated oncolytic adenoviruses.

ACKNOWLEDGEMENTS

This work was carried out at the Cancer Gene Therapy Group (CGTG), which is a part of the Molecular Cancer Biology Program, HUSLAB, Transplantational Laboratory, Haartman Institute and Finnish Institute of Molecular Medicine at the University of Helsinki and the Helsinki University Central Hospital, during 2003 through 2009. I wish to express my sincere gratitude to a large number of people who contributed to these studies.

I want to thank the former head of the Transplantation Laboratory, Professor Pekka Häyry, the former coordinator of the Rational Drug Design Program and current head of the Transplantation Laboratory, Professor Risto Renkonen, the former and current directors of the Molecular Cancer Biology Program, Professors Kari Alitalo, Jorma Keski-Oja, Marikki Laiho, the head of the Haartman Institute, Seppo Meri, the Heads of HUSLAB Professor Lasse Viinikka and Docent Lasse Lehtonen, head of FIMM Professor Olli Kallioniemi, and the head of the Biomedicum and Faculty Research Programs, Professor Olli Jänne, for providing excellent research facilities.

Professor Kari Airenne and Docent Mikko Savontaus are warmly thanked for the careful revision of my thesis and their valuable comments on it.

Most importantly, I am deeply indebted to my supervisor Akseli Hemminki for giving me a possibility to work in a best group possible, and all the support he has given me with great patience.

I wish to thank all members of the Cancer Gene Therapy Group. I thank my mentor Tanja Hakkarainen, and all our PhDs for showing me way with my projects. Furthermore, I want to express my gratitude to co-authors and collaborators.

For the final support I wish to acknowledge: Mum, HUCH Research Funds (EVO), Finnish Cancer Society, University of Helsinki Funds, K. Albin Johansson Foundation, Orion-Farmos Research Foundation, Finnish Cultural Foundation, Finnish Foundation for Research on Viral Disease, Finnish Gastroenterology Foundation, Oskar Öflund Foundation, Finnish-Norwegian Medical Foundation, Biomedicum Helsinki Foundation, Otto A. Malm Foundation, Bayer-Shering Pharma Foundation, Juliana von Wendt Foundation and Irja Karvonen Cancer Foundation.

Helsinki, January 2010 Lotta Kangasniemi

REFERENCES

Al-Hajj, M., M. S. Wicha, et al. (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A 100(7): 3983-8.

Alemany, R. and D. T. Curiel (2001). "CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors." Gene Ther 8(17): 1347-53.

Alemany, R., K. Suzuki, et al. (2000). "Blood clearance rates of adenovirus type 5 in mice." J Gen Virol 81(Pt 11): 2605-9.

Alfthan, H., J. Schroder, et al. (1988). "Choriogonadotropin and its beta subunit separated by hydrophobic-interaction chromatography and quantified in serum during pregnancy by time-resolved immunofluorometric assays." Clin Chem 34(9): 1758-62.

Asada, T. (1974). "Treatment of human cancer with mumps virus." Cancer 34(6): 1907-28.

Balachandran, S. and G. N. Barber (2007). "PKR in innate immunity, cancer, and viral oncolysis." Methods Mol Biol 383: 277-301.

Banerjee, N. S., A. A. Rivera, et al. (2004). "Analyses of melanoma-targeted oncolytic adenoviruses with tyrosinase enhancer/promoter-driven E1A, E4, or both in submerged cells and organotypic cultures." Mol Cancer Ther 3(4): 437-49.

Barber, G. N. (2004). "Vesicular stomatitis virus as an oncolytic vector." Viral Immunol 17(4): 516-27.

Bauerschmitz, G. J., S. D. Barker, et al. (2002). "Adenoviral gene therapy for cancer: from vectors to targeted and replication competent agents (review)." Int J Oncol 21(6): 1161-74.

Bauerschmitz, G. J., K. Guse, et al. (2006). "Triple-targeted oncolytic adenoviruses featuring the cox2 promoter, E1A transcomplementation, and serotype chimerism for enhanced selectivity for ovarian cancer cells." Mol Ther 14(2): 164-74.

Bauerschmitz, G. J., T. Ranki, et al. (2008). "Tissue-specific promoters active in CD44+CD24-/low breast cancer cells." Cancer Res 68(14): 5533-9.

Bergelson, J. M., J. A. Cunningham, et al. (1997). "Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5." Science 275(5304): 1320-3.

Berget, S. M., C. Moore, et al. (1977). "Spliced segments at the 5' terminus of adenovirus 2 late mRNA." Proc Natl Acad Sci U S A 74(8): 3171-5.

Berk, A. J. and P. A. Sharp (1978). "Structure of the adenovirus 2 early mRNAs." Cell 14(3): 695-711.

Bernhard, J., D. Dietrich, et al. (2008). "Clinical benefit and quality of life in patients with advanced pancreatic cancer receiving gemcitabine plus capecitabine versus gemcitabine alone: a randomized multicenter phase III clinical trial--SAKK 44/00-CECOG/PAN.1.3.001." J Clin Oncol 26(22): 3695-701.

Bett, A. J., L. Prevec, et al. (1993). "Packaging capacity and stability of human adenovirus type 5 vectors." J Virol 67(10): 5911-21.

Bieler, A., K. Mantwill, et al. (2006). "Novel three-pronged strategy to enhance cancer cell killing in glioblastoma cell lines: histone deacetylase inhibitor, chemotherapy, and oncolytic adenovirus dl520." Hum Gene Ther 17(1): 55-70.

Bierman, H. R., D. M. Crile, et al. (1953). "Remissions in leukemia of childhood following acute infectious disease: staphylococcus and streptococcus, varicella, and feline panleukopenia." Cancer 6(3): 591-605.

Bischoff, J. R., D. H. Kirn, et al. (1996). "An adenovirus mutant that replicates selectively in p53-deficient human tumor cells." Science 274(5286): 373-6.

Bishop, J. M., Weinberg, R. A., eds. (1996). "Molecular Oncology (New York: Scientific American, Inc.)."

Breidenbach, M., D. T. Rein, et al. (2004). "Genetic replacement of the adenovirus shaft fiber reduces liver tropism in ovarian cancer gene therapy." Hum Gene Ther 15(5): 509-18.

Brunori, M., M. Malerba, et al. (2001). "Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway." J Virol 75(6): 2857-65.

Calabrese, C., H. Poppleton, et al. (2007). "A perivascular niche for brain tumor stem cells." Cancer Cell 11(1): 69-82.

Cascallo, M., G. Capella, et al. (2003). "Ras-dependent oncolysis with an adenovirus VAI mutant." Cancer Res 63(17): 5544-50.

Chen, Y., D. C. Yu, et al. (2000). "Pre-existent adenovirus antibody inhibits systemic toxicity and antitumor activity of CN706 in the nude mouse LNCaP xenograft model: implications and proposals for human therapy." Hum Gene Ther 11(11): 1553-67.

Cheng, J., H. Sauthoff, et al. (2007). "Human matrix metalloproteinase-8 gene delivery increases the oncolytic activity of a replicating adenovirus." Mol Ther 15(11): 1982-90.

Cheon, J., S. C. Ko, et al. (1997). "Chemogene therapy: osteocalcin promoter-based suicide gene therapy in combination with methotrexate in a murine osteosarcoma model." Cancer Gene Ther 4(6): 359-65.

Connelly, S. (1999). "Adenoviral vectors for liver-directed gene therapy." Curr Opin Mol Ther 1(5): 565-72.

Coradin, T., M. Boissiere, et al. (2006). "Sol-gel chemistry in medicinal science." Curr Med Chem 13(1): 99-108.

Coughlan, L., S. Vallath, et al. (2009). "In vivo retargeting of adenovirus type 5 to alphavbeta6 integrin results in reduced hepatotoxicity and improved tumor uptake following systemic delivery." J Virol 83(13): 6416-28.

Coukos, G., A. Makrigiannakis, et al. (2000). "Oncolytic herpes simplex virus-1 lacking ICP34.5 induces p53-independent death and is efficacious against chemotherapy-resistant ovarian cancer." Clin Cancer Res 6(8): 3342-53.

Crompton, A. M. and D. H. Kirn (2007). "From ONYX-015 to armed vaccinia viruses: the education and evolution of oncolytic virus development." Curr Cancer Drug Targets 7(2): 133-9.

Curiel, D. T. and J. T. Douglas (2002). "Adenoviral vectors for gene therapy."

Czarnobaj, K. and J. Lukasiak (2004). "In vitro release of cisplatin from sol-gel processed porous silica xerogels." Drug Deliv 11(6): 341-4.

Dean, M., T. Fojo, et al. (2005). "Tumour stem cells and drug resistance." Nat Rev Cancer 5(4): 275-84.

DeWeese, T. L., H. van der Poel, et al. (2001). "A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy." Cancer Res 61(20): 7464-72.

Di Paolo, N. C., S. Tuve, et al. (2006). "Effect of adenovirus-mediated heat shock protein expression and oncolysis in combination with low-dose cyclophosphamide treatment on antitumor immune responses." Cancer Res 66(2): 960-9.

Dmitriev, I., E. Kashentseva, et al. (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(15): 6875-84.

Dmitriev, I., V. Krasnykh, et al. (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(12): 9706-13.

Doronin, K., M. Kuppuswamy, et al. (2001). "Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy." J Virol 75(7): 3314-24.

Douglas, J. T., B. E. Rogers, et al. (1996). "Targeted gene delivery by tropism-modified adenoviral vectors." Nat Biotechnol 14(11): 1574-8.

Edelstein, M. L., M. R. Abedi, et al. (2007). "Gene therapy clinical trials worldwide to 2007--an update." J Gene Med 9(10): 833-42.

Edelstein, M. L., M. R. Abedi, et al. (2004). "Gene therapy clinical trials worldwide 1989-2004-an overview." J Gene Med 6(6): 597-602.

Eriksson, M., K. Guse, et al. (2007). "Oncolytic adenoviruses kill breast cancer initiating CD44+CD24-/low cells." Mol Ther 15(12): 2088-93.

Freimuth, P. (1996). "A human cell line selected for resistance to adenovirus infection has reduced levels of the virus receptor." J Virol 70(6): 4081-5.

Freytag, S. O., D. Paielli, et al. (2002). "Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model." Int J Radiat Oncol Biol Phys 54(3): 873-85.

Freytag, S. O., H. Stricker, et al. (2003). "Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer." Cancer Res 63(21): 7497-506.

Fueyo, J., R. Alemany, et al. (2003). "Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway." J Natl Cancer Inst 95(9): 652-60.

Fueyo, J., C. Gomez-Manzano, et al. (2000). "A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo." Oncogene 19(1): 2-12.

Gaggar, A., D. M. Shayakhmetov, et al. (2003). "CD46 is a cellular receptor for group B adenoviruses." Nat Med 9(11): 1408-12.

Ganly, I., D. Kirn, et al. (2000). "A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer." Clin Cancer Res 6(3): 798-806.

Garcia-Castro, J., J. Martinez-Palacio, et al. (2005). "Tumor cells as cellular vehicles to deliver gene therapies to metastatic tumors." Cancer Gene Ther 12(4): 341-9.

Georgiades, J., T. Zielinski, et al. (1959). "Research on the oncolytic effect of APC viruses in cancer of the cervix uteri; preliminary report." Biul Inst Med Morsk Gdansk 10: 49-57.

Girardin, S. E., P. J. Sansonetti, et al. (2002). "Intracellular vs extracellular recognition of pathogens--common concepts in mammals and flies." Trends Microbiol 10(4): 193-9.

Glasgow, J. N., M. Everts, et al. (2006). "Transductional targeting of adenovirus vectors for gene therapy." Cancer Gene Ther 13(9): 830-44.

Goldsmith, M. E., M. Kitazono, et al. (2003). "The histone deacetylase inhibitor FK228 preferentially enhances adenovirus transgene expression in malignant cells." Clin Cancer Res 9(14): 5394-401.

Goodrum, F. D. and D. A. Ornelles (1998). "p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection." J Virol 72(12): 9479-90.

Goodrum, F. D. and D. A. Ornelles (1999). "Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication." J Virol 73(9): 7474-88.

Gordon, J. W. (2001). "Direct exposure of mouse ovaries and oocytes to high doses of an adenovirus gene therapy vector fails to lead to germ cell transduction." Mol Ther 3(4): 557-64.

Graat, H. C., M. A. Witlox, et al. (2006). "Different susceptibility of osteosarcoma cell lines and primary cells to treatment with oncolytic adenovirus and doxorubicin or cisplatin." Br J Cancer 94(12): 1837-44.

Greber, U. F., P. Webster, et al. (1996). "The role of the adenovirus protease on virus entry into cells." EMBO J 15(8): 1766-77.

Grill, J., V. W. Van Beusechem, et al. (2001). "Combined targeting of adenoviruses to integrins and epidermal growth factor receptors increases gene transfer into primary glioma cells and spheroids." Clin Cancer Res 7(3): 641-50.

Grzesiak, J. J., J. C. Ho, et al. (2007). "The integrin-extracellular matrix axis in pancreatic cancer." Pancreas 35(4): 293-301.

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

Gupta, V., W. Wang, et al. (2006). "Fibroblast growth factor-2-retargeted adenoviral vector for selective transduction of primary glioblastoma multiforme endothelial cells." Neurosurg Focus 20(4): E26.

Guse, K., I. Diaconu, et al. (2009). "Ad5/3-9HIF-Delta24-VEGFR-1-Ig, an infectivity enhanced, dual-targeted and antiangiogenic oncolytic adenovirus for kidney cancer treatment." Gene Ther 16(8): 1009-20.

Guse, K., T. Ranki, et al. (2007). "Treatment of metastatic renal cancer with capsid-modified oncolytic adenoviruses." Mol Cancer Ther 6(10): 2728-36.

Haisma, H. J., J. A. Kamps, et al. (2008). "Polyinosinic acid enhances delivery of adenovirus vectors in vivo by preventing sequestration in liver macrophages." J Gen Virol 89(Pt 5): 1097-105.

Hakkarainen, T., A. Kanerva, et al. (2005). "[Adenoviruses in cancer therapy]." Duodecim 121(20): 2195-203.

Hakkarainen, T., M. Sarkioja, et al. (2007). "Human mesenchymal stem cells lack tumor tropism but enhance the antitumor activity of oncolytic adenoviruses in orthotopic lung and breast tumors." Hum Gene Ther 18(7): 627-41.

Haley, K. P., J. Overhauser, et al. (1984). "Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products." Proc Natl Acad Sci U S A 81(18): 5734-8.

Han, J., D. Modha, et al. (1998). "Interaction of E1B 19K with Bax is required to block Bax-induced loss of mitochondrial membrane potential and apoptosis." Oncogene 17(23): 2993-3005.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell 100(1): 57-70.

Harada, J. N. and A. J. Berk (1999). "p53-Independent and -dependent requirements for E1B-55K in adenovirus type 5 replication." J Virol 73(7): 5333-44.

Hardcastle, J., K. Kurozumi, et al. (2007). "Oncolytic viruses driven by tumor-specific promoters." Curr Cancer Drug Targets 7(2): 181-9.

Hay, R. T., A. Freeman, et al. (1995). "Molecular interactions during adenovirus DNA replication." Curr Top Microbiol Immunol 199 (Pt 2): 31-48.

Heise, C., T. Hermiston, et al. (2000). "An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy." Nat Med 6(10): 1134-9.

Heise, C., A. Sampson-Johannes, et al. (1997). "ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents." Nat Med 3(6): 639-45.

Hemmi, S., R. Geertsen, et al. (1998). "The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures." Hum Gene Ther 9(16): 2363-73.

Hemminki, A. (2002). "From molecular changes to customised therapy." Eur J Cancer 38(3): 333-8.

Hernando, E., Z. Nahle, et al. (2004). "Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control." Nature 430(7001): 797-802.

Hill, R. P. and R. Perris (2007). ""Destemming" cancer stem cells." J Natl Cancer Inst 99(19): 1435-40.
Hitt, M. M. and F. L. Graham (1990). "Adenovirus E1A under the control of heterologous promoters: wide variation in E1A expression levels has little effect on virus replication." Virology 179(2): 667-78.

Horikawa, I., P. L. Cable, et al. (1999). "Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene." Cancer Res 59(4): 826-30.

Hoster, H. A., R. P. Zanes, Jr., et al. (1949). "Studies in Hodgkin's syndrome; the association of viral hepatitis and Hodgkin's disease; a preliminary report." Cancer Res 9(8): 473-80.

Huebner, R. J., W. P. Rowe, et al. (1956). "Studies on the use of viruses in the treatment of carcinoma of the cervix." Cancer 9(6): 1211-8.

Jiang, H., C. Gomez-Manzano, et al. (2007). "Examination of the therapeutic potential of Delta-24-RGD in brain tumor stem cells: role of autophagic cell death." J Natl Cancer Inst 99(18): 1410-4.

Johansson, C., M. Jonsson, et al. (2007). "Adenoviruses use lactoferrin as a bridge for CAR-independent binding to and infection of epithelial cells." J Virol 81(2): 954-63.

Johnson, L., A. Shen, et al. (2002). "Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents." Cancer Cell 1(4): 325-37.

Jokinen, M., M. Koskinen, et al. (2007). "Method for storing silica-based material, package produced with the method and use of package for packaging of silica-based products." WIPO Patent number WO 2007/135224.

Jonsson, M. I., A. E. Lenman, et al. (2009). "Coagulation factors IX and X enhance binding and infection of adenovirus types 5 and 31 in human epithelial cells." J Virol 83(8): 3816-25.

Jordan, C. T., M. L. Guzman, et al. (2006). "Cancer stem cells." N Engl J Med 355(12): 1253-61.

Kalyuzhniy, O., N. C. Di Paolo, et al. (2008). "Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo." Proc Natl Acad Sci U S A 105(14): 5483-8.

Kanerva, A. and A. Hemminki (2005). "Adenoviruses for treatment of cancer." Ann Med 37(1): 33-43.
Kanerva, A., S. Lavilla-Alonso, et al. (2008). "Systemic therapy for cervical cancer with potentially regulatable oncolytic adenoviruses." PLoS ONE 3(8): e2917.

Kanerva, A., G. V. Mikheeva, et al. (2002). "Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells." Clin Cancer Res 8(1): 275-80.

Kanerva, A., K. R. Zinn, et al. (2003). "Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus." Mol Ther 8(3): 449-58.

Kass-Eisler, A., L. Leinwand, et al. (1996). "Circumventing the immune response to adenovirus-mediated gene therapy." Gene Ther 3(2): 154-62.

Kawashima, A., S. Tsugawa, et al. (2003). "Expression of alphav integrin family in gastric carcinomas: increased alphavbeta6 is associated with lymph node metastasis." Pathol Res Pract 199(2): 57-64.

Kelly, E. and S. J. Russell (2007). "History of oncolytic viruses: genesis to genetic engineering." Mol Ther 15(4): 651-9.

Kelly, P. N., A. Dakic, et al. (2007). "Tumor growth need not be driven by rare cancer stem cells." Science 317(5836): 337.

Khuri, F. R., J. Nemunaitis, et al. (2000). "a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer." Nat Med 6(8): 879-85.

Kim, J. H., Y. S. Lee, et al. (2006). "Relaxin expression from tumor-targeting adenoviruses and its intratumoral spread, apoptosis induction, and efficacy." J Natl Cancer Inst 98(20): 1482-93.

Kitazono, M., M. E. Goldsmith, et al. (2001). "Enhanced adenovirus transgene expression in malignant cells treated with the histone deacetylase inhibitor FR901228." Cancer Res 61(17): 6328-30.

Ko, D., L. Hawkins, et al. (2005). "Development of transcriptionally regulated oncolytic adenoviruses." Oncogene 24(52): 7763-74.

Koizumi, N., K. Kawabata, et al. (2006). "Modified adenoviral vectors ablated for coxsackievirus-adenovirus receptor, alphav integrin, and heparan sulfate binding reduce in vivo tissue transduction and toxicity." Hum Gene Ther 17(3): 264-79.

Korn, T., D. M. Nettelbeck, et al. (2004). "Recombinant bispecific antibodies for the targeting of adenoviruses to CEA-expressing tumour cells: a comparative analysis of bacterially expressed single-chain diabody and tandem scFv." J Gene Med 6(6): 642-51.

Koski, A., M. Rajecki, et al. (2009). "Systemic adenoviral gene delivery to orthotopic murine breast tumors with ablation of coagulation factors, thrombocytes and Kupffer cells." J Gene Med.

Kuhn, I., P. Harden, et al. (2008). "Directed evolution generates a novel oncolytic virus for the treatment of colon cancer." PLoS ONE 3(6): e2409.

Kuo, C. J., F. Farnebo, et al. (2001). "Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer." Proc Natl Acad Sci U S A 98(8): 4605-10.

Laitinen, M., K. Makinen, et al. (1998). "Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia." Hum Gene Ther 9(10): 1481-6.

Lamfers, M. L., G. Fulci, et al. (2006). "Cyclophosphamide increases transgene expression mediated by an oncolytic adenovirus in glioma-bearing mice monitored by bioluminescence imaging." Mol Ther 14(6): 779-88.

Lamfers, M. L., J. Grill, et al. (2002). "Potential of the conditionally replicative adenovirus Ad5-Delta24RGD in the treatment of malignant gliomas and its enhanced effect with radiotherapy." Cancer Res 62(20): 5736-42.

Lamfers, M. L., S. Idema, et al. (2007). "Differential effects of combined Ad5- delta 24RGD and radiation therapy in in vitro versus in vivo models of malignant glioma." Clin Cancer Res 13(24): 7451-8.

Leen, A. M., A. Christin, et al. (2008). "Identification of hexon-specific CD4 and CD8 T-cell epitopes for vaccine and immunotherapy." J Virol 82(1): 546-54.

Lenaerts, L., E. De Clercq, et al. (2008). "Clinical features and treatment of adenovirus infections." Rev Med Virol 18(6): 357-74.

Li, X., J. Zhang, et al. (2005). "Transcriptional targeting modalities in breast cancer gene therapy using adenovirus vectors controlled by alpha-lactalbumin promoter." Mol Cancer Ther 4(12): 1850-9.

Li, X., Y. P. Zhang, et al. (2005). "Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer." Cancer Res 65(5): 1941-51.

Li, Y., Y. Chen, et al. (2003). "Carcinoembryonic antigen-producing cell-specific oncolytic adenovirus, OV798, for colorectal cancer therapy." Mol Cancer Ther 2(10): 1003-9.

Li, Y., D. C. Yu, et al. (2001). "A hepatocellular carcinoma-specific adenovirus variant, CV890, eliminates distant human liver tumors in combination with doxorubicin." Cancer Res 61(17): 6428-36.

Liu, Y., H. Wang, et al. (2009). "Transduction of liver metastases after intravenous injection of Ad5/35 or Ad35 vectors with and without factor X-binding protein pretreatment." Hum Gene Ther.

Louhimo, J., A. Kokkola, et al. (2004). "Preoperative hCGbeta and CA 72-4 are prognostic factors in gastric cancer." Int J Cancer 111(6): 929-33.

Lukas, J., H. Muller, et al. (1994). "DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1." J Cell Biol 125(3): 625-38.

Lukashok, S. A. and M. S. Horwitz (1998). "New perspectives in adenoviruses." Curr Clin Top Infect Dis 18: 286-305.

Martuza, R. L., A. Malick, et al. (1991). "Experimental therapy of human glioma by means of a genetically engineered virus mutant." Science 252(5007): 854-6.

Mastrangeli, A., B. G. Harvey, et al. (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(1): 79-87.

McKee, T. D., P. Grandi, et al. (2006). "Degradation of fibrillar collagen in a human melanoma xenograft improves the efficacy of an oncolytic herpes simplex virus vector." Cancer Res 66(5): 2509-13.

Mizuguchi, H., T. Sasaki, et al. (2005). "Fiber-modified adenovirus vectors mediate efficient gene transfer into undifferentiated and adipogenic-differentiated human mesenchymal stem cells." Biochem Biophys Res Commun 332(4): 1101-6.

Mok, W., Y. Boucher, et al. (2007). "Matrix metalloproteinases-1 and -8 improve the distribution and efficacy of an oncolytic virus." Cancer Res 67(22): 10664-8.

Muruve, D. A. (2004). "The innate immune response to adenovirus vectors." Hum Gene Ther 15(12): 1157-66.
Muruve, D. A., V. Petrilli, et al. (2008). "The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response." Nature 452(7183): 103-7.

Nemunaitis, J., I. Ganly, et al. (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(22): 6359-66.

Nettelbeck, D. M. (2008). "Cellular genetic tools to control oncolytic adenoviruses for virotherapy of cancer." J Mol Med 86(4): 363-77.

Nettelbeck, D. M., A. A. Rivera, et al. (2002). "Novel oncolytic adenoviruses targeted to melanoma: specific viral replication and cytolysis by expression of E1A mutants from the tyrosinase enhancer/promoter." Cancer Res 62(16): 4663-70.

O'Riordan, C. R., A. Lachapelle, et al. (1999). "PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo." Hum Gene Ther 10(8): 1349-58.

O'Shea, C. C., L. Johnson, et al. (2004). "Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity." Cancer Cell 6(6): 611-23.

O'Shea, C. C., C. Soria, et al. (2005). "Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy." Cancer Cell 8(1): 61-74.

Oosterhoff, D., H. M. Pinedo, et al. (2005). "Gene-directed enzyme prodrug therapy with carboxylesterase enhances the anticancer efficacy of the conditionally replicating adenovirus AdDelta24." Gene Ther 12(12): 1011-8.

Pan, J. J., S. W. Zhang, et al. (2009). "Effect of recombinant adenovirus-p53 combined with radiotherapy on long-term prognosis of advanced nasopharyngeal carcinoma." J Clin Oncol 27(5): 799-804.

Parker, A. L., S. N. Waddington, et al. (2006). "Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes." Blood 108(8): 2554-61.

Parkin, D. M. (2001). "Global cancer statistics in the year 2000." Lancet Oncol 2(9): 533-43.

Parkin, D. M., F. Bray, et al. (2005). "Global cancer statistics, 2002." CA Cancer J Clin 55(2): 74-108.

Pasqualini, R., E. Koivunen, et al. (1997). "Alpha v integrins as receptors for tumor targeting by circulating ligands." Nat Biotechnol 15(6): 542-6.

Peter, I., C. Graf, et al. (2003). "A novel attenuated replication-competent adenovirus for melanoma therapy." Gene Ther 10(7): 530-9.

Pong, R. C., Y. J. Lai, et al. (2003). "Epigenetic regulation of coxsackie and adenovirus receptor (CAR) gene promoter in urogenital cancer cells." Cancer Res 63(24): 8680-6.

Prestwich, R. J., F. Errington, et al. (2009). "The case of oncolytic viruses versus the immune system: waiting on the judgment of Solomon." Hum Gene Ther 20(10): 1119-32.

Prestwich, R. J., K. J. Harrington, et al. (2008). "Oncolytic viruses: a novel form of immunotherapy." Expert Rev Anticancer Ther 8(10): 1581-8.

Prokopowicz, M. (2007). "In-vitro controlled release of doxorubicin from silica xerogels." J Pharm Pharmacol 59(10): 1365-73.

Quintanar-Guerrero, D., A. Ganem-Quintanar, et al. (2009). "Silica xerogels as pharmaceutical drug carriers." Expert Opin Drug Deliv 6(5): 485-98.

Rajecki, M., A. Kanerva, et al. (2007). "Treatment of prostate cancer with Ad5/3Delta24hCG allows non-invasive detection of the magnitude and persistence of virus replication in vivo." Mol Cancer Ther 6(2): 742-51.

Raki, M., T. Hakkarainen, et al. (2007). "Utility of TK/GCV in the context of highly effective oncolysis mediated by a serotype 3 receptor targeted oncolytic adenovirus." Gene Ther 14(19): 1380-8.

Raki, M., A. Kanerva, et al. (2005). "Combination of gemcitabine and Ad5/3-Delta24, a tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer." Gene Ther 12(15): 1198-205.

Raki, M., M. Sarkioja, et al. (2008). "Oncolytic adenovirus Ad5/3-delta24 and chemotherapy for treatment of orthotopic ovarian cancer." Gynecol Oncol 108(1): 166-72.

Ranki, T., A. Kanerva, et al. (2007). "A heparan sulfate-targeted conditionally replicative adenovirus, Ad5.pk7-Delta24, for the treatment of advanced breast cancer." Gene Ther 14(1): 58-67.

Rao, L., M. Debbas, et al. (1992). "The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins." Proc Natl Acad Sci U S A 89(16): 7742-6.

Raper, S. E., N. Chirmule, et al. (2003). "Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer." Mol Genet Metab 80(1-2): 148-58.

Reddy, P. S., K. D. Burroughs, et al. (2007). "Seneca Valley virus, a systemically deliverable oncolytic picornavirus, and the treatment of neuroendocrine cancers." J Natl Cancer Inst 99(21): 1623-33.

Reid, T., R. Warren, et al. (2002). "Intravascular adenoviral agents in cancer patients: lessons from clinical trials." Cancer Gene Ther 9(12): 979-86.

Reya, T., S. J. Morrison, et al. (2001). "Stem cells, cancer, and cancer stem cells." Nature 414(6859): 105-11.

Reynolds, P. N., K. R. Zinn, et al. (2000). "A targetable, injectable adenoviral vector for selective gene delivery to pulmonary endothelium in vivo." Mol Ther 2(6): 562-78.

Ribacka, C. and A. Hemminki (2008). "Virotherapy as an approach against cancer stem cells." Curr Gene Ther 8(2): 88-96.

Ribacka, C., S. Pesonen, et al. (2008). "Cancer, stem cells, and oncolytic viruses." Ann Med 40(7): 496-505.
Rich, J. N. (2007). "Cancer stem cells in radiation resistance." Cancer Res 67(19): 8980-4.

Ries, S. and W. M. Korn (2002). "ONYX-015: mechanisms of action and clinical potential of a replication-selective adenovirus." Br J Cancer 86(1): 5-11.

Rodriguez, R., E. R. Schuur, et al. (1997). "Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells." Cancer Res 57(13): 2559-63.

Rogulski, K. R., S. O. Freytag, et al. (2000). "In vivo antitumor activity of ONYX-015 is influenced by p53 status and is augmented by radiotherapy." Cancer Res 60(5): 1193-6.

Rogulski, K. R., M. S. Wing, et al. (2000). "Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization." Hum Gene Ther 11(1): 67-76.

Russell, W. C. (2000). "Update on adenovirus and its vectors." J Gen Virol 81(Pt 11): 2573-604.

Russell, W. C. (2009). "Adenoviruses: update on structure and function." J Gen Virol 90(Pt 1): 1-20.

Sarkioja, M., S. Pesonen, et al. (2008). "Changing the adenovirus fiber for retaining gene delivery efficacy in the presence of neutralizing antibodies." Gene Ther 15(12): 921-9.

Saukkonen, K. and A. Hemminki (2004). "Tissue-specific promoters for cancer gene therapy." Expert Opin Biol Ther 4(5): 683-96.

Savontaus, M. J., B. V. Sauter, et al. (2002). "Transcriptional targeting of conditionally replicating adenovirus to dividing endothelial cells." Gene Ther 9(14): 972-9.

Shao, R., D. Karunagaran, et al. (1997). "Inhibition of nuclear factor-kappaB activity is involved in E1A-mediated sensitization of radiation-induced apoptosis." J Biol Chem 272(52): 32739-42.

Shayakhmetov, D. M., A. Gaggar, et al. (2005). "Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity." J Virol 79(12): 7478-91.

Shayakhmetov, D. M., Z. Y. Li, et al. (2004). "Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors." J Virol 78(10): 5368-81.

Shen, B. H., M. Bauzon, et al. (2006). "The effect of hypoxia on the uptake, replication and lytic potential of group B adenovirus type 3 (Ad3) and type 11p (Ad11p)." Gene Ther 13(12): 986-90.

Shen, Y. (2006). "Viral vectors and their applications." In Fields Virology, D. M. Knipe, ed. (Lippincott Williams & Wilkins), pp. 539-564.

Sherr, C. J. (1996). "Cancer cell cycles." Science 274(5293): 1672-7.

Sinkovics, J. and J. Horvath (1993). "New developments in the virus therapy of cancer: a historical review." Intervirology 36(4): 193-214.

Sinkovics, J. G. and J. C. Horvath (2000). "Newcastle disease virus (NDV): brief history of its oncolytic strains." J Clin Virol 16(1): 1-15.

Smith, T. A., N. Idamakanti, et al. (2003). "Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice." Hum Gene Ther 14(8): 777-87.

Smyth, J. W., M. N. Fleeton, et al. (2005). "Treatment of rapidly growing K-BALB and CT26 mouse tumours using Semliki Forest virus and its derived vector." Gene Ther 12(2): 147-59.

Southam, C. M. and A. E. Moore (1952). "Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus." Cancer 5(5): 1025-34.

Stewart, P. L., C. Y. Chiu, et al. (1997). "Cryo-EM visualization of an exposed RGD epitope on adenovirus that escapes antibody neutralization." EMBO J 16(6): 1189-98.

Stewart, P. L., S. D. Fuller, et al. (1993). "Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy." EMBO J 12(7): 2589-99.

Stevenson, S. C., M. Rollence, et al. (1995). "Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain." J Virol 69(5): 2850-7.

Stoff-Khalili, M. A., A. Stoff, et al. (2005). "Gene transfer to carcinoma of the breast with fiber-modified adenoviral vectors in a tissue slice model system." Cancer Biol Ther 4(11): 1203-10.

Stone, D., Y. Liu, et al. (2007). "Adenovirus-platelet interaction in blood causes virus sequestration to the reticuloendothelial system of the liver." J Virol 81(9): 4866-71.

Stubdal, H., N. Perin, et al. (2003). "A prodrug strategy using ONYX-015-based replicating adenoviruses to deliver rabbit carboxylesterase to tumor cells for conversion of CPT-11 to SN-38." Cancer Res 63(20): 6900-8.

Suzuki, K., J. Fueyo, et al. (2001). "A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency." Clin Cancer Res 7(1): 120-6.

Tanner, M., A. I. Kapanen, et al. (2004). "Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer." Mol Cancer Ther 3(12): 1585-92.

Tao, N., G. P. Gao, et al. (2001). "Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver." Mol Ther 3(1): 28-35.

Tekant, Y., J. Davydova, et al. (2005). "Oncolytic adenoviral therapy in gallbladder carcinoma." Surgery 137(5): 527-35.

Templeton, N. S. (2009). "Gene and cell therapy. Therapeutic mechanisms and strategies." CRC Press 3rd edition.

Thorne, S. H., T. H. Hwang, et al. (2005). "Vaccinia virus and oncolytic virotherapy of cancer." Curr Opin Mol Ther 7(4): 359-65.

Thorne, S. H., R. S. Negrin, et al. (2006). "Synergistic antitumor effects of immune cell-viral biotherapy." Science 311(5768): 1780-4.

Thorne, S. H., B. Y. Tam, et al. (2006). "Selective intratumoral amplification of an antiangiogenic vector by an oncolytic virus produces enhanced antivascular and anti-tumor efficacy." Mol Ther 13(5): 938-46.

Tomko, R. P., R. Xu, et al. (1997). "HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses." Proc Natl Acad Sci U S A 94(7): 3352-6.

Toth, K., H. Djeha, et al. (2004). "An oncolytic adenovirus vector combining enhanced cell-to-cell spreading, mediated by the ADP cytolytic protein, with selective replication in cancer cells with deregulated wnt signaling." Cancer Res 64(10): 3638-44.

Tsai, V., D. E. Johnson, et al. (2004). "Impact of human neutralizing antibodies on antitumor efficacy of an oncolytic adenovirus in a murine model." Clin Cancer Res 10(21): 7199-206.

Tuve, S., Y. Liu, et al. (2009). "In situ adenovirus vaccination engages T effector cells against cancer." Vaccine.

Uil, T. G., T. Seki, et al. (2003). "Generation of an adenoviral vector containing an addition of a heterologous ligand to the serotype 3 fiber knob." Cancer Gene Ther 10(2): 121-4.

Waddington, S. N., J. H. McVey, et al. (2008). "Adenovirus serotype 5 hexon mediates liver gene transfer." Cell 132(3): 397-409.

Walters, R. W., P. Freimuth, et al. (2002). "Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape." Cell 110(6): 789-99.

Walther, W., J. Wendt, et al. (1997). "Employment of the mdr1 promoter for the chemotherapy-inducible expression of therapeutic genes in cancer gene therapy." Gene Ther 4(6): 544-52.

Van Dyke, T. A. (1994). "Analysis of viral-host protein interactions and tumorigenesis in transgenic mice." Semin Cancer Biol 5(1): 47-60.

Wang, G., J. W. Barrett, et al. (2006). "Infection of human cancer cells with myxoma virus requires Akt activation via interaction with a viral ankyrin-repeat host range factor." Proc Natl Acad Sci U S A 103(12): 4640-5.

Wang, G., G. Li, et al. (2009). "E1B 55-kDa deleted, Ad5/F35 fiber chimeric adenovirus, a potential oncolytic agent for B-lymphocytic malignancies." J Gene Med.

Wang, K., S. Huang, et al. (1998). "Adenovirus internalization and infection require dynamin." J Virol 72(4): 3455-8.

Wang, Y., S. A. Xue, et al. (2005). "Virus-associated RNA I-deleted adenovirus, a potential oncolytic agent targeting EBV-associated tumors." Cancer Res 65(4): 1523-31.

Varghese, R., Y. Mikyas, et al. (2004). "Postentry neutralization of adenovirus type 5 by an antihexon antibody." J Virol 78(22): 12320-32.

Varghese, S. and S. D. Rabkin (2002). "Oncolytic herpes simplex virus vectors for cancer virotherapy." Cancer Gene Ther 9(12): 967-78.

Varnavski, A. N., R. Calcedo, et al. (2005). "Evaluation of toxicity from high-dose systemic administration of recombinant adenovirus vector in vector-naive and pre-immunized mice." Gene Ther 12(5): 427-36.

Wickham, T. J., P. Mathias, et al. (1993). "Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment." Cell 73(2): 309-19.

Wickham, T. J., E. Tzeng, et al. (1997). "Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins." J Virol 71(11): 8221-9.

Vigne, E., J. F. Dedieu, et al. (2003). "Genetic manipulations of adenovirus type 5 fiber resulting in liver tropism attenuation." Gene Ther 10(2): 153-62.

Viitala, R., M. Jokinen, et al. (2007). "Mechanistic studies on release of large and small molecules from biodegradable SiO2." Int J Pharm 336(2): 382-90.

Wilkinson, G. W. and A. Akrigg (1992). "Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector." Nucleic Acids Res 20(9): 2233-9.

Vlachaki, M. T., A. Hernandez-Garcia, et al. (2002). "Impact of preimmunization on adenoviral vector expression and toxicity in a subcutaneous mouse cancer model." Mol Ther 6(3): 342-8.

Worgall, S., G. Wolff, et al. (1997). "Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration." Hum Gene Ther 8(1): 37-44.

Wu, H., T. Seki, et al. (2002). "Double modification of adenovirus fiber with RGD and polylysine motifs improves coxsackievirus-adenovirus receptor-independent gene transfer efficiency." Hum Gene Ther 13(13): 1647-53.

Xu, Z., J. Tian, et al. (2008). "Clearance of adenovirus by Kupffer cells is mediated by scavenger receptors, natural antibodies, and complement." J Virol 82(23): 11705-13.

Yamamoto, M., R. Alemany, et al. (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(3): 385-94.

Yamamoto, M., J. Davydova, et al. (2003). "Infectivity enhanced, cyclooxygenase-2 promoter-based conditionally replicative adenovirus for pancreatic cancer." Gastroenterology 125(4): 1203-18.

Yang, Y., Q. Li, et al. (1995). "Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses." J Virol 69(4): 2004-15.

Yew, P. R. and A. J. Berk (1992). "Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein." Nature 357(6373): 82-5.

Yotnda, P., C. Zompeta, et al. (2004). "Comparison of the efficiency of transduction of leukemic cells by fiber-modified adenoviruses." Hum Gene Ther 15(12): 1229-42.

You, L., C. T. Yang, et al. (2000). "ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients." Cancer Res 60(4): 1009-13.

Yu, D. C., Y. Chen, et al. (2001). "Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel." Cancer Res 61(2): 517-25.

Yu, W. and H. Fang (2007). "Clinical trials with oncolytic adenovirus in China." Curr Cancer Drug Targets 7(2): 141-8.

Zaiss, A. K., H. B. Machado, et al. (2009). "The influence of innate and pre-existing immunity on adenovirus therapy." J Cell Biochem 108(4): 778-90.

Zhang, Z., W. Zou, et al. (2005). "Suppression of tumor growth by oncolytic adenovirus-mediated delivery of an antiangiogenic gene, soluble Flt-1." Mol Ther 11(4): 553-62.

Zheng, S., I. V. Ulasov, et al. (2007). "Fiber-knob modifications enhance adenoviral tropism and gene transfer in malignant glioma." J Gene Med 9(3): 151-60.

Zhu, H., Y. Su, et al. (2007). "Immune analysis on mtHSV mediated tumor therapy in HSV-1 seropositive mice." Cancer Biol Ther 6(5): 724-31.