

ADENOVIRAL GENE THERAPY FOR OVARIAN CANCER

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The most beautiful thing we can experience is the mysterious.

It is the source of all true art and science.

Albert Einstein (1879-1955)

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LIST OF ORIGINAL PUBLICATIONS

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

- I Kanerva A, Mikheeva GV, Krasnykh V, Coolidge CJ, Lam JT, Mahasreshti PJ, Barker SD, Straughn M, Barnes MN, Alvarez RD, Hemminki A, Curiel DT. Targeting Adenovirus to the Serotype 3 Receptor Increases Gene Transfer Efficiency to Ovarian Cancer Cells. *Clin. Cancer Res.*, 8: 275-280, 2002.
- II Kanerva A, Wang M, Bauerschmitz GJ, Lam JT, Desmond RA, Bhoola SM, Barnes MN, Alvarez RD, Siegal GP, Curiel DT, Hemminki A. Gene Transfer to Ovarian Cancer versus Normal Tissues with Fiber Modified Adenoviruses. *Mol. Ther.*, 5: 695-704, 2002.
- III Kanerva A, Zinn KR, Chaudhuri TR, Lam JT, Suzuki K, Uil TG, Hakkarainen T, Bauerschmitz GJ, Wang M, Liu B, Cao Z, Alvarez RD, Curiel DT, Hemminki A. Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor targeted oncolytic adenovirus. *Mol. Ther.*, 8: 449-458, 2003.
- IV Kanerva A, Zinn KR, Peng K-W, Chaudhuri TR, Desmond RA, Wang M, Takayama K, Hakkarainen T, Curiel DT, Hemminki A. Non-invasive dual modality *in vivo* monitoring of the persistence and potency of a tumor targeted conditionally replicating adenovirus. *Submitted*.

ABBREVIATIONS

Ad3	adenovirus serotype 3
Ad5	adenovirus serotype 5
ADP	adenoviral death protein
AFP	α -fetoprotein
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATCC	American Type Culture Collection
CAR	coxsackie-adenovirus receptor
CCD	charge-coupled device
CD	cytosine deaminase
cGMP	current good manufacturing practice
cox-2	cyclooxygenase-2
CR2	constant region 2
CRAd	conditionally replicating adenovirus
C-terminal	carboxy-terminal
CTL	cytotoxic T-lymphocyte
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF2	basic fibroblast growth factor
FITC	fluorescein isothiocyanate
GM	growth medium
GCV	ganciclovir
hCEA	human carcinoembryogenic antigen
H&E	hematoxylin-eosin
6-His	six histidine amino acid residue
HSG	heparan sulfate glycosaminoglycan
HSV-TK	herpes simplex virus type I thymidine kinase
hTERT	human telomerase reverse transcriptase
IFN	interferon
Ig	immunoglobulin
i.p.	intraperitoneal
i.v.	intravenous
kb	kilobase
kD	kiloDalton
luc	firefly luciferase
MHC I	major histocompatibility complex I
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
pfu	plaque forming unit
PKR	protein kinase R
PSA	prostate specific antigen
Rb	retinoblastoma
RGD(-4C)	arginine-glycine-aspartic acid
s.c.	subcutaneous
sCAR	soluble coxsackie-adenovirus receptor
SCCHN	squamous cell carcinoma of the head and neck
SLPI	secretory leukoprotease inhibitor
TAG-72	tumor associated glycoprotein 72
TSP	tumor/tissue specific promoter
UTR	untranslated region
VA RNA	virus associated RNA
vp	viral particle

ABSTRACT

Most cases of cancer, when detected at an advanced stage, cannot be cured with conventional therapeutic modalities. Therefore, novel approaches such as gene therapy are needed. Nevertheless, while the safety record of gene therapy for cancer has been excellent, clinical efficacy has so far been limited. Moreover, it has become evident that clinical efficacy is directly determined by gene delivery efficacy. Most adenoviruses used for gene therapy have been based on serotype 5 (Ad5). Unfortunately, expression of the primary receptor for Ad5 (coxsackie-adenovirus receptor, CAR) is highly variable on ovarian and other cancer cells, resulting in resistance to infection. Consequently, various strategies have been evaluated to modify adenovirus tropism in order to circumvent CAR deficiency, including retargeting complexes or genetic capsid modifications.

By performing genetic fiber pseudotyping, we created Ad5/3 chimeric vectors possessing the receptor binding fiber knob protein from serotype 3 in the otherwise Ad5 derived capsid. This genetically modified virus is therefore retargeted to the distinct but currently unknown Ad3 receptor. We found high expression of the Ad3 receptor on ovarian cancer cells, and importantly, this correlated with the enhanced infectivity of the target cells as compared to a virus with the serotype 5 capsid.

Further, the murine liver toxicity, blood clearance and biodistribution profiles were comparable to Ad5. These might be favorable features, as Ad5 based viruses have displayed excellent safety profile in clinical cancer gene therapy trials. Importantly, Ad5/3 chimerism could be useful as a means for circumventing preexisting neutralizing anti-adenovirus antibodies present in serum and ascites fluid.

To further improve tumor penetration and local amplification on the anti-tumor effect, selectively oncolytic agents, *i.e.* conditionally replicating adenoviruses (CRAds), have been constructed. Infection of tumor cells results in replication, oncolysis, and subsequent release of the virus progeny. Normal tissue is spared due to lack of replication. Further, these viruses are potentially attractive therapeutics for cancer because they selectively amplify the input treatment dose in target cells. However, the oncolytic potency of replicating agents is directly determined by their capability of infecting target cells.

Thus, we created Ad5/3- Δ 24, the first selectively oncolytic adenovirus which does not bind CAR. This virus has a 24-bp deletion in the E1A gene, and therefore, the expressed E1A protein is unable to bind retinoblastoma (Rb) protein, which normally allows S-phase entry, needed for virus replication. Thus, this virus preferentially replicates in and lyses cells with an inactive Rb/p16 pathway. Ad5/3 chimerism resulted in dramatically enhanced infectivity of ovarian cancer cells,

which translated into increased oncolysis of target cells. Replication was also analyzed with quantitative PCR on three-dimensional primary tumor cell spheroids purified fresh from patient samples. Further, the anti-tumor efficacy in orthotopic animal models of ovarian cancer was impressive. Finally, Ad5/3- Δ 24 achieved a significant anti-tumor effect as assessed by non-invasive, *in vivo* bioluminescence imaging.

Next, we hypothesized that a CRAd expressing an inert soluble protein, which could be monitored in growth medium or serum, might give information about the persistence or antitumor efficacy of CRAds. Consequently, we constructed Ad5/3- Δ 24-hCEA, which features a secreted marker protein, soluble human carcinoembryonic antigen (hCEA). We found that virus replication closely correlated with hCEA secretion. Further, antitumor efficacy and persistence of the virus could be deduced from plasma hCEA levels. Finally, using *in vivo* bioluminescence imaging, we were able to detect effective tumor cell killing by the virus, which led to enhanced therapeutic efficacy and increased survival of mice with disseminated ovarian cancer.

The preclinical therapeutic efficacy of Ad5/3- Δ 24 is improved over the respective CAR-binding controls. Taken together with promising biodistribution and toxicity data, this approach could translate into successful clinical interventions for ovarian cancer patients.

REVIEW OF THE LITERATURE

1. Introduction

Adenovirus-mediated gene therapy has been proposed as a treatment alternative for advanced cancers refractory to traditional therapies (Russell. 2000). Adenoviruses are attractive vectors for cancer due to their unparalleled capacity for gene transfer and stability *in vivo*. Also, the production of high titers of current good manufacturing practices (cGMP) quality adenoviruses is well established. From a safety standpoint, it is important that the wild-type virus usually causes only mild, well characterized human disease. Additionally, adenoviruses have been well studied since their description in the 1950s, and in contrast to many other types of viruses proposed for utilization in gene therapy, we have a reasonable understanding of adenovirus biology (Russell. 2000). Further, adenovirus DNA is not integrated into the host genome, and the risk of insertional mutagenesis is low. Adenovirus cannot infect oocytes, and therefore, no female germ-line transduction has been noted (Gordon. 2001).

The human adenovirus is a non-enveloped icosahedral particle that encapsulates a 36 kb double-stranded DNA genome (Figure 1a). Hexon is the most abundant structural protein. Penton base units are located at each of the twelve vertices of the capsid, and the twelve fiber homo-trimers protrude from the penton bases. Hexon appears to play a structural role as a coating protein, while the penton base and fiber are responsible for cellular attachment and internalization. As shown in Figure 1b, initial high-affinity binding of the most common adenoviral gene therapy vector, serotype 5 (Ad5), occurs via direct binding of the fiber knob domain to the primary receptor, the coxsackie-adenovirus receptor (CAR) (Bergelson et al. 1997; Tomko et al. 1997). Cell surface binding is followed by internalization of the virus, mediated by the interaction of a penton base arginine-glycine-aspartic acid (RGD) motif and cellular $\alpha_v\beta$ integrins (Wickham et al. 1993), which triggers endocytosis of the virion via clathrin-coated pits (Wang et al. 1998). In the endosome, the virus is disassembled followed by endosomal lysis. Thereafter, viral DNA is transported to the nucleus through a microtubule-mediated process, and viral genes are expressed (Leopold et al. 2000). The adenoviral genome can be divided into immediately early (*E1A*), early (*E1B*, *E2*, *E3*, *E4*), intermediate (*IX*, *IVa2*) and late genes (Figure 2). The latter encode structural proteins, while the early genes encode mainly regulatory proteins that prepare the host cell for virus DNA replication and block antiviral mechanisms. The E1A protein and its binding to the retinoblastoma (Rb) protein leads to the release of E2F family transcription factors, which force the host cell into S-phase. Further, to prevent apoptosis the adenovirus utilizes E1B proteins. Proteins encoded from the *E3* region regulate host immune responses and enhances cell lysis and release of virus progeny.

Recently, it has been suggested that the binding of adenovirus to its primary receptor may be an important rate-limiting step for gene transfer, *i.e.* lack of CAR could make target tissues refractory (Bauerschmitz et al. 2002a; Hemminki and Alvarez. 2002a; Miller et al. 1998; Zabner et al. 1997; Seidman et al. 2001; Okegawa et al. 2001; Cohen et al. 2001; Shayakhmetov et al. 2002; Li et al. 1999; Cripe et al. 2001; Nalbantoglu et al. 1999; You et al. 2001). Indeed, recent data suggests that although CAR is expressed ubiquitously on most normal epithelial cells, CAR expression in tumors may be highly variable resulting in resistance to Ad5 infection (Bauerschmitz et al. 2002a; Miller et al. 1998; Shayakhmetov et al. 2002; Li et al. 1999; Cripe et al. 2001; Dmitriev et al. 1998; Kasono et al. 1999; Hemmi et al. 1998; Rauen et al. 2002). Specifically, resistance may be due to low expression levels, or aberrant localization at the cellular or tissue level (Anders et al. 2003a). Further, there may be inverse correlation with tumor grade, and also between activity of the RAS/MAPK pathway and CAR expression (Anders et al. 2003b). The function of CAR is not fully understood, but its localization in the tight junctions suggests a role in cell adhesion (Cohen et al. 2001). Interestingly, it might have tumor suppressing activity (Okegawa et al. 2001).

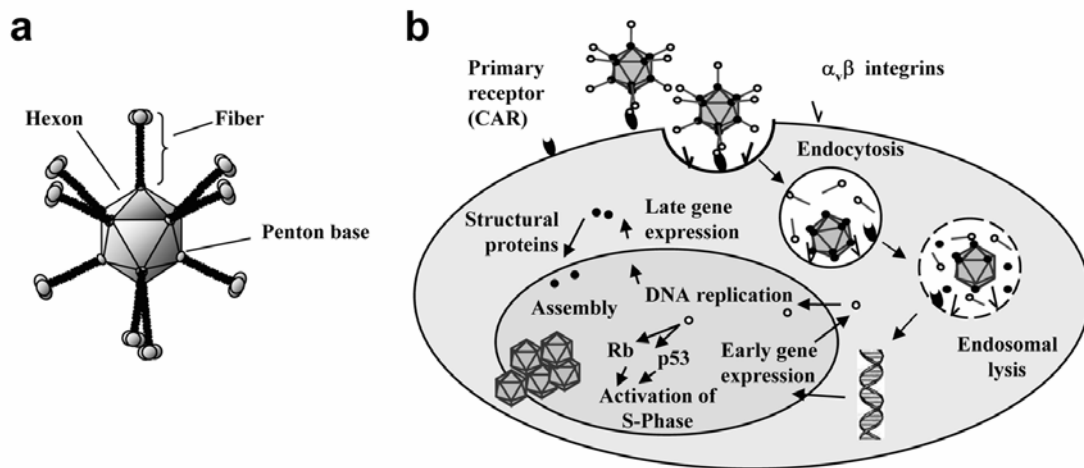


Figure 1. a) Adenovirus virion. Major structural components of a wild-type Ad5 are shown. Adenovirus capsid contains up to 36 kb double-stranded DNA genome. b) Adenovirus infection pathway. Cell entry is initiated by high-affinity binding of the fiber knob domain to its primary receptor, CAR. CAR-binding is followed by endocytosis mediated by penton base RGD interaction with cellular α, β integrins. After endosomal lysis, viral DNA is transported to the nucleus through a microtubule-mediated process, and viral genes or transgenes are expressed.

Despite exciting preclinical data, adenoviral cancer gene therapy approaches utilizing various strategies have yet to display definitive clinical breakthroughs. In general, this result has been attributed to insufficient transduction of tumor cells. Understanding of the adenoviral cellular entry pathway gave rise to the strategy of transductional targeting. Consequently, various approaches

have been evaluated to modify adenovirus tropism in order to circumvent CAR deficiency, including retargeting complexes or genetic capsid modifications. Further, certain chemical agents have shown to increase CAR expression *in vitro* and *in vivo*. Specifically, these reagents affect cell cycle or cell adhesion (Hemminki et al. 2003a).

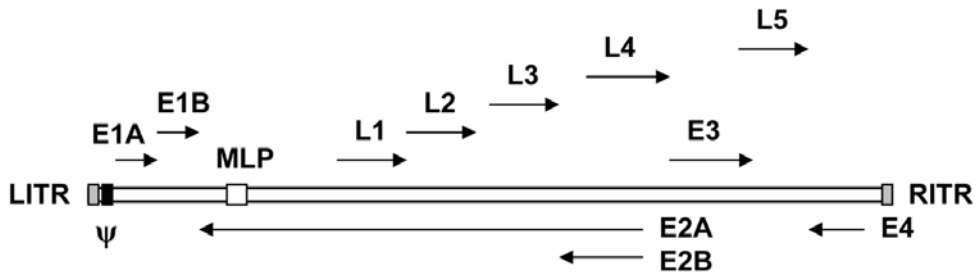


Figure 2. Generalized map of adenovirus genome. Adenovirus transcription can be defined largely as a two-phase event, early and late. The early genes (E1-E4) regulate adenovirus transcription, induct cell growth, prevent apoptosis, modulate the host immune response to adenovirus infection and promote virion release. Late phase gene expression is driven primarily by the major late promoter. A complex series of splicing events results in five gene clusters (L1-L5) encoding mostly virion proteins. LITR = left inverted terminal repeat, Ψ = packaging signal, MLP = major late promoter, RITR = right inverted terminal repeat.

However, adenovirus biodistribution is not determined by CAR expression alone. In fact, intravascular adenovirus delivery results in accumulation mainly in the liver, spleen, heart, lung and kidneys of mice, although these tissues may not necessarily be the highest in CAR expression (Fechner et al. 1999). Instead, the degree of blood flow, and the structure of the vasculature in each organ probably contribute to the biodistribution. Importantly, tissue macrophages, such as Kupffer cells of the liver, have a major role in clearing adenovirus from blood (Worgall et al. 1997; Alemany et al. 2000). This is a non-CAR mediated process, and results in rapid blood-clearance and virus degradation. However, Kupffer cells can be saturated with *ca.* $1-2 \times 10^{10}$ viral particles (vp) in mice, and thereafter virus delivery to other organs is increased (Tao et al. 2001). Alternatively, Kupffer cells can be depleted. It is currently unknown, if these aspects are similar in humans (Worgall et al. 1997; Alemany et al. 2000; Wolff et al. 1997).

Finally, adenovirus may be able to utilize other cellular receptors besides CAR and $\alpha_v\beta$ integrins for cell entry. Recent studies have suggested that major histocompatibility complex I (MHC I) (Hong et al. 1997) and heparan sulfate glycosaminoglycans (HSG) (Dechecchi et al. 2001; Smith et al. 2003) may be involved in virus binding. Importantly, HSG-binding by the fiber shaft Lysine-Lysine-

Threonine-Lysine (KKTK) motif might influence *in vivo* hepatocyte transduction (Smith et al. 2003).

2. Transductionally targeted adenoviruses

Transductional targeting of adenovirus aims at enhanced or specific transduction of the target cell. The goal is to delete the broad tropism of Ad5 towards normal epithelial cells, and/or enhance virus infectivity of CAR-deficient tumor cells. Strategies to redirect adenovirus binding to other cellular receptors than CAR include bispecific molecules that block the interaction with CAR and redirects the virus to a novel receptor (Figure 3b), and genetic retargeting, in which the virus particle is genetically modified (Figure 3c).

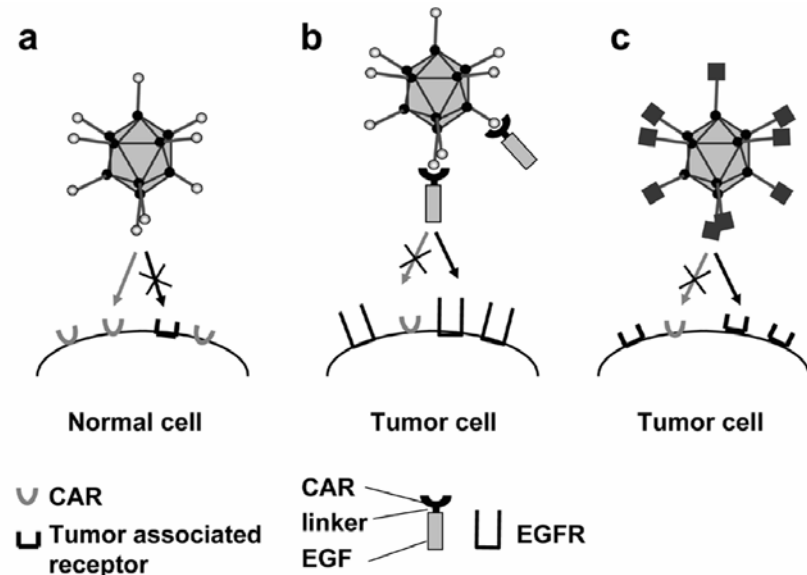


Figure 3. Transductional retargeting. **a)** Ad5 binds to CAR in the normal cells. **b)** Adapter molecule can retarget the adenovirus binding to the alternative receptor. sCAR-EGF fusion protein targets the binding to EGFR overexpressed in many cancers (Hemminki et al. 2001a). **c)** Receptor binding knob domain can be genetically modified to achieve CAR-independent binding to a tumor associated receptor.

2.1. Conjugate-based retargeting

In the two-component conjugate-based strategy, adapters typically have been designed to have specific recognition for the knob domain of the adenovirus fiber. Various chemical conjugates between the Fab fragment of an anti-knob monoclonal antibody and natural ligands specific for cell surface receptors [folate (Douglas et al. 1996) or basic fibroblast growth factor (FGF2) (Goldman et

al. 1997; Rancourt et al. 1998; Gu et al. 1999; Printz et al. 2000)] have been used successfully. Also, anti-receptor antibodies [anti-epidermal growth factor receptor (anti-EGFR) (Miller et al. 1998), anti-epithelial cellular adhesion molecule (anti-EpCAM) (Haisma et al. 1999), anti-tumor associated glycoprotein 72 (TAG-72) (Kelly et al. 2000), anti-CD40 (Tillman et al. 2000)] have been conjugated to anti-knob Fab.

The initial *in vitro* proof of principle study was performed by Douglas *et al.*, with a Fab-folate conjugate. This retargeting resulted in CAR-independent, folate receptor-mediated transduction of cancer cells highly expressing the folate receptor (Douglas et al. 1996). A Fab-FGF2 adapter was utilized to target adenoviruses to FGF receptor-positive ovarian cancer cells *in vitro*. It was also used to retarget herpes simplex virus type I thymidine kinase (HSV-TK) expressing adenovirus to ovarian cancer cells, resulting in enhanced survival of ovarian cancer xenograft-bearing mice (Rancourt et al. 1998). These findings led to a clinical trial protocol for intraperitoneal (i.p.) treatment of ovarian cancer patients with peritoneally disseminated disease with a Fab-FGF2 targeted adenovirus coding for HSV-TK (A Hemminki, personal communication). Importantly, the antibody mediated targeting method has been evaluated *in vivo* with systemic administration, utilizing a bispecific antibody between the adenovirus fiber and angiotensin-converting enzyme (Reynolds et al. 2000). This vector showed a more than 20-fold increase in transgene expression and virus DNA localization in the lungs. Finally, bispecific antibodies have been designed to bind to FLAG-modified penton base (Wickham et al. 1996) or hexon protein (Yoon et al. 2000) instead of knob.

Recent studies have utilized a truncated, soluble form of CAR (sCAR) as a knob-binding component in a recombinant fusion molecule consisting of sCAR fused to epidermal growth factor (EGF) (Dmitriev et al. 2000) (Figure 3b). Up to a 9-fold increase in reporter gene expression was achieved in several EGFR-overexpressing cancer cell lines compared to untargeted adenovirus *in vitro* (Dmitriev et al. 2000). To further increase adenovirus-sCAR-ligand complex stability, a trimeric sCAR-fibritin-anti-*erbB2* single chain antibody targeting molecule was created, and it increased gene transfer in c-*erbB2*-positive breast and ovarian cancer cell lines *in vitro* (Kashentseva et al. 2002).

2.2. Genetic targeting strategies

Two-component retargeting strategies add complexity to the system, which might be disadvantageous in clinical trials. Further, there might be variations in the amount of retargeting conjugates bound to each virion, and the stability of adenovirus-conjugate-complexes in humans is not known. Therefore, genetic modifications of the adenovirus capsid for incorporation of targeting

moieties have been created (Figure 3c). This results in homogenous population of retargeted vectors.

Wickham *et al.* modified the knob domain of the fiber protein by adding peptides to the C-terminus of the protein (Wickham *et al.* 1997) (Figure 4b). Adenoviruses with C-terminal $\alpha_v\beta$ integrin-binding RGD motifs and heparan sulfate-binding polylysine residues have yielded some promising results *in vitro* and *in vivo*, but other, larger peptides result in inefficient packaging of the virion, possibly due to inability of the modified fiber to trimerize (Wickham *et al.* 1997; Hong and Engler. 1996). After the crystal structure of the Ad5 fiber was revealed (Xia *et al.* 1994) (Figure 5), another knob location was found to possibly possess favorable features for genetic modification. There is an exposed HI loop (*i.e.* between β -strands H and I) in the knob monomer, which can tolerate peptide insertions up to 100 amino acids, often with minimal negative effects on virion integrity (Belousova *et al.* 2002) (Figure 4c). First, Krasnykh *et al.* incorporated a FLAG octapeptide to validate the strategy (Krasnykh *et al.* 1998). Subsequently, other retargeting motifs in the HI loop have been evaluated. An RGD-4C peptide (Dmitriev *et al.* 1998) enhanced the infectivity of wide range of tumor cells *in vitro* and *in vivo* (Dmitriev *et al.* 1998; Vanderkwaak *et al.* 1999; Kasono *et al.* 1999; Wesseling *et al.* 2001a; Hemminki *et al.* 2001b; Hemminki *et al.* 2002b; Grill *et al.* 2001; Cripe *et al.* 2001), as $\alpha_v\beta$ integrins are often highly expressed in tumor cells and tumor vasculature. Other targeting peptides incorporated into HI loop include Asparagine-Glycine-Arginine (NGR) (Mizuguchi *et al.* 2001) and Serine-Isoleucine-Glycine-Tyrosine-Leucine-Proline-Leucine-Proline (SIGYLPLP) (Nicklin *et al.* 2001). Of note, the combination of C-terminal (polylysine) and HI loop (RGD-4C) targeting has also been evaluated (Wu *et al.* 2002a) (Figure 4d). This double-modification achieved enhanced transduction over single-modifications.

However, mere incorporation of heterologous targeting ligands into the knob domain might result in vectors with enhanced cell transduction, as opposed to actual retargeting, since the knob domain is still able to bind CAR. Nevertheless, if ligand incorporation is combined with ablation of CAR-binding, as is a case with an endothelial cell binding SIGYLPLP (Nicklin *et al.* 2001), CAR-independent retargeting is achieved. Recently, the CAR-binding amino acid residues of the knob were identified. The binding site consists of a non-linear amino acid sequence in the AB loop, B β -sheet and DE loop, and the mutation of these residues can ablate CAR-binding (Roelvink *et al.* 1999).

The complete knob can be deleted, and replaced with a retargeting ligand (Figure 4e). However, the chimeric fiber protein must be able to assemble into a trimer, as the knob domain normally initiates trimerization (Hong and Engler. 1996), which is required for proper fiber function. Further, these chimeric fibers must be able to localize to the nucleus for capsid assembly. Magnusson *et al.* solved

the problem with an external trimerization signal, a neck region peptide (NRP) of human surfactant protein D, which had the RGD ligand attached with a linker (Magnusson et al. 2001). This modification resulted in selective infection of integrin-expressing cell lines *in vitro*. Another strategy for deknobbing used the phage T4 fibrin as a fiber protein (Krasnykh et al. 2001). The Ad5 tail and immediately proximal part of the shaft were fused with the C-terminal T4 fibrin, which is able to trimerize. A six histidine (6-His) targeting motif was fused via a short peptide linker. This retargeted adenovirus lacks the ability to interact with CAR and showed up to a 100-fold increase in reporter gene expression in cells presenting an artificial 6-His binding receptor.

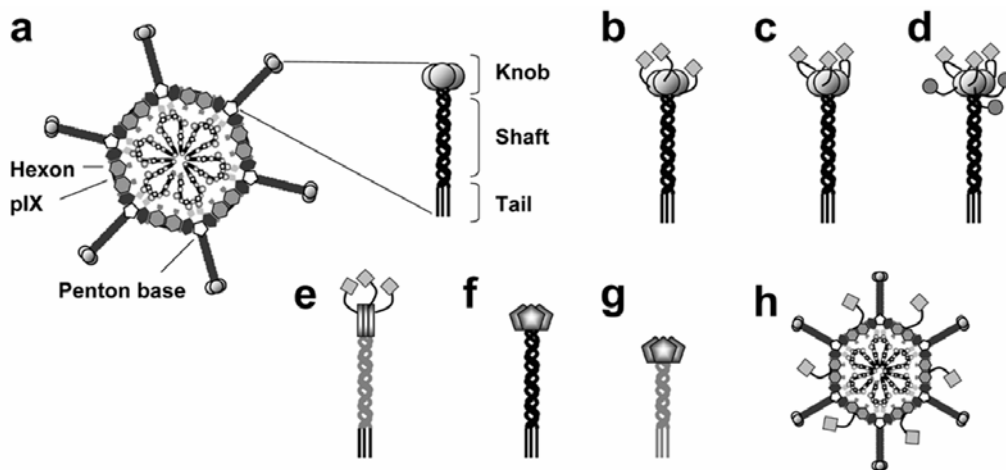


Figure 4. Genetic capsid targeting modifications. **a)** Adenovirus capsid proteins. **b)** C-terminal ligand in the fiber knob. **c)** Modification of the HI loop. **d)** The combination of C-terminus and HI loop targeting. **e)** “Deknocking”. **f)** Pseudotyping, knob chimerism. **g)** Pseudotyping, fiber chimerism. **h)** Targeting ligands in other capsid proteins (pIX, hexon or penton base).

Adenovirus fiber pseudotyping, *i.e.* alteration of the virus tropism by substituting the receptor-binding proteins (knob/fiber) with those from other serotypes, has been evaluated (Figure 4f, g). Of special interest have been Ad5 based vectors with the knob or fiber from subgroup B adenoviruses, which bind other cellular receptors than CAR (Gall et al. 1996; Shayakhmetov et al. 2000; Shayakhmetov et al. 2002; Havenga et al. 2002; Mizuguchi and Hayakawa. 2002; Krasnykh et al. 1996). Resultant vectors have displayed CAR-independent transduction and enhanced infectivity of low-CAR target cells. Initially, Karsnykh *et al.* created an Ad5 vector that expressed a chimeric fiber displaying the adenovirus serotype 3 knob domain (Ad5/3) (Krasnykh et al. 1996). Ad5/3 effectively transduced low-CAR cancer cell lines *in vitro*, whereas these cells were refractory to Ad5 infection. This knob chimerism retargeted the virus to the distinct but currently unknown Ad3 receptor (Stevenson et al. 1995). Also, other serotype chimeras have been evaluated. Specifically,

Ad5/35 chimeras displayed enhanced infectivity of cancer cells and favorable biodistribution profile (Mizuguchi and Hayakawa. 2002; Bernt et al. 2003; Shayakhmetov et al. 2002; Knaan-Shanzer et al. 2001; Havenga et al. 2002). Recent preliminary data suggests that CD46 could be the Ad35 receptor, and it might serve as a common receptor for many subgroup B adenoviruses (Gaggar et al. 2003). The CD46 membrane cofactor protein is expressed on the surface of all nucleated human cells examined to date (Liszewski et al. 1991). CD46 is a ubiquitous type-1 glycoprotein that is a member of a family of regulators of complement activation, and therefore prevents spontaneous activation of complement on autologous cells. Interestingly, CD46 has been identified as a primate-specific cellular receptor for human herpesvirus 6 (HHV-6) (Santoro et al. 1999) and vaccine strains of measles virus (Dorig et al. 1993).



Figure 5. Structure of the adenovirus fiber knob domain. The knob trimer resembles a three-bladed propeller formed by two sheets of β -strands connected with loops and turns. The exposed HI loop is circled (picture adapted from Xia et al. 1994).

Attempts to target adenoviruses have also involved genetic modifications of other capsid proteins such as penton base, hexon or pIX. Penton base wild-type RGD has been replaced with retargeting peptide (Wickham et al. 1995). Hexon hypervariable region 5 (HVR5) is a loop structure localized on the surface of the virion, and it was successfully used for incorporation of an RGD motif (Vigne et al. 1999). pIX is a minor capsid component whose function may involve stabilization. The C-terminus of pIX is exposed on the outer surface of the virus capsid. Dmitriev *et al.* incorporated a polylysine motif at the pIX C-terminus, resulting in augmented, CAR-independent gene transfer via binding to cellular heparan sulfate moieties (Dmitriev et al. 2002) (Figure 4h). Finally, serotype switching has been used to create hexon chimeras, which circumvented murine pre-existing neutralizing Ad5 antibodies (Wu et al. 2002b).

3. Transcriptionally targeted adenoviruses

Transductional targeting approaches attempt to increase vector entry into target cells while reducing gene transfer to non-target cells. In contrast, transcriptional targeting does not change the tropism of viruses but restricts gene expression to target cells. Transcriptional targeting can be achieved by placing a viral gene or transgene under control of a tissue- or tumor-specific promoter (TSP). Such viruses are able to infect various cell types, but the viral gene or transgene are expressed only in the cell types, which actively express the transcription factors needed for activity of the TSP.

Numerous TSPs have been evaluated with promising preclinical results (Bauerschmitz et al. 2002a). For example, the secretory leukoprotease inhibitor (SLPI) gene is expressed in several different carcinomas. Its expression in normal organs, such as the liver, is low (Abe et al. 1997). Therefore, the SLPI promoter was utilized to drive transgene expression in ovarian cancer cell lines and primary tumor cells. The promoter retained its fidelity in an adenovirus context. Further, a murine orthotopic model of peritoneally disseminated ovarian cancer was used to demonstrate high tumor gene expression *versus* low liver expression with the SLPI promoter, and that adenovirus-delivered HSV-TK under the control of the SLPI promoter was able to increase survival (Barker et al. 2003a).

The cyclooxygenase-2 (cox-2) promoter has been explored in the context of gastric cancer, pancreatic carcinoma and ovarian cancer (Barker et al. 2003a; Yamamoto et al. 2001; Wesseling et al. 2001b; Casado et al. 2001). The cox-2 promoter was active in a panel of epithelial cancer cell lines and primary cells. Furthermore, its activity was low in liver and normal mesothelial cells, which may be important from a safety standpoint.

Gene expression from certain promoters can be regulated with radiation. For example, the early growth response gene 1 (*egr-1*) enhancer/promoter, which is radiation inducible, has been used as a TSP for specific expression of HSV-TK in glioma cells (Manome et al. 1998). Another regulation strategy is the use of hypoxia-inducible promoters (Binley et al. 2003), and finally, a combination of these two modalities (Greco et al. 2002). Further, regulation can be achieved with chemically inducible promoters. For example, a tetracycline-activated promoter can be used to regulate gene expression and subsequent protein production by oral tetracycline (Fechner et al. 2003). Withdrawal of the drug rapidly abrogates gene expression.

4. Double-targeted adenoviruses

TSPs can reduce the side effects of cancer gene therapy approaches, as transgene/viral gene expression is restricted to cells capable of activating the TSP, which thereby mitigates toxicity to normal cells. As the liver is the organ responsible for uptake and degradation of the majority of

adenovirus, regardless of the route of administration, it is also the most relevant organ with regard to evaluation of a candidate TSP. This is compounded by the Pennsylvania gene therapy fatality, which was probably caused by a Kupffer cells launching an aberrant hyperimmune reaction to a very large dose of adenovirus delivered via the hepatic artery into a liver already damaged by the underlying disease (Raper et al. 2003). Nevertheless, it is remarkable that more than 1000 cancer patients have been treated with adenovirus, without mortality attributable to the treatment (Hemminki and Alvarez. 2002a). Transductional and transcriptional targeting can be combined to create double-targeted viruses. Conceivably, this approach could be synergistic with regard to safety and efficacy. Double targeting for ovarian cancer has been achieved *in vitro* and *in vivo*. Transductional targeting with a sCAR-fibritin-anti-*erbB2* adapter was able to increase gene transfer to target cells while reducing transduction of non-target cells (Kashentseva et al. 2002). When combined with transcriptional targeting with the SLPI promoter, an increase in selectivity was seen (Barker et al. 2003b). Reynolds *et al.* combined the conjugate-based pulmonary vascular targeting, *i.e.* the bispecific antibody between the adenovirus fiber and angiotensin-converting enzyme, with the use of an endothelial cell-specific promoter *flt-1*. The dual-targeting resulted in a synergistic improvement in the specificity of transgene expression in the pulmonary target cells (over 300 000-fold improvement compared with untargeted vector) (Reynolds et al. 2001).

5. Conditionally replicating adenoviruses (CRAds)

Although chemotherapeutics and radiation therapy target various different cellular structures and pathways, most of them kill cancer cells by inducing apoptosis and are only effective for cycling cells. As malignant cells are characterized by an impressive ability to adapt to the environment, apoptosis-resistant clones frequently develop during treatment. Each cancer cell has a statistical possibility for gaining resistance and therefore the number of malignant cells directly determines the overall likelihood of relapse. Furthermore, during subsequent treatment regimens, resistance usually occurs more rapidly, and there is a tendency for cross resistance between agents. Therefore, new approaches are needed. Importantly, new agents should have novel mechanisms of action, thereby lacking cross resistance with currently available treatments. Tumor-targeted oncolytic viruses might prove useful in this regard. These viruses have a cytolytic nature, *i.e.* the replicative life cycle of the virus results in host cell destruction.

The first clinical cancer trial with replicating adenoviruses was done in 1956 with various wild-type strains (Smith et al. 1956). More recently, the increased understanding of adenovirus replication and its interactions with cellular proteins have inspired the construction of CRAds. Infection of tumor cells results in replication, oncolysis, and subsequent release of the virus progeny. Normal tissue is

spared due to lack of replication (Figure 6). Importantly, this replication cycle allows dramatic local amplification of the input dose, and in theory, a CRAd would replicate until all cancer cells are lysed. Conceivably, CRAd released from the tumor tissue might disseminate and infect distant metastases. Furthermore, the immunogenicity of adenovirus mediated tumor cell killing could be useful for eradication of distant metastases (Todo et al. 1999), and long term anti-tumor immune surveillance, but needs to be studied further.

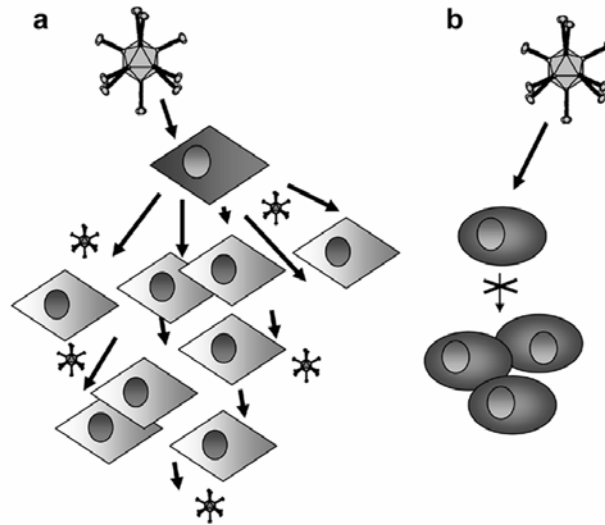


Figure 6. Conditionally replicating adenoviruses. **a)** Infection of tumor cells results in replication, cell lysis, and subsequent release of virus progeny. Importantly, replication allows local amplification of the input dose. **b)** Benign cells are spared due to lack of replication.

5.1. Deletion mutant CRAds

Type I CRAds feature loss-of-function mutations in the virus genome, which are compensated by cellular factors present in cancer but not normal cells. For example, this can be achieved by incorporating deletions in the immediately-early (*E1A*) or early (*E1B*) adenoviral genes resulting in mutant E1 proteins unable to bind the cellular proteins necessary for viral replication in normal cells, but not in cancer cells.

The first published CRAd was ONYX-015 (a virus was initially reported as dl1520), which has two mutations in the gene coding for the E1B-55 kD protein (Bischoff et al. 1996). The purpose of this protein is binding and inactivation of p53 in infected cells, for induction of S-phase, which is required for effective virus replication. Thus, this virus should only replicate in cells with an aberrant p53-p14^{ARF} pathway, a common feature in human tumors (Ries et al. 2000). While this is

still subject to debate, initial studies suggested that this agent replicates more effectively in tumor than in normal cells. However, other adenoviral proteins also have direct effects on p53 inhibition (e.g. E4orf, E1B-19kD, E1A). Further, E1B-55kD has other viral functions unrelated to p53 binding such as viral mRNA transport, which might result in inefficient replication of ONYX-015 in comparison to wild-type adenovirus (Dix et al. 2001).

Ad5- Δ 24 contains a 24-bp deletion in the constant region 2 (CR2) of *E1A*, and the modified protein is unable to bind the cellular Rb protein for induction of S-phase (Fueyo et al. 2000). Therefore, viruses with this type of deletion have reduced ability to overcome the G₁-S checkpoint and replicate efficiently only in cells where this interaction is not necessary, e.g. tumor cells defective in the Rb-p16 pathway. It has been suggested that all human cancers may be deficient in this crucial pathway (Sherr. 1996).

In normal cells, virus associated (VA) RNAs I and II inactivate the RNA-dependent protein kinase R (PKR), which otherwise would block protein translation in response to infection. However, an activated RAS/MAPK pathway can also inactivate PKR. Cascallo *et al.* studied a deletion mutant CRAd, dl331, which has a deletion in the VAI region, and therefore is unable to replicate efficiently in normal cells, but retains RAS/MAPK-dependent replication (Cascallo et al. 2003).

5.2. Promoter-inducible CRAds

In type II CRAds, TSPs replace endogenous viral promoters. This restricts viral replication to target tissues actively expressing the transcription factors that stimulate the TSP. Usually, a TSP is placed to control *E1A*, but alternatively or in addition, other early genes can also be regulated. Various promoters have been used to control viral replication. AvE1a04i, containing the E1A gene under the control of α -fetoprotein (AFP) promoter, selectively replicates in hepatocellular carcinomas that express AFP (Hallenbeck et al. 1999). The same approach was used for breast cancer with the DF3/MUC1 gene promoter (Kurihara et al. 2000), a truncated L-plastin promoter (Zhang et al. 2002) and estrogen-responsive elements from pS2 gene promoter (Hernandez-Alcoceba et al. 2000).

The IAI.3B gene encoding the B-pox protein is highly expressed in ovarian cancer cells. Hamada *et al.* utilized the IAI.3B promoter to control E1A expression (Hamada et al. 2003). The truncated L-plastin promoter was evaluated in ovarian carcinoma (Zhang et al. 2002). Also, the cox-2 promoter has been explored in the context of pancreatic carcinoma and ovarian cancer (Yamamoto et al. 2003; Kanerva et al. 2004). Telomerase activity is present in almost all human tumors. Therefore, tumor-specific human telomerase reverse transcriptase (hTERT) regulated CRAds were shown to have oncolytic activity in various types of tumors (Wirth et al. 2003).

The midkine differentiation factor promoter was used for advanced neuroblastoma and Ewing's sarcoma (Adachi et al. 2001). OV798 has the carcinoembryonic antigen (CEA) promoter driving *E1A* expression. It displayed oncolysis of CEA expressing colorectal cancer cells *in vitro* and *in vivo* (Li et al. 2003). Another interesting approach for treating colorectal cancer was described by Fuerer *et al.* They introduced Tcf transcription factor binding sites in multiple early adenovirus promoters, which therefore target the cells with activated wnt signaling pathway (Fuerer and Iggo. 2002).

Human prostate specific antigen (PSA) promoter and rat probasin promoters have been utilized for prostate cancer specific replication. CV706 has the PSA promoter and enhancer controlling *E1A* expression (Rodriguez et al. 1997), while CV787 has the rat probasin promoter controlling *E1A* and the PSA promoter and enhancer driving *E1B* (Yu et al. 1999). Further, the replication has been controlled by the noncollagenous bone matrix protein osteocalcin (OC) promoter. Ad-OC-E1a has been used for treatment of both androgen-dependent and androgen-independent prostate cancer bone metastasis (Matsubara et al. 2001).

Ahmed *et al.* described a novel CRAd, whose tumor selectivity was based on control of gene expression at the level of mRNA stability. They ligated the *cox-2* 3' untranslated region (UTR) downstream of the *E1A* gene. This results in mRNA stabilization regulated by an activated RAS/MAPK pathway (Ahmed et al. 2003). Of note, also regulatable promoters, such as hypoxia- or chemically inducible promoters have been evaluated in the context of CRAds (Cuevas et al. 2003; Fechner et al. 2003). Furthermore, CRAds can be transcriptionally targeted to dividing endothelial cells, which are present in the tumor vasculature. This offers an alternative approach to anti-angiogenic therapy for cancer (Savontaus et al. 2002).

Finally, a novel CRAd combining both type I and type II approaches featured a melanoma-specific tyrosinase promoter driving a CR2 deletion-mutated *E1A* (Nettelbeck et al. 2002). Also, this deletion has been combined to E2F1 promoter controlled *E1A* and *E4* expression (Johnson et al. 2002).

6. CRAds in combination with traditional therapeutics

Oncolytic tumor killing differs from conventional anticancer therapies, providing a possibility for additive or synergistic interactions in a multimodal antitumor approach. Further, the toxicity profiles may be different, which could result in enhanced efficacy without significantly increased adverse effects.

There are several studies suggesting enhanced cell killing activity when CRAbs and chemotherapeutics were combined. ONYX-015 has been combined *in vitro* and *in vivo* with 5-fluorouracil for treatment of squamous cell carcinoma of the head and neck (SCCHN) and colon carcinoma (Heise et al. 1997). Also, ONYX-015 was combined with 5-fluorouracil + cisplatin (SCCHN, ovarian cancer) (Heise et al. 2000), cisplatin (SCCHN) (Heise et al. 1997) and cisplatin + paclitaxel (non small cell lung cancer) (You et al. 2000). These results suggested potentially synergistic interactions with chemotherapeutics. Interestingly, Heise *et al.* suggested that the efficacy is highly dependent on the sequencing of the agents, *i.e.* simultaneous treatment or administration of the virus before 5-fluorouracil + cisplatin were superior to chemotherapy followed by virus (Heise et al. 2000). Also, the combination of prostate cancer specific CV787 and paclitaxel or docetaxel displayed synergistic efficacy both *in vitro* and *in vivo* (Yu et al. 2001).

Combined ONYX-015 and radiotherapy exhibited additive antitumor cell killing effects in glioma (Georger et al. 2003), colon cancer (Rogulski et al. 2000a) and cervical cancer (Rogulski et al. 2000b) xenograft models. Importantly, viral replication *in vitro* after radiation was not significantly inhibited. A $\Delta 24$ -based virus, Ad5- $\Delta 24$ RGD, displayed enhanced oncolysis for glioma in combination with radiation *in vitro* and *in vivo* (Lamfers et al. 2002). Further, type II CRAb CV706 exhibited synergistic antitumor efficacy in combination with radiotherapy (Chen et al. 2001).

The mechanism of additive or synergistic activity in the multimodal therapy is not known. However, few hypotheses have been suggested. First, chemotherapy or radiotherapy might augment viral replication. There are some data supporting this view (Yu et al. 2001; Chen et al. 2001), while other studies reported no significant effect on the amount of virus produced (Georger et al. 2003). Secondly, the CRAb might enhance the anti-tumor activity of chemotherapeutic agents or radiotherapy. E1A gene expression has been shown to increase cellular sensitivity to chemotherapy and radiation through both p53 dependent and independent mechanism (Sanchez-Prieto et al. 1996; Duque et al. 1998). Interestingly, certain chemotherapeutic agents have shown to increase CAR expression *in vitro* and *in vivo* on cancer cells (Hemminki et al. 2003a). Finally, each agent may be working independently on different cell populations within the tumor tissue.

7. Armed and infectivity enhanced CRAbs

To further increase the oncolytic effect, transgenes for cytokines (Bauzon et al. 2003) or prodrug-activating enzymes have been included in CRAbs. Such “armed CRAbs” couple the lytic capability of the virus with the capacity to deliver therapeutic factors into tumor cells. In the prodrug-based strategy, genes encoding prodrug activating enzyme are utilized. Common approaches include HSV-TK and *Escherichia coli* cytosine deaminase (CD), which convert systemically administrable

and relatively nontoxic prodrugs [ganciclovir (GCV) and 5-fluorocytosine, respectively] into toxic products. The activated drugs can spread into surrounding cells (local bystander effect). The HSV-TK strategy may also allow non-invasive imaging (Hemminki et al. 2002b) and abrogation of virus replication in case of toxicity. This approach has led to an enhanced antitumor effect compared to virus alone (Rogulski et al. 2000b; Freytag et al. 1998; Wildner et al. 1999; Akbulut et al. 2003; Fuerer and Iggo. 2004). However, other studies have suggested that addition of GCV did not increase oncolysis, possibly due to the inhibition of viral replication by GCV (Lambright et al. 2001). Freytag *et al.* introduced a TK/CD fusion gene in the deleted *E1B-55 kD* region of ONYX-015. Further, they combined this double suicide gene therapy to radiotherapy with encouraging results (Freytag et al. 1998).

Most published CRAds rely on CAR for entry into cells. Recently, it has been demonstrated that the oncolytic potency of replicating agents is directly determined by their capability for infecting target cells (Hemminki et al. 2001a; Douglas et al. 2001). Thus, variable CAR expression on cancer cells could hinder CRAd mediated oncolysis. Therefore, methods to circumvent CAR-deficiency and improve cell killing have been evaluated in the context of CRAds.

Ad5- Δ 24RGD features the 24-bp *E1A* CR2 deletion, and an RGD-4C modification of the fiber (Suzuki et al. 2001). The combination resulted in similar or enhanced oncolytic potency in comparison to wild-type virus in various cancer cells *in vitro* and *in vivo* (Cripe et al. 2001; Fueyo et al. 2003; Witlox et al. 2004). Further, this virus was able to replicate in ovarian cancer primary cell spheroids and resulted in significantly prolonged survival in an aggressive orthotopic ovarian cancer model (Bauerschmitz et al. 2002b; Lam et al. 2003a). Also, an *E1B-55 kD*-deleted CRAd has been modified with the heparan sulfate-binding polylysine residue at the C-terminus of the fiber (Shinoura et al. 1999). The first TSP-controlled, infectivity enhanced CRAd has recently been constructed and tested on ovarian and pancreatic cancer substrates (Yamamoto et al. 2003; Kanerva et al. 2004). Replicative specificity was achieved with the *cox-2* promoter controlling expression of *E1A*, while the fiber was modified with RGD-4C.

Targeting with adapter molecules has been tested in the context of CRAds. Co-infection of a replication-deficient virus coding sCAR-EGF and Ad5- Δ 24 resulted in enhanced oncolysis (Hemminki et al. 2001a). However, this approach did not increase infectivity as a single-component oncolytic virus, D24sCAR-EGF, which incorporates the sCAR-EGF fusion protein in the deleted *E3* region (Hemminki et al. 2003b). This suggests that the expression of biologically active adapter proteins can interfere with virus production and oncolysis. In contrast, van Beusechem *et al.* introduced a bispecific single chain (sFv) antibody 425-S11 (recognizes EGFR and the fiber knob)

into the *E3* region of Ad5- Δ 24. This secretory retargeting moiety increased the killing of CAR-deficient glioma cells *in vitro* and *in vivo* (van Beusechem et al. 2003).

8. Clinical trials with oncolytic viruses

8.1. Other oncolytic viruses

Both naturally occurring and genetically engineered oncolytic viruses have been described. Specifically, it has been suggested that some viruses, such as reovirus and Newcastle disease virus, might have intrinsic selectivity for replication in tumors. In contrast, to achieve tumor selective replication and subsequent oncolysis, HSV-1, vaccinia and adenovirus (CRAd) can be genetically attenuated to preferentially replicate in malignant cells.

Reovirus and Newcastle disease viruses are RNA viruses. During their replication cycle, double-stranded RNA, a stimulator of PKR, is formed. PKR inhibits protein synthesis and promotes apoptosis, thereby controlling the spread of the virus infection. Double-stranded RNA can also stimulate release of interferons (IFNs), which activate PKR in adjacent, uninfected cells. Importantly, tumors are frequently defective in the PKR signaling pathway, allowing the replication of these viruses in malignant cells. Reoviruses are commonly isolated from the human respiratory and gastrointestinal tracts, although they seem to be nonpathogenic. Recently, it has been shown that reoviruses replicate in cancer cells with the activated RAS signaling pathway. Therefore, up to 80% of tumors might be susceptible to reovirus replication. Newcastle disease virus causes respiratory and central nervous system infections to fowl. However, to humans it is a mild pathogen causing conjunctivitis. Tissue culture-adapted strains of the virus (PV701) show potent oncolytic activity in human cancer cells, possibly due to a defect in the IFN signaling pathway.

HSV-1 and vaccinia virus are double-stranded DNA viruses. HSV-1 is a natural human pathogen that can cause recurrent oropharyngeal or genital sores. Pathogenicity is reduced by mutating one or more of the crucial virulence genes (*e.g. ICP6, γ 34.5, UL24, UL56*) resulting in replication competence only in cycling cells. Due to their neurotropism, oncolytic HSV-1 viruses were initially constructed for brain tumor therapy. Nevertheless, preclinical studies have shown efficacy against various solid tumors *in vitro* and *in vivo*. Vaccinia virus is a member of the poxvirus family. It is related to smallpox, and therefore it has been used as a smallpox vaccine. However, vaccinia is a mild pathogen, and it may cause rash, fever and body aches. Most genetically modified vaccinia viruses have the TK gene deleted, which might help to give selectivity for dividing cells. This deletion makes the virus dependent on host cell nucleotides, which are more available in dividing cells. Also other viral genes have been mutated to achieve tumor selectivity.

8.2. Clinical trials with oncolytic viruses

The most advanced clinical results are reported for CRAAd, and ONYX-015 is the most comprehensively clinically evaluated CRAAd (Table I). Safety data has been excellent, but demonstration of efficacy has been limited. ONYX-015 has been well tolerated at doses up to 2×10^{13} vp by i.p., intravenous (i.v.), intratumoral and intra-arterial routes. In a phase II study of intratumorally administered ONYX-015 in 40 patients with head and neck cancer, 3 complete and 2 partial responses were reported (Nemunaitis et al. 2001a). In contrast, when the same virus was given in combination with 5-fluorouracil and cisplatin, 27% and 36% of patients had complete and partial responses (Khuri et al. 2000). There were 11 patients with several tumors, but only the largest one was injected with the virus. Thus, the trial included internal control tumors. Only 17% of injected tumors had progressed 6 months after treatment initiation, while all the control tumors treated with chemotherapy alone had progressed. The phase III trial of this combination for patients with recurrent head and neck cancer has been started.

These results suggested that initial successful clinical applications may feature combination treatments. However, most completed trials have employed CRAAd such as ONYX-015, with low replicativity and therefore low oncolytic potency. Of note, Onyx Pharmaceuticals discontinued their therapeutic virus program in order to concentrate in the development of the small molecules (<http://www.onyx-pharm.com/onyxtech>). However, other biotechnology companies have continued cGMP quality production and research of ONYX-015 and other CRAAd. Further, single agent efficacy may be more impressive with more potent viruses. This was well demonstrated by the high rate of PSA responses in a preliminary report of a trial featuring systemic treatment of disseminated prostate cancer with CG7870 (formerly CV787) (DeWeese et al. 2003).

All completed trials have evaluated CAR-binding CRAAd. As CAR-deficiency may be a frequent phenomenon associated with carcinogenesis, this may have decreased the efficacy of approaches utilized thus far. The first trial featuring a transductionally targeted CRAAd, Ad5- Δ 24RGD (Suzuki et al. 2001), has received National Cancer Institute funding and may soon start enrolling glioma and ovarian cancer patients (A Hemminki, personal communication). Further, patient selection has a major impact on the displayed efficacy in phase I/II trials. The enrolled patients have been heavily pretreated and very often end stage patients, which may weaken the potential for detecting responses. In contrast, the patients with minimal residual disease with the goal being prevention of tumor recurrence might display significant efficacy.

Several cancer trials have been performed recently with viruses other than adenovirus (Nemunaitis. 2003a; Hermiston and Kuhn. 2002; Hawkins et al. 2002). Phase I dose-escalation trials of intratumoral injection of reovirus (Reolysin) in patients with recurrent malignant glioma and

SCCHN are in progress (Kirn et al. 2001; Shah et al. 2003). Passage attenuated Newcastle disease virus strain PV701 has been evaluated in a phase I trial of i.v. administration of involving 79 patients with advanced solid cancers that were unresponsive to standard therapy (Pecora et al. 2002). The most common side effects were we fever, chills, nausea and vomiting. There were one complete and one partial response, and 14 patients had stable disease 4 months. Vaccinia virus has been utilized for inducing antitumoral immune responses in addition to tumor selective replication. However, no objective systemic antitumoral responses were seen in patients with advanced disease (Hawkins et al. 2002; Shah et al. 2003). Furthermore, HSV-1 has been widely evaluated in patients with glioma. In two phase I studies no significant toxic effects were reported, but unfortunately, there were no responses detected. However, phase II studies are ongoing (Shah et al. 2003; Varghese and Rabkin. 2002).

All of these early phase trials with other oncolytic viruses reported good safety data, while efficacy seemed modest at best. Thus, CRAds are currently the most promising oncolytic agents. Nevertheless, armed variants of the other oncolytic viruses could improve their efficacy.

Table 1. Clinical trials with CRAds^a

Virus	Genetic alterations/ Concurrent treatment	Ph^b	Pts	Route^c	Tumor targets^d	Results^e	Refs
dl1520 (same virus as ONYX-015)	<i>E1B-55 kD</i> deletion	I	22	i.t.	SCCHN	1x10 ¹¹ pfu, 2 PR	(Ganly et al. 2000)
ONYX-015	<i>E1B-55 kD</i> deletion	I	23	i.t.	Pancreatic ca.	1x10 ¹¹ pfu, no responses	(Mulvihill et al. 2001)
ONYX-015	<i>E1B-55 kD</i> deletion	I	10	i.v.	Metastatic solid tumor	2x10 ¹³ vp, no responses	(Nemunaitis et al. 2001b)
ONYX-015	<i>E1B-55 kD</i> deletion	I	16	i.p.	Ovarian ca.	1x10 ¹¹ pfu/d x 5d, no responses	(Vasey et al. 2002)
ONYX-015	<i>E1B-55 kD</i> deletion +/- 5-fluorouracil and leucovorin	I	11	i.ha.	Colorectal ca. metastatic to liver	2x10 ¹² vp, 1 PR with combination therapy	(Reid et al. 2001)

ONYX-015	<i>E1B-55 kD</i> deletion	I	22	m.w.	Premalignant oral dysplasia	1x10 ¹¹ pfu/d x 5d, followed by 1x/wk, 2 CR, 1 PR	(Rudin et al. 2003)
ONYX-015	<i>E1B-55 kD</i> deletion + irinotecan and 5-fluorouracil	I	5	i.v.	Colon ca.	2x10 ¹² vp, no responses	(Nemunaitis et al. 2003b)
ONYX-015	<i>E1B-55 kD</i> deletion + interleukin 2	I	5	i.v.	Solid tumor	2x10 ¹¹ vp, no responses	(Nemunaitis et al. 2003b)
CV706	PSA promoter-enhancer controlling <i>E1A</i>	I	20	i.t.	Prostate ca.	1x10 ¹³ vp, ≥50% PSA decrease in 5/20 pts	(DeWeese et al. 2001)
Ad5-CD/TK rep	<i>E1B-55 kD</i> deletion, insertion of TK/CD + GCV/5-fluorocytosine	I	16	i.t.	Recurrent prostate ca.	1x10 ¹² vp, ≥50% PSA decrease in 3/16 pts	(Freytag et al. 2002)
Ad5-CD/TK rep	<i>E1B-55 kD</i> deletion, insertion of TK/CD + GCV/5-fluorocytosine and radiation	I	15	i.t.	Newly diagnosed prostate ca.	1x10 ¹² vp, significant decline in PSA level in all pts	(Freytag et al. 2003)
ONYX-015	<i>E1B-55 kD</i> deletion + 5-fluorouracil (in phase II)	I-II	16	i.t., i.ha., i.v.	HCC and colorectal ca. metastatic to liver	3x10 ¹¹ pfu, no responses, in phase II 50% CEA decrease in 3/7 pts	(Habib et al. 2001)
ONYX-015	<i>E1B-55 kD</i> deletion + 5-fluorouracil and leucovorin	II	27	i.ha.	Gastro-intestinal ca. metastatic to liver	2x10 ¹² vp, 3 PR	(Reid et al. 2002)
ONYX-015	<i>E1B-55 kD</i> deletion	II	40	i.t.	SCCHN	2x10 ¹¹ vp for 10 d, 2 PR, 3 CR	(Nemunaitis et al. 2001a)

ONYX-015	<i>E1B-55 kD</i> deletion + cisplatin and 5-fluorouracil	II	37	i.t.	SSCHN	1x10 ¹⁰ pfu/d x 5d, 11 PR, 8 CR	(Khuri et al. 2000)
ONYX-015	<i>E1B-55 kD</i> deletion +/- gemcitabine	I-II	21	i.t.	Pancreatic ca.	2x10 ¹¹ vp, 1x/wk, 8 cycles 2 PR	(Hecht et al. 2003)
ONYX-015	<i>E1B-55 kD</i> deletion	II	18	i.v.	Metastatic colorectal ca.	2x10 ¹² vp every 2 weeks, no responses	(Hamid et al. 2003)
CG7870	Rat probasin promoter controlling <i>E1A</i> , PSA promoter and enhancer driving <i>E1B</i>	I-II	20	i.t.	Locally recurrent prostate ca.	1x10 ¹³ vp, 25-50% PSA decrease in 8/12 evaluable pts	(DeWeese et al. 2003)

^a Includes clinical cancer gene therapy trials that have completed patient enrollment; ^b Ph = phase; ^c i.t. = intratumoral, i.v. = intravenous, i.p. = intraperitoneal, i.h.a. = intrahepatic artery, m.w. = mouthwash; ^d SCCHN = squamous cell carcinoma of the head and neck, HCC = hepatocellular carcinoma; ^e vp = viral particle, pfu = plaque forming unit, PR = partial response, CR = complete response

9. Adenoviral gene therapy trials for ovarian cancer

9.1. Ovarian cancer

Ovarian cancer is the fourth most common cancer among women in Finland with 599 new cases (including 199 borderline tumors) in 2001. The age-standardized incidence rate was 13.4 per 100 000 person-years. The median age at diagnosis is 62 years (The Finnish Cancer Registry; <http://www.cancerregistry.fi>). Most cancers of ovary are of epithelial origin, *i.e.* carcinomas. The most common histological type is serous (over 50%), followed by mucinous and endometrioid types (15% each). The prognosis of ovarian cancer is poor as approximately 75% of patients are at International Federation of Gynecology and Obstetrics (FIGO) stages II-IV at the time of diagnosis. The overall 5-year survival rate is 47%, which makes ovarian cancer a leading cause of gynecological cancer mortality. However, in women with low-risk stage I epithelial ovarian cancer, 5-year survival rates can be over 90%. Unfortunately, these rates fall progressively as the disease becomes more advanced (to <10% in patients with stage IV malignancy) (Knopf and Kohn. 2001).

In addition to FIGO stage, important prognostic factors include histological type and grade, residual disease and patient's performance status and age (Friedlander. 1998).

The initial approach to the treatment of ovarian cancer is almost always surgery. The purpose of surgery is to establish the diagnosis, to surgically stage the disease, and - in the event of advanced disease - to remove as much cancer tissue as possible, *i.e.* cytoreduce the tumor. Chemotherapeutics has been regarded as a standard therapy for majority of patients (Marsden et al. 2000; McGuire and Markman. 2003). Lately, the combination of either cisplatin or carboplatin with paclitaxel has been a first-line therapy. Also, alternative taxanes, such as docetaxel, have been studied (McGuire and Markman. 2003). Further, alternative delivery route via i.p. catheter has been investigated for patients with minimal residual disease. However, this approach has not found widespread acceptance (Marsden et al. 2000).

9.2. Adenoviral gene therapy trials for ovarian cancer

Clearly, novel treatment strategies are needed for this disease. Adenoviral gene therapy is an attractive modality for treatment of ovarian cancer because ovarian cancer tends to remain localized in the peritoneal cavity, allowing for regional delivery of the vector or virus. In the setting of ovarian cancer, Ad21 was among the first clinically evaluated adenoviral gene therapy approaches. Ad21 is an *E1/E3*-deleted adenovirus, which encodes an endoplasmic reticulum localizing anti-*erbB2* single chain intrabody. It was hypothesized that the expressed intrabody traps a cancer associated receptor *erbB2* to endoplasmic reticulum, and therefore, down-regulates the cell surface expression of otherwise overexpressed protein. This approach resulted in induction of apoptosis and ovarian cancer cytotoxicity *in vitro*, and enhanced anti-tumor activity and survival in ovarian cancer animal models (Deshane et al. 1994; Deshane et al. 1995a; Deshane et al. 1995b; Deshane et al. 1996; Deshane et al. 1997). Alvarez *et al.* analyzed the feasibility of the strategy in phase I ovarian cancer trial (Alvarez et al. 2000a). The treatment was well tolerated up to 10^{11} pfu without dose-limiting toxicity. Importantly, PCR and RT-PCR analyses from ascites samples demonstrated the presence of vector and expression of transgene, however, there was no data identifying the infected cells. Further, there were no responses detected. The major disadvantage of this kind of approach is a requirement of infecting all cancer cells.

The prodrug-based strategy utilizes genes encoding prodrug activating enzymes, which convert systemically administrable and relatively nontoxic prodrugs into toxic compounds. The HSV-TK-based suicide gene strategy has a clear advantage as compared to anti-*erbB2* single chain intrabody approach. The activated drugs can spread into surrounding cells via gap junctions, *i.e.* the so-called local bystander effect. In phase I clinical ovarian cancer trial 14 patients were treated i.p. with AdHSV-TK in single dosages from 1×10^9 to 1×10^{11} pfu, which was followed by 14 days of i.v.

GCV (Alvarez et al. 2000b). Transient vector-associated fever was experienced by 4/14 (29%) patients, however, there were no dose-limiting virus-related side effects. Again, the HSV-TK gene transfer and transgene expression were detected in peritoneal aspirates. However, there was no objective response to treatment, though 5/14 (38%) had a stable disease. Even with the bystander effect, the anti-tumor effect could be limited to surface layers of residual tumor. Another phase I study combined i.p. delivered HSV-TK-encoding adenovirus to i.v. administered acyclovir and concomitant topotecan (Hasenburger et al. 2000). They reported grade 3-4 thrombocytopenia and neutropenia, which were most likely related to chemotherapy. Again, vector-associated temperature elevations were noted. Further, 5/10 patients underwent second-look exploration 20 to 40 days after adenovirus delivery. None of the peritoneal biopsies showed residual adenoviral DNA. The median survival time was 18.5 months, which compares favorably to previously reported second- and third-line chemotherapy trials (Hasenburger et al. 2001).

Mutation of the p53 tumor suppressor gene is one of the most frequent molecular genetic changes in cancer. Nearly 70% of advanced stage ovarian cancers contain p53 mutations (Wen et al. 1999; Shahin et al. 2000). Subsequently, *E1/E3*-deleted Ad p53 (SCH 58500) encoding human, wild-type p53 was evaluated in phase I/II ovarian cancer trial (Buller et al. 2002). In phase I patients received single i.p. injection of virus, while in phase I/II they were treated with multiple doses of the virus up to 7.5×10^{13} vp on 5 consecutive days. Further, in multiple dose schema patients received 3 cycles of treatment, and last two of them were in combination with chemotherapy. Treatment was well tolerated. The RT-PCR transgene expression data was generated from ascited fluid cell pellets and also tissue biopsies, which again might contain non-malignant cells also. However, the presence of viral DNA in tumor cells was demonstrated with *in situ* PCR on a cancer samples from a single patient. Finally, 8 of 16 patients in multidose schema demonstrated >50% decrease in serum CA125 level.

The poor penetration of the adenoviral vector into solid tumor mass is one restricting factor for achieving clinical benefit (Kirn et al. 2001). Replicating viruses can dramatically improve tumor penetration. Thus, many CRAds have been preclinically evaluated also for treatment of ovarian cancer (Zhang et al. 2002; Hamada et al. 2003; Kanerva et al. 2004; Heise et al. 2000; Bauerschmitz et al. 2002b; Lam et al. 2003a; Akbulut et al. 2003; Tsukuda et al. 2002) However, only ONYX-015 has entered a phase I clinical trial (Vasey et al. 2002). Sixteen patients received from 1 to 4 cycles of ONYX-015 on 5 consecutive days at doses from 1×10^9 to 1×10^{11} pfu. One patient developed dose-limiting, grade 3 abdominal pain and diarrhea, nevertheless, the maximum-tolerated dose was not reached. Using PCR, the presence of ONYX-015 DNA in cell-free fraction of peritoneal aspirates was demonstrated. However, *in situ* hybridization on smeared ascites cell pellets demonstrated only once viral DNA, and unfortunately, the positive cells did not appear

malignant based on size and nuclear morphology. There was no clinical or radiological response in any of the patient.

Importantly, none of the studies above were randomized. Further, there were no clinical complete responses. It might be necessary to target adenovirus to non-CAR receptors to achieve reasonable transduction levels in clinical setting. Various strategies to retarget adenovirus binding or transgene expression in ovarian cancer cells have been analyzed *in vitro* and *in vivo*. Rancourt *et al.* utilized a Fab-FGF2 adapter to target HSV-TK expressing adenovirus to FGF receptor-positive ovarian cancer cells, which resulted in enhanced survival of ovarian cancer xenograft-bearing mice (Rancourt et al. 1998). Consequently, a clinical trial for i.p. treatment of ovarian cancer patients with peritoneally disseminated disease with this virus will soon start enrolling patients (A Hemminki, personal communication). Incorporation of an RGD-4C peptide in the HI loop of the adenovirus vector enhanced the infectivity of ovarian cancer cells *in vitro* and *in vivo* (Dmitriev et al. 1998; Vanderkwaak et al. 1999; Hemminki et al. 2001b; Hemminki et al. 2002b). Further, RGD-4C-modified CRAd, Ad5- Δ 24RGD, was able to replicate in ovarian cancer primary cell spheroids and resulted in significantly prolonged survival in an aggressive orthotopic ovarian cancer model (Bauerschmitz et al. 2002b; Lam et al. 2003a). Of note, also Ad5- Δ 24RGD is entering a multi-center phase I clinical trial for treatment of ovarian cancer (A Hemminki, personal communication).

AIMS OF THE STUDY

1. To measure cell surface expression of CAR and the Ad3 receptor on ovarian cancer cells, and to evaluate if there is a correlation with transgene expression mediated by adenoviruses that bind to the respective receptors. **(I)**
2. To evaluate murine liver toxicity, blood clearance and biodistribution, and to assess gene transfer efficiency to human primary ovarian cancer cells and in a murine model of ovarian cancer with the Ad3 receptor targeted adenovirus. **(II)**
3. To construct a CRAd retargeted to the Ad3 receptor, Ad5/3- Δ 24, and to evaluate its oncolytic potency *in vitro* and *in vivo*. **(III)**
4. To construct Ad5/3- Δ 24-hCEA encoding a marker peptide (soluble hCEA) measurable in tissue culture fluid or in plasma, and to evaluate replication kinetics of the virus *in vitro* and *in vivo*. To combine non-invasive imaging of the tumor with the analysis of the marker peptide to achieve dual-modality monitoring of virus efficacy. **(IV)**

MATERIALS AND METHODS

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

1. Cell lines and primary ovarian cancer cells (I-IV)

Table 2. The list of human cell lines used in this study

Cell line	Description	Source	Used in
293	Transformed embryonic kidney cells	Microbix (Toronto, Canada)	I, II, III, IV
911	Transformed embryonic retinoblasts	Dr. AJ van der Eb (University of Leiden, the Netherlands)	III, IV
AG07086A	Mesothelial cells	Coriell Cell Repositories (Gamden, NJ)	II
AG07090B	Mesothelial cells	Coriell Cell Repositories	II
A549 (CCL-185)	Lung adenocarcinoma	ATCC (Manassas, VA)	III, IV
PA-1 (CRL-1572)	Ovarian teratocarcinoma	ATCC	I
ES-2 (CRL-1978)	Ovarian adenocarcinoma	ATCC	III
OV-3 (HTB-75)	Ovarian adenocarcinoma	ATCC	III
SKOV3.ip1	Ovarian adenocarcinoma	Dr. J Price (M.D. Anderson Cancer Center, Houston, TX)	I, II, III, IV
Hey	Ovarian adenocarcinoma	Dr. J Wolf (M.D. Anderson Cancer Center, Houston, TX)	I, II, III
OV-4	Ovarian adenocarcinoma	Dr. TJ Eberlein (Harvard Medical School, Boston, MA)	I, II, III
SKOV3-luc	Ovarian adenocarcinoma cells expressing firefly luciferase	Dr. R Negrin (Stanford Medical School, CA)	III, IV

Primary ovarian adenocarcinoma cells were purified by an immunomagnetic-based method from malignant ascites fluid samples from patients undergoing a procedure for ovarian cancer at the University of Alabama at Birmingham Hospital (Barker et al. 2001). Briefly, ovarian cancer cells were bound with a murine anti-TAG-72-antibody (CC-49, a generous gift from Dr. J Schlom, National Institute of Health, Bethesda, MD) and then collected with magnetic beads coated with anti-mouse-IgG (Pan Mouse IgG Dynabead, Dynal AS, Oslo, Norway) (I, II, III).

2. The adenovirus vectors and replicating adenoviruses (I-IV)

Table 3. The list of the adenovirus vectors used in this study

Virus	Description	Source	Used in
Ad5luc1	<i>E1/E3</i> -deleted, a luc gene under the CMV promoter in place of <i>E1</i>	(Krasnykh et al. 2001)	I, II
Ad5/3luc1	<i>E1/E3</i> -deleted, a luc gene under the CMV promoter in place of <i>E1</i> , chimeric fiber with the tail and shaft from Ad5 and the knob domain from Ad3	This study (I)	I, II
Ad5lucRGD	<i>E1/E3</i> -deleted, a luc gene under the CMV promoter in place of <i>E1</i> , RGD-4C modification in the HI loop of the knob domain	(Dmitriev et al. 1998)	II
AdCMVHSV-TK	<i>E1/E3</i> -deleted, the HSV-TK gene under the CMV promoter in place of <i>E1</i>	(Rosenfeld et al. 1995)	III

Table 4. The list of the CRAbs used in this study

Virus	Description	Source	Used in
Ad5- Δ 24E3	24-bp deletion in CR2 of <i>E1A</i>	(Suzuki et al. 2002)	III
Ad5- Δ 24RGD	24-bp deletion in CR2 of <i>E1A</i> , RGD-4C modification in the HI loop of the knob domain	(Suzuki et al. 2001)	III
Ad5/3- Δ 24	24-bp deletion in CR2 of <i>E1A</i> , chimeric fiber with the tail and shaft from Ad5 and the knob domain from Ad3	This study (III)	III

Ad5/3- Δ 24-hCEA	24-bp deletion in CR2 of <i>E1A</i> , chimeric fibers with tail and shaft from Ad5 and knob domain from Ad3, hCEA cDNA in a partially deleted <i>E3</i> region	This study (IV)	IV
Ad5/3- Δ 24 Δ E3	24-bp deletion in CR2 of <i>E1A</i> , chimeric fibers with tail and shaft from Ad5 and knob domain from Ad3, <i>E3</i> region deleted	This study (IV)	IV

Table 5. The plasmids used for cloning of the viruses

Plasmid	Description	Source	Used in
pNEB.PK.F5/3	A fiber shuttle vector, containing an Ad5 tail and shaft and Ad3 knob	(Krasnykh et al. 1996)	I, III
pTU.5/3	<i>E1</i> -deleted Ad5 genome with the chimeric fiber	(Uil et al. 2003)	III
pShuttle Δ 24	A shuttle plasmid containing 24-bp deletion in CR2 of <i>E1A</i>	(Suzuki et al. 2002)	III, IV
pAdEasy-1.5/3	<i>E1/E3</i> -deleted Ad5 genome with the chimeric fiber	This study (IV)	IV
pAdEasy-1.5/3- Δ 24 Δ E3	<i>E3</i> -deleted Ad5 genome with 24-bp deletion in CR2 of <i>E1A</i> and the chimeric fiber	This study (IV)	IV
pTHSN	A shuttle plasmid containing the <i>E3</i> region	This study (IV)	IV
pTHSN-hCEA	A shuttle plasmid containing a cDNA encoding hCEA inserted in the partially deleted <i>E3</i> region	This study (IV)	IV
pAdEasy-1.5/3- Δ 24-hCEA	Ad5 genome with 24-bp deletion in CR2 of <i>E1A</i> , cDNA encoding hCEA inserted in the partially deleted <i>E3</i> region, and the chimeric fiber	This study (IV)	IV

2.1. Construction of Ad5/3- Δ 24 (III)

pNEB.PK.F5/3 was digested with *PacI* and *KpnI*, followed by cotransformation into *Escherichia coli* for homologous recombination with a *SwaI*-linearized plasmid containing an *E1*-deleted Ad5

genome. This resulted in pTU.5/3. In order to create Ad5/3- Δ 24, pShuttle Δ 24 was linearized with *PacI/PmeI* and cotransfected into 911 cells with *PacI*-linearized pTU.5/3 to rescue Ad5/3- Δ 24.

2.2. Construction of Ad5/3- Δ 24 Δ E3 and Ad5/3- Δ 24-hCEA (IV)

pShuttle Δ 24 was linearized with *PmeI* and homologous recombination in *E.coli* was performed with pAdEasy-1.5/3. The Ad5/3- Δ 24 Δ E3 virus genome was released by *PacI* digestion and transfection to 911 cells. In order to insert hCEA gene into *E3* region, pTHSN was digested with *SunI/MunI* creating a 965-bp deletion in *E3* region. cDNA encoding the extracellular domain of hCEA (2031-bp) was amplified by PCR generating *SunI/MunI* restriction enzymes sites flanking the gene (CEA(F): 5'-ACGTCGTACGATGGAGTCTCCCTCGGCCCT-3', CEA(R): 5'-TGTGCAATTGCTATGCAGAGACTGTGATGCTCTTG-3') and then inserted into *SunI/MunI*-digested pTHSN. pAdEasy-1.5/3- Δ 24-hCEA was generated by homologous recombination in *E.coli* between *FspI*-linearized pTHSN-hCEA and *SrfI*-linearized pAdEasy-1.5/3- Δ 24. Ad5/3- Δ 24-hCEA was rescued as above.

2.3. High titer production of the viruses

Propagation of the adenovirus vectors and CRAds was performed on 293 and A549 cells, respectively. All viruses were purified on cesium chloride gradients. The vp concentration was determined at 260 nm, and standard plaque assay on 293 cells was performed to determine infectious particles.

3. In vitro experiments in this study

3.1. Adenovirus-mediated gene transfer assays (I, II)

Cells were infected for 30 min at room temperature. Cells were washed once, and growth medium was added. After 24 hrs of incubation at 37°C, luciferase assay was performed (Luciferase Assay System, Promega, Madison, WI). The protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) to allow normalization of the gene expression data for the amount of protein. In knob-blocking experiment, monolayers of SKOV3.ip1 cells were preincubated with increasing concentrations of Ad5 or Ad3 knob for 10 min at room temperature. To analyze the effect of pre-existing neutralizing antibodies, viruses were preincubated for 10 min at room temperature with malignant ascites. Then, cell monolayers were infected for 1 hr at 37°C, followed by incubation and luciferase assay as above.

3.2. Determination of receptor expression by flow cytometry (I)

Cells were incubated with either Ad5 or Ad3 recombinant knob protein with 6-His tag. Thereafter, the knob-binding was detected with primary Tetra-His-Antibody (Qiagen, Valencia, CA) and secondary fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma, St Louis, MO). 2.6 µg/ml Propidium Iodide (Sigma) was added to sort out dead cells from the sample, then 2×10^4 cells (OV-4) or 10^4 cells (other cell lines) were analyzed immediately by flow cytometry at the University of Alabama at Birmingham FACS Core Facility.

3.3. In vitro cytotoxicity assay (III)

Cells were infected with CRAds and E1-deleted control virus for 1 h at 37°C. Thereafter, cells were incubated with growth medium with 5% fetal bovine serum until almost complete cell killing was visually evident at the lowest dose of any virus. Cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay, Promega).

3.4. Quantitating virus replication (III)

Primary ovarian cancer cells were purified and cultured as three dimensional spheroids overnight as described (Lam et al. 2003a). Briefly, cells were suspended in growth medium in 3% agar coated flasks and incubated on a rocking platform. The next day, spheroids were infected for 1 h at 37°C. Cells and growth medium were harvested and frozen at indicated time-points. To quantitate the viral copy number, DNA was purified from spheroid suspension (cellular and growth medium fractions together) using a DNeasy Tissue Kit (Qiagen), and the *E4* copy number and human β -actin were determined with quantitative PCR as described (Hemminki et al. 2001b). The background values (uninfected spheroids) were subtracted at each time point. To estimate total virus production by the spheroids, cumulative virus copy number was calculated.

Table 6. Primers and probes used for quantitative PCR

<i>Adenoviral E4</i>		Used in
Forward:	5'-GGAGTGCGCCGAGACAAC-3'	II, III, IV
Reverse:	5'-ACTACGTCCGGCGTTCCAT-3'	II, III, IV
Probe:	5'-TGGCATGACACTACGACCAACACGATCT-3'	II, III, IV
<i>Human β-actin</i>		
Forward:	5'-TAAGTAGGCGCACAGTAGGTCTGA-3'	III, IV
Reverse:	5'-AAAGTGCAAAGAACACGGCTAAGT-3'	III, IV
Probe:	5'-CAGACTCCCCATCCCAAGACCCCA-3'	III, IV

<i>Mouse β-actin</i>		
Forward:	5'-CGAGCGGTTCCGATGC-3'	II
Reverse:	5'-TGGATGCCACAGGATTCCAT-3'	II
Probe:	5'-AGGCTCTTTCCAGCCTTCCTTCTGG-3'	II

3.5. *In vitro* growth kinetics of Ad5/3- Δ 24-hCEA (IV)

Cells were infected with Ad5/3- Δ 24-hCEA for 1 h at 37°C. At indicated time points supernatant was collected, and hCEA level was measured in Mayo Clinic Central Clinical Laboratory using the Bayer Centaur Immunoassay System as described (Peng et al. 2002a). Cells were collected and virus copy number was quantitated as above.

4. Preclinical, *in vivo* evaluation of the viruses (II, III, IV)

Mice were obtained from University of Alabama at Birmingham CFAR SCID Mouse Core Facility or Charles River Laboratories (Wilmington, MA) at 3-4 weeks age and quarantined for 2 weeks. Mice were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of University of Alabama at Birmingham.

Table 7. *In vivo* experiments performed with female mice

Study	Route	Dose (vp/ml)	Mice	Method	Used in
Liver toxicity, ALT, AST release	i.v.	5 x 10 ¹⁰	C57BL/6	GO&GP-Trans-aminase Kit, Sigma	II
Liver toxicity, histopathology	i.v.	5 x 10 ¹⁰	C57BL/6	H&E staining	II
Liver toxicity, liver transduction	i.v.	5 x 10 ¹⁰	C57BL/6	Luciferase analysis	II
Blood clearance	i.v.	5 x 10 ¹⁰	C57BL/6	Luciferase analysis from cells infected with plasma	II
Biodistribution	i.p.	5 x 10 ¹⁰	CB17 SCID	Luciferase analysis from organs	II
Liver transduction	i.p.	5 x 10 ¹⁰	CB17 SCID	Quantitative PCR for viral DNA	II

Transduction, s.c. tumor	i.t.	2.5 x 10 ⁹	CD-1 nude	Luciferase analysis	II
Therapeutic efficacy, i.p. tumor	i.p.	1x 3 x 10 ⁷	CB17 SCID	Survival	III
Therapeutic efficacy, i.p. tumor	i.p.	3x 1 x 10 ⁸	CB17 SCID	Survival	III
Imaging of therapeutic response	i.p.	3 x 10 ⁷	CB17 SCID	Bioluminescence imaging	III
Virus replication kinetics	i.p.	4x 1x 10 ⁸	CB17 SCID	Plasma hCEA level, ELISA	IV
Imaging of therapeutic response, viral replication kinetics	i.p.	4x 1x 10 ⁸	CB17 SCID	Bioluminescence imaging, plasma hCEA level, ELISA	IV

ALT = alanine aminotransferase, AST = asparate aminotransferase, i.p. = intraperitoneal, i.t. = intratumoral, i.v. = intravenous, s.c. = subcutaneous

5. Statistics (II, III, IV)

All statistical analyses were performed using statistical analysis software SAS v.8.2 or v.9.0 (SAS Institute, Cary, NY). *P* value of <0.05 was deemed statistically significant. The differences among groups were assessed with the Kruskal-Wallis test. When significant differences were detected, pair-wise comparisons between groups were performed with the Wilcoxon two-sample test (II). The results with Ad5/3-Δ24 group in MTS assay were compared to the other groups using two-tailed T-test (III). Survival data were plotted on a Kaplan-Meier curve, and the comparison between groups by the log-rank procedure and χ^2 testing (III, IV). For comparison of tumor size with bioluminescence data we constructed a repeated measures linear model that tested for the effects of time, treatment group and the interaction of treatment group and time on emitted light. For comparison of mean tumor bioluminescence within each treatment group on each time-point with baseline, we used the Wilcoxon-rank sum test (IV).

RESULTS AND DISCUSSION

1. Expression of adenovirus serotype 5 and 3 receptors on ovarian cancer cells (I)

To evaluate the receptor binding properties of adenovirus vectors containing a firefly luciferase transgene cassette in the deleted *E1*-region with either the native Ad5 fiber protein (Ad5luc1) or a chimeric fiber with the knob from Ad3 fiber (Ad5/3luc1), infections of SKOV3.ip1 cells were performed in the presence of purified, trimeric recombinant Ad5 and Ad3 knob proteins (Figure 1 in Study I). The relevant knob protein was able to block transgene expression in a dose-dependent manner up to 85%, while the irrelevant knob had only minimal effects. Our results support the existence of a distinct receptor for Ad3, as suggested previously (Stevenson et al. 1995; Roelvink et al. 1998). More importantly, these results confirm that Ad5/3luc1 is retargeted to the Ad3 receptor. Recently, it has been suggested that there is a common receptor for subgroup B adenoviruses, which Ad3 also belongs to (Segerman et al. 2003a). Specifically, the CD46 membrane cofactor protein is proposed as a cellular receptor for many group B serotypes (Segerman et al. 2003b; Gaggar et al. 2003). In contrast, recent publication suggests that Ad3 does not use CD46 as an attachment receptor (Gaggar et al. 2003). CD46 is a member of a family of glycoproteins acting as regulators of complement activation. Interestingly, other members of the this family also serve as viral receptors, *i.e.* CD21 for Epstein-Barr virus (Fingeroth et al. 1984) and CD55 for several echoviruses (Bergelson et al. 1994) and coxsackieviruses (Bergelson et al. 1995).

As the Ad3 receptor is not yet identified, we developed a novel knob binding assay to quantify the cell surface expression of CAR and the Ad3 receptor on human ovarian cancer cell lines (Figure 2 in Study I). For this analysis cells were incubated with recombinant, 6-His-tagged Ad5 knob or Ad3 knob, followed by flow cytometric analysis. 293 cells were included as a CAR-positive control (Dmitriev et al. 1998). In contrast, OV-4 and SKOV3.ip1 have been shown to display moderate or low levels of CAR (Dmitriev et al. 1998; Dmitriev et al. 2000). This knob binding assay suggested that 293 cells express larger amount of CAR than the Ad3 receptor (38% of cells were FITC positive after incubation with Ad5 knob *versus* 17% with Ad3 knob). In human ovarian adenocarcinoma cell lines OV-4 and SKOV3.ip1, the Ad3 receptor was detected in 95% and 48% of cells, whereas 32% and 0.7%, respectively, were positive for CAR. These results suggest higher expression of the Ad3 receptor relative to CAR on human ovarian adenocarcinoma lines. Subsequently, high expression of the Ad3 receptor on cancer cells has been demonstrated in the context of renal cancer and melanoma using same method (Haviv et al. 2002; Volk et al. 2003). Importantly, the primary melanoma cells from patients demonstrated lower CAR expression than the analyzed melanoma cell lines (Volk et al. 2003).

2. Infectivity of ovarian cancer cells with modified adenoviruses (I, II)

We hypothesized that differential expression of CAR and Ad3 receptor would correlate with infectivity of wild-type capsid Ad5 and Ad5/3 possessing chimeric fibers. First, we analyzed transduction of ovarian carcinoma cell lines *in vitro* (Figure 3 in Study I). Non-adenocarcinoma cell lines expressing slightly more CAR than Ad3 receptor in the knob binding assay (PA-1 teratocarcinoma and control cell line 293) demonstrated *ca.* two-fold more luciferase with the chimeric virus in the gene transfer assay. Further, transgene expression in adenocarcinoma cell lines (OV-4, SKOV3.ip1 and Hey) was two orders of magnitude higher with Ad5/3luc1. Specifically, the most prominent infectivity enhancement was seen with OV-4 cells, as Ad5/3 displayed 280-fold higher transgene expression. Importantly, the receptor density, as estimated by the knob binding assay, roughly correlated with reporter gene expression. Thus, the binding to the primary receptor appears to be important factor determining the efficiency of adenovirus-based gene delivery to target cells. Nevertheless, there are other receptors, which might mediate the initial binding of Ad5. Recent studies have suggested that MHC I (Hong et al. 1997) and HSG (Dechecchi et al. 2001; Smith et al. 2003) may be involved in the virus binding. Furthermore, expression of $\alpha_v\beta$ or $\alpha_3\beta_1$ integrins may affect the infectivity of cells by adenovirus (Mathias et al. 1998; Salone et al. 2003). Similarly, Ad5/3 fiber knob binds to the Ad3 receptor, but there might be more interactions with other cellular receptors. Further, Shayakhmetov *et al.* concluded that the interaction between the knob protein and the primary receptor determines the intracellular trafficking route (Shayakhmetov et al. 2003). Thus, the significant enhancement in transgene expression might partly reflect faster or more efficient intracellular behavior of the chimeric virus. However, there are earlier studies showing the importance of the whole fiber protein (Miyazawa et al. 1999). Further, the fiber shaft length has an impact on virus tropism. Seki *et al.* showed that the artificial extension of the Ad5 shaft inhibits infectivity of CAR-expressing cells (Seki et al. 2002). The Ad5 shaft might be beneficial for internalization (allowing easy interaction of penton base RGD and cellular integrins) and intracellular trafficking of the virus. Nevertheless, our promising results with the Ad5/3 chimeric vectors displaying Ad3 knob/Ad5 shaft suggest that it may be a useful combination for avoiding the problems with CAR deficiency while retaining the high gene transfer capacity of Ad5.

An important recent revelation has been that primary tumor cells express highly variable and often low amounts of CAR, although the corresponding cancer cell lines might be transducible with Ad5 (reviewed in Bauerschmitz et al. 2002a). Thus, it is crucial to analyze clinical samples for reliable preclinical estimation of efficacy. For this purpose, we have developed a purification method that typically yields >95% pure population of cancer cells (Barker et al. 2001). We analyzed the purified

primary cells without passaging which could help avoid confounding due to geno- and phenotypic changes involved in the clonal selection process. Altogether nine patient samples used in this study demonstrated from 5- to 50-fold higher transgene expression when infected with Ad5/3luc1 in comparison to Ad5luc1 (Figure 4 in Study I, Figure 4 and Table 2 in Study II). Importantly, in all cases, Ad5/3luc1 was superior to Ad5lucRGD, which has displayed enhanced infectivity in various carcinomas (Dmitriev et al. 1998; Vanderkwaak et al. 1999; Kasono et al. 1999; Wesseling et al. 2001a; Hemminki et al. 2001b; Hemminki et al. 2002b; Grill et al. 2001; Cripe et al. 2001). Thus, an augmentation of luciferase activity was observed with the modified vector, but to somewhat smaller extent than with ovarian adenocarcinoma cell lines.

There are increasing recent data obtained suggesting low CAR expression on ovarian cancer cells (You et al. 2001; Dmitriev et al. 1998; Kelly et al. 2000; Vanderkwaak et al. 1999; Zeimet et al. 2002). As with ovarian cancer, variable expression of CAR is documented in many other cancer types such as glioma, melanoma, bladder cancer, rhabdomyosarcoma, pancreatic cancer, SCCHN, prostate cancer, osteosarcoma and colorectal cancer (Miller et al. 1998; Li et al. 1999; Cripe et al. 2001; Hemmi et al. 1998; Kelly et al. 2000; Fechner et al. 2000; Rauen et al. 2002; Kasono et al. 1999; Grill et al. 2001; Wesseling et al. 2001a; Witlox et al. 2002). Interestingly, previous studies suggest that CAR may act as a tumor suppressor, which could be linked to the frequent down-regulation seen in highly tumorigenic cells (Okegawa et al. 2000). Our results suggest that expression of CAR *versus* the Ad3 receptor is different on human ovarian cancer cells, and the density of Ad3 receptor is often higher. Although the receptor and its function are unknown, this might suggest that the Ad3 receptor is unrelated to carcinogenesis. Malignant progression might not affect its expression level, and thus, Ad3 receptor mediated gene transfer could be advantageous in the context of advanced cancer.

In an animal model of ovarian cancer, Ad5luc1, Ad5/3luc1 or Ad5lucRGD were injected into established s.c. Hey cell tumors (Figure 6 in Study II). Both fiber modified vectors demonstrated significantly enhanced transgene expression in comparison to Ad5luc1 (*versus* Ad5/3luc1, $P = 0.0064$; *versus* Ad5lucRGD, $P = 0.0014$). Of note, the difference between Ad5/3 and Ad5 *in vivo* was 4-fold compared to over 100-fold increase *in vitro*. However, xenografts contain variable amounts of host stromal cells, whose receptor expression levels affect the infectivity of the xenograft as a whole.

These results indicate that modification of the Ad5 fiber with an RGD-4C motif in the HI-loop or swapping the knob for a serotype 3 knob can lead to significant enhancements in transduction of purified primary ovarian cancer cells and *in vivo*. Also, as the 5/3 chimera was superior to the RGD-4C, it is the best available genetic retargeting moiety for ovarian cancer. Subsequently, the

same phenomenon has been noted in the context of renal cancer and melanoma (Haviv et al. 2002; Volk et al. 2003).

At diagnosis, most ovarian cancer patients have ascites fluid. Previous studies have suggested partial escape of RGD-4C modified viruses from pre-existing neutralizing anti-adenovirus antibodies present in malignant ascites (Blackwell et al. 2000; Hemminki et al. 2001b). Here, we showed that also Ad5/3luc1 is able to circumvent neutralization (Figure 5 and Table 3 in Study II). In fact, in the presence of ascites, gene transfer was improved up to 3 orders of magnitude in comparison to Ad5luc1. The level of neutralizing anti-Ad3 antibodies in ascites is unknown, but in the general population, the prevalence of total serum anti-Ad3 antibodies seems to be comparable to that of anti-Ad5 (Vogels et al. 2003). However, in parallel to what has been suggested for RGD-4C modified adenoviruses, the chimeric nature of the Ad5/3luc1 fiber may allow escape from many of the anti-Ad3-fiber neutralizing antibodies, as such antibodies are often conformation sensitive. Further, it has been suggested that effective neutralization requires concerted action of anti-fiber, anti-penton and anti-hexon antibodies (Hemminki et al. 2002c). This could help explain the impressive escape from neutralization seen in our experiments.

3. Liver toxicity and blood clearance rates of fiber modified adenoviruses (II)

In mice, the liver is the major organ responsible for adenovirus clearance, and could therefore be an important organ in regard to potential clinical toxicity in humans (Lieber et al. 1997; Zhang et al. 2001; Tao et al. 2001). Kupffer cells have an important role in clearing adenovirus from the blood but their uptake capacity can be saturated which leads to a non-linear dose effect at approximately 2×10^{10} vp in mice (Tao et al. 2001). Only after saturation is achieved, are hepatocytes and other tissues transduced effectively. To evaluate immediate and early liver toxicity, we injected 5×10^{10} vp of Ad5luc1, Ad5/3luc1 or Ad5lucRGD, and analyzed serum transaminases, liver histopathology and transgene expression at 72 hours (Figure 1 and Table 1 in Study II). In general, only minor differences were seen, suggesting that the toxicity of tropism modified viruses does not significantly differ from Ad5. Ad5 has been administered i.v., i.p., intra-arterially and intratumorally in large doses in clinical trials without significant toxicity (Hemminki and Alvarez. 2002a). Thus, toxicity similar to Ad5 could predict a good safety profile for these tropism modified viruses.

All viruses caused moderate release of transaminases, with values 3-fold higher than without virus. There were no significant differences between the groups. Histopathological analysis revealed fewer cytomorphologic hepatic findings with Ad5/3luc1. In the Ad5luc1 group, mild abnormalities were found, a few portal triads displayed mild chronic inflammation, some vascular leakage, and

mild scattered hepatocyte drop-out and necrosis within the parenchyma. Ad5/3luc1 caused only minimal changes, rare portal triad chronic inflammation, mild scattered parenchymal inflammation, and focal hepatocyte necrosis. Mice injected with Ad5lucRGD had focal hepatocyte necrosis, congestion, and moderate portal triad chronic inflammation, but no parenchymal inflammation. Transgene expression in the livers was slightly lower after injection with Ad5lucRGD and Ad5/3luc1 in comparison to Ad5luc1 ($P = 0.0472$ and not significant, respectively), which probably reflects differences in functional titers, as the ratio of vp/infectious particles was 5.2, 46 and 51 for Ad5luc1, Ad5/3luc1 and Ad5lucRGD, respectively.

Viruses and other foreign particles in the blood are cleared mostly by Kupffer cells (Worgall et al. 1997; Alemany et al. 2000). Further, the blood clearance of adenovirus is effective, with a half-life of less than 2 min after a single i.v. injection (Alemany et al. 2000). We compared the blood clearance rates of the fiber modified viruses to Ad5luc1 by collecting plasma samples 1-60 min after i.v. virus injection, and found no differences (Figure 1G in Study II). Further, the clearance rate of Ad5luc1 was similar to what has been reported by other groups (Alemany et al. 2000; Sakurai et al. 2003).

4. Biodistribution of fiber modified adenoviral vectors (II)

To obtain preclinical data on the biodistribution of the fiber modified viruses in the context of i.p. administration, we performed i.p. injection into orthotopic ovarian cancer tumor bearing mice (Figure 2 in Study II). At 48 hours, liver, spleen, kidneys, heart, lungs, peritoneum, brain and ovaries were harvested, and luciferase activity and protein concentration of tissue lysates were measured. Also, blood samples were collected, and blood cells separated and analyzed. When transgene expression was normalized to the amount of tissue analyzed (mg protein), the highest transgene expression was seen in the spleen, ovaries, peritoneum and liver. In the liver, luciferase activity was higher with Ad5/3luc1 in comparison to Ad5luc1, but the opposite was true after i.v. injection. This might result from the lower peritoneal uptake of Ad5/3luc1, subsequently allowing entry of Ad5/3luc1 into the circulation and eventually into the liver. We estimated the total uptake of virus by organ. The liver had the highest total transgene expression, followed by spleen, kidneys and ovaries. These results are in accordance with previous reports describing liver as the main organ expressing transgene in mice after i.v. injection of adenovirus (Wood et al. 1999; Reynolds et al. 1999). Further, the pattern for i.p. biodistribution of Ad5 has been subsequently corroborated by Barker *et al.* (Barker et al. 2003b).

Quantitative PCR was performed to detect virus copies in the liver. In contrast to the transgene expression data, fewer copies of Ad5/3luc1 were detected in the liver in comparison to Ad5luc1.

These findings could reflect differences in uptake by Kupffer cells *versus* hepatocyte transduction. Kupffer cells are generally reported to be ineffective in transgene expression (Tao et al. 2001; Wolff et al. 1997). Thus, if Ad5luc1 has higher relative tropism for Kupffer cells in comparison to Ad5/3luc1, the result could be less transgene expression but higher copy number. This is supported by the higher liver toxicity seen with Ad5luc1. Kupffer cells are the main cells mediating immunological responses in the liver and lower uptake could result in less inflammation (Zhang et al. 2001; Wolff et al. 1997). Clearly, this needs to be investigated in more detail in animals and more importantly, in humans. Contributing factors to the findings could include capsid dependent differences during post-entry steps. It is not well understood how adenovirus induces endosomal lysis and transport of its DNA to the nucleus, but it seems likely that the fiber plays a role (Shayakhmetov et al. 2003). Conceivably, the rate of degradation of adenovirus by innate immune mechanisms could also be a contributing factor. Importantly, the Ad3 receptor targeted virus is not circumventing the hepatocyte transduction. Therefore, the mitigation of liver toxicity needs other means for detargeting liver (transcriptional targeting).

All ovarian cancer gene therapy trials published so far have relied on i.p. administration of the agent. Thus, the normal tissue that is most closely in immediate contact with the agent is the peritoneal lining. We analyzed the infectivity of two human established mesothelial cell lines and three fresh primary peritoneum samples *in vitro* (Figure 3 in Study II). Data obtained in murine i.p. biodistribution experiment suggested significantly reduced peritoneal transduction with Ad5/3 chimera. However, the human mesothelial samples displayed increased infectivity with Ad5/3luc1 and in some cases, also with Ad5lucRGD. For CAR, it has been shown that the localization of the receptor in the context of the three dimensional structure is crucial in determining transduction (Walters et al. 1999). The situation could be similar for the Ad3 receptor. In the intact peritoneum, the receptor may not be accessible and thus infectivity is low, while in the subconfluent cell monolayer, the opposite might be true. Finally, it is likely that human and mouse mesothelial cells display different expression patterns of the Ad3 receptor and/or CAR. In any case, it is unclear if transduction of the peritoneum is likely to cause severe side effects to patients. In the ovarian cancer trials published for far, doses of up to 7.5×10^{13} vp of untargeted virus has been administered, and although abdominal pain was reported regularly, it was not dose limiting (Buller et al. 2002).

5. *In vitro* replication and ovarian cancer cell killing efficacy of Ad5/3-Δ24 (III)

The oncolytic potency of replicating agents is directly determined by the capability of infecting target cells (Douglas et al. 2001; Hemminki et al. 2001b). Consequently, infectivity enhanced CRAds have been constructed. With the aid of additional cellular receptors the oncolytic potency

has been increased in preclinical studies. Ad5- Δ 24RGD (Suzuki et al. 2001) features an RGD-4C modification of the HI loop of the knob, which allows binding to $\alpha_v\beta$ integrins, that are regularly expressed and often overexpressed on ovarian cancer cells (Dmitriev et al. 1998; Vanderkwaak et al. 1999; Hemminki et al. 2001b) and tumor vasculature (Arap et al. 1998). In the context of ovarian cancer, Ad5- Δ 24RGD demonstrated impressive oncolytic potential, and significantly increased survival in an animal model (Bauerschmitz et al. 2002b). Consequently, clinical trials utilizing this virus for ovarian cancer and glioma are in development (A. Hemminki, personal communication). Nevertheless, Ad5- Δ 24RGD continues to bind CAR, and is therefore not retargeted but infectivity enhanced instead.

In this study, we used fiber chimerism as a retargeting strategy. The Ad5/3- Δ 24 fiber features the knob from serotype 3, and this chimerism results also in enhanced infectivity, which translated into increased oncolysis of target cells. In all ovarian adenocarcinoma lines, the quantitative cell killing assay showed oncolysis with Ad5/3- Δ 24, while Ad5- Δ 24E3 (the isogenic control with the Ad5 fiber) caused minimal or no cell killing (Figure 2 in Study III). At the highest viral dose, the percentage of viable cells remaining with Ad5/3- Δ 24 was 0.5%, 5.6%, 15%, 2.7% and 5.5% for SKOV3.ip1, OV-4, OV-3, Hey and ES-2, respectively, as compared to uninfected wells. On all cell lines, oncolysis was significantly improved with fiber-modified Ad5/3- Δ 24 in comparison to Ad5- Δ 24E3 (all $P < 0.0033$). AdCMVHSV-TK was included as an *E1*-deleted control, and it did not cause oncolysis.

Ad5/3- Δ 24 was also compared to Ad5- Δ 24RGD on SKOV3.ip1 cells (Figure 6 in Study III). At the lower dose, Ad5- Δ 24RGD did not cause significant cell killing, while the percentage of surviving cells with Ad5/3- Δ 24 was 76%, 12% and 1.7% on days 5, 9 and 13 after infection, respectively. The difference was significant at every time point (all $P \leq 0.0048$). At the higher viral dose, the cell viability with Ad5/3- Δ 24 was 17%, 0.3% and 0.5% at the same time points, while with Ad5- Δ 24RGD the respective percentages were 92%, 35% and 1.2%. Statistically significant differences were found on days 5 and 9 ($P \leq 0.0022$). Oncolytic efficacy was evaluated also on two human primary ovarian cancer patient samples cultures as three dimensional spheroids. With patient sample A, there was no statistical significance between Ad5/3- Δ 24 and Ad5- Δ 24RGD. On patient sample B, Ad5/3- Δ 24 showed earlier cell killing than Ad5- Δ 24RGD, and the viabilities were 66% *versus* 84% ($P = 0.0020$) on day 8. In order to achieve effective oncolysis in patient sample B, higher viral dose was used (1 vp/cell *versus* 10 000 vp/cell). Lam *et al.* have reported similar results when they compared a panel of CRAds in the context of ovarian cancer (Lam et al. 2003b). Variation of CRAd DNA replication between different patient samples suggests that target tissue features, such as surface receptors and endogenous transcription factors, may affect CRAd

infectivity and replicativity. Evaluation of such factors may become important in optimizing cancer therapy for individual patients.

Further, Ad5/3- Δ 24 was able to replicate in ovarian cancer primary cell spheroids as measured with quantitative PCR (Figure 3 in Study III). Importantly, Ad5/3- Δ 24 was superior to control viruses in all patient samples. Therefore, the Ad3 receptor retargeted CRAAd effectively replicates in primary cancer cells and the replication kinetics are not adversely affected by the genetic fiber modification. This is always a danger related to modification of the virus genome, well demonstrated by ONYX-015, which expresses mutated E1B-55 kD protein unable to bind and inactivate p53 (Bischoff et al. 1996). E1B-55 kD has other functions than p53 binding, and therefore, the replication and oncolytic potency of ONYX-015 in comparison to wild-type adenovirus is low (Dix et al. 2001).

6. Therapeutic efficacy of Ad5/3- Δ 24 in an orthotopic ovarian cancer model (III)

In order to mimic a clinical situation of ovarian cancer, we inoculated advanced i.p. carcinomatosis into female CB17 SCID mice, followed by i.p. administration of viruses (Figure 4 in Study III). With a single injection of the virus, the median survival was not reached for Ad5/3- Δ 24, and 50% of mice were alive at the end of the experiment on day 135. For Ad5- Δ 24E3, AdCMVHSV-TK and no virus, the median survival times of mice were 96.5, 32 and 33 days, respectively. In comparison to the other groups, the overall survival of mice treated with Ad5/3- Δ 24 was statistically significantly improved (log-rank test $P < 0.0001$). Further, Ad5/3- Δ 24 allowed 80% survival on day 114, when all the control mice were dead.

A schema of three injections on consecutive days was also tested. Again, the median survival of mice treated with Ad5/3- Δ 24 was not reached, and 50% of mice were alive on day 135. For Ad5- Δ 24E3, AdCMVHSV-TK and no virus, the median survivals were 41.5, 33 and 32 days, respectively. The overall survival was significantly better in mice treated with Ad5/3- Δ 24 (log-rank test $P < 0.0001$).

The results obtained here could compare favorably to other reports (Bauerschmitz et al. 2002b; Hemminki et al. 2002b; Peng et al. 2002b), though the direct comparison to the other experiments is difficult due to different study designs. Interestingly, the survival with Ad5/3- Δ 24 using single injection *versus* multiple injections was not significantly different. However, the larger dose of Ad5- Δ 24E3 seemed to result in less anti-tumor efficacy in addition to giving increased toxicity. This might be due to liver toxicity, as we have seen the same phenomenon with a wild-type Ad5 (Kanerva et al. 2004). Converted weight/weight into humans, the smaller dose used here would

equal *ca.* 9×10^{10} vp. This is well below the 2×10^{12} vp daily for 5 consecutive days used in a CRAd trial, where the maximal tolerated dose was not reached (Vasey et al. 2002).

7. Ad5/3- Δ 24-hCEA replication *in vitro* and *in vivo* (IV)

As both safety and efficacy relate to persistence and replication of the CRAd, a secretory marker protein whose expression correlates with replication might allow non-invasive, repeatable detection of these features. Consequently, we constructed a retargeted CRAd featuring a secreted marker protein, Ad5/3- Δ 24-hCEA. We utilized soluble hCEA lacking the hydrophobic C-terminus, which would anchor the glycoprotein to the cell membrane. Trackable proteins have been evaluated in the context of oncolytic measles viruses (Peng et al. 2002a; Peng et al. 2002b). The results with hCEA expressing viruses have been promising *in vitro* and *in vivo*. Thus, we wanted to test the approach in the setting of replicating adenoviruses.

The hCEA is a 180 kD glycoprotein, which is expressed by many cancers, and widely used as a tumor marker (Goldenberg et al. 1981). Some patients with mucinous ovarian cancer or with recurring or stage III disease have detectable levels of hCEA in serum. However, most epithelial ovarian cancers do not express hCEA (Panza et al. 1988; Meier et al. 1997). Several possible functions have been suggested for the membrane bound form of hCEA. It plays a role in cell adhesion, might inhibit cell differentiation and promotes entering G_0 phase (Berinstein. 2002). It is not known if secreted hCEA has a biological function, but it is widely used as a routine clinical marker, which facilitates possible clinical utilization.

We introduced the hCEA gene into a partially deleted *E3* region of the virus (Figure 7). The *E3* region of adenovirus is an early transcription unit encoding at least seven proteins. Interestingly, the *E3* region is non-essential for viral replication, but the *E3* proteins have an important role in the regulation of host immune response. Specifically, they inhibit both innate and specific immune responses. Further, it is known that deletion of the *E3* region results in increased lymphocyte, macrophage and monocyte inflammatory responses (Horwitz. 2001). The gp19K binds and sequesters MHC I molecules in the endoplasmic reticulum, and therefore prevents the recognition of infected cells by cytotoxic T-lymphocytes (CTLs) (Burgert and Kvist. 1985). This reduces CTL mediated cytotoxicity of infected cells. Interestingly, this is another feature shared between the adenovirus life cycle and carcinogenesis, as also tumor cells can evade CTL lysis with various methods. Thus, a gp19K deleted virus, such as ours, might allow infection and subsequent productive replication preferentially in tumor cells, as infected normal cells would be effectively recognized by CTLs. This could provide an additional level of selectivity, in addition to the 24-bp deletion in *E1A*, which restricts virus replication to Rb/p16 pathway deficient cells.

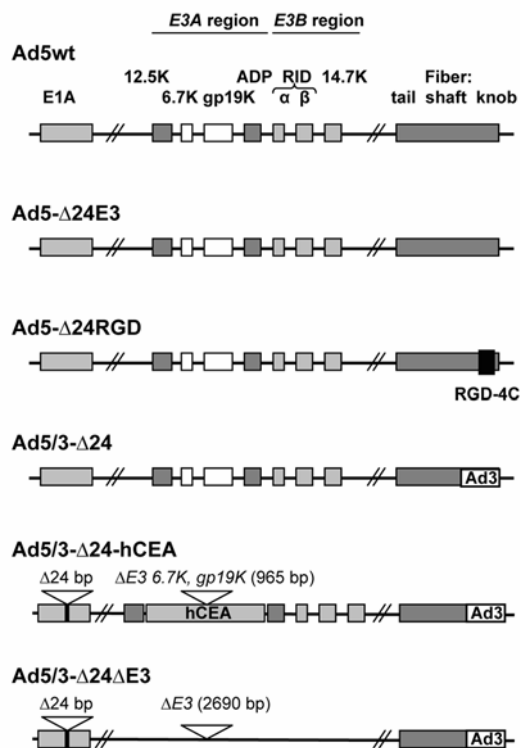


Figure 7. Schematic illustration of the CRAds used in this study. Viruses have a 24-bp deletion in the CR2 of the E1A gene, corresponding to the region responsible for Rb protein binding. This results in an agent replication competent and oncolytic in cells defective in Rb/p16 pathway. Ad5-Δ24RGD has an RGD-4C motif in the HI loop of the fiber knob. The fiber of Ad5/3-Δ24, Ad5/3-Δ24-hCEA and Ad5/3-Δ24ΔE3 is modified to incorporate the serotype 3 knob, while retaining the Ad5 shaft and tail. In addition, Ad5/3-Δ24-hCEA has an hCEA gene inserted under control of endogenous viral expression signals in the partially deleted *E3A* region, while Ad5/3-Δ24ΔE3 has the complete *E3* region deleted. Ad5-Δ24RGD, Ad5-Δ24E3 and Ad5/3-Δ24 have an intact *E3* region. For comparison, wild-type adenovirus serotype 5 is also shown.

Another protein deleted in our construct is 6.7K, which is expressed on cellular surface. It induces, as a complex with other E3 proteins, down-regulation of TNF-related apoptosis inducing ligand (TRAIL) receptor 2 (Benedict et al. 2001). Lack of this protein could further restrict replication of the virus in normal cells. The *6.7K/gp19K*-deletion has been demonstrated to allow effective expression the remaining E3, especially adenoviral death protein (ADP), and structural proteins (Hawkins et al. 2001). ADP is expressed later during the virus cycle, and it contributes to cell lysis and release of virus progeny (Tollefson et al. 1996). All the E3B proteins inhibit inflammation after adenovirus infection *in vivo*. Importantly, by creating a partial deletion we may be able to enhance the selectivity of replication while retaining functions which are conducive for effective oncolysis, such as ADP expression.

In this study, we saw correlation between Ad5/3-Δ24-hCEA replication and hCEA production *in vitro* (Figure 1 in Study IV). Further, the 2 kb transgene did not affect replication significantly as compared to an *E3* deleted control. Importantly, we were able to follow the virus kinetics *in vivo* in i.p. tumor bearing mice (Figure 2 in Study IV). Furthermore, none of the analyzed mice treated with a control virus Ad5/3-Δ24ΔE3 had measurable hCEA concentrations in plasma. All mice treated with Ad5/3-Δ24-hCEA survived the duration of the experiment, while all untreated mice died due to progressive carcinomatosis. In comparison to the untreated mice, the overall survival of mice

treated with Ad5/3- Δ 24-hCEA was improved (log-rank test $P < 0.0001$). However, none of the Ad5/3- Δ 24-hCEA treated mice were tumor free on day 59 at the end of the experiment. Residual i.p. tumors were harvested and virus copies were measured with quantitative PCR. All of the Ad5/3- Δ 24-hCEA treated mice had residual tumors showing up to 2×10^6 E4 copies per ng of cellular β -actin, which suggested virus replication. Only four out of seven mice had residual i.p. tumors in Ad5/3- Δ 24 Δ E3 treated group. Again, virus was detected in the tumors at high levels (up to 3×10^7 copies).

Nevertheless, most of the mice treated with Ad5/3- Δ 24-hCEA displayed increase in plasma concentration of hCEA after each virus injection, suggesting productive replication and concomitant anti-tumor efficacy. Further, some of them had high plasma hCEA levels during the last week of the experiment suggesting strong replication. Interestingly, the hCEA curves of these mice were comparable to the others during the first 5 weeks of the experiments. Therefore, it seems possible that a balance between virus replication and tumor cell growth was achieved. Alternatively, although episomal persistence of adenoviral DNA has been reported (Ehrhardt et al. 2003), our results suggest that in some cases, tumor cells can acquire resistance to CRAds. Thus, it may be possible that although few tumor cells were remaining, they had gained resistance to oncolysis but continued to allow Ad5/3- Δ 24-hCEA DNA replication and subsequent hCEA expression. Acquired resistance to cytolysis in ovarian cancer cells has been reported with ONYX-015 (Kim et al. 2003). Ten weeks after infection of the initially sensitive cells, intracellular hexon protein was found in immunofluorescence and FACS analyses. Plaque assay from cell lysate demonstrated that cells resistant to cytolysis contained infectious viruses. Further, these cells were resistant to the oncolysis of a wild-type Ad2, as well as ONYX-015. However, these cells were transducible with *E1*-deleted adenovirus expressing a lacZ transgene. Thus, there might be a block at the lytic stage in these cells (Kim et al. 2003).

8. Dual modality monitoring of CRAAd efficacy *in vivo* (III, IV)

Non-invasive imaging technologies provide fundamental safety and efficacy information on experimental therapy approaches such as gene therapy (Gambhir et al. 1999; Min and Gambhir. 2004). They may allow more efficient utilization of orthotopic animal models, which otherwise are problematic as tumors are not easily accessible to measurements. Further, another important feature of non-invasive imaging is the possibility of performing repeated measurements. Therefore, various imaging systems have been evaluated (Gambhir et al. 1999; Min and Gambhir. 2004). For example, expression of somatostatin receptor subtype 2, coded by an adenovirus vector, can be imaged with radioisotope gamma camera after administration of the somatostatin analogue ^{99m}Tc -P2045

(Hemminki et al. 2002b). Also, optical charge-coupled device (CCD)-imaging has been used to detect bioluminescence emitted from D-luciferin reacting with firefly luciferase, coded by an adenovirus vector. Other approaches include magnetic resonance and positron-emission tomography imaging of positron-emitting ligands such as imaging of sodium/iodide symporter transgene after systemic injection of ^{124}I (Groot-Wassink et al. 2004). Furthermore, cancer cells expressing reporter genes such as firefly luciferase and green fluorescence protein are useful means of following tumor growth.

We used an orthotopic ovarian cancer model with SKOV3-luc cells, which emit light after i.p. administration of D-luciferin (Figure 5 in Study III). Using *in vivo* bioluminescence imaging, we were able to detect and measure i.p. tumor cell killing by the virus. Oncolytic killing of tumor cells corresponded with reduction of signal in comparison to control animals. Ad5/3- Δ 24 treated mice initially responded, but then relapsed with s.c. tumors in the needle-tract, an intrinsic defect of this animal model (Bauerschmitz et al. 2002b). Nevertheless, the sensitivity of the assays was sufficient to allow detection of i.p. cell killing. A further round of imaging was performed without the abdominal wall to distinguish the anti-tumor without the confounding s.c. tumors, and found that light emitted from the peritoneal cavity was only 4% of the untreated group, suggesting effective killing of tumor cells by the virus.

When plasma hCEA measurements were combined with non-invasive bioluminescence imaging, it was possible to correlate virus replication to anti-tumor efficacy (Figure 3 in Study IV). The level of emitted light in the Ad5/3- Δ 24-hCEA treated group did not increase during the experiment, and there were no significant differences in mean photon count at any time point *versus* baseline on day 7. However, the untreated mice emitted significantly more light over the time period. On day 35 photon counts had increased up to 131-fold ($P = 0.0082$, as compared to day 7). Light emitted from the peritoneal cavity of Ad5/3- Δ 24-hCEA treated mice was only 0.4% of the signal of the untreated group on day 35 ($P = 0.0097$, compared to untreated) suggesting effective killing of tumor cells by the virus. An overall increase in amount of emitted light over time in the untreated group compared to the Ad5/3- Δ 24-hCEA group was significant ($P = 0.0069$).

Concomitant analysis of hCEA levels suggested strong early replication, when the tumors were larger. Then, two weeks later the replication was already slowing down, but the photon count was at its highest. Finally, both values decreased to baseline in two out of three mice. This suggests that hCEA secretion was primarily an early event and at its maximum during the intratumoral and i.p. dissemination of the virus, while the actual dying of the cells was a slower process during which bioluminescent imaging was still possible. Of note, the detected hCEA in plasma was expressed during virus replication in the tumor tissue, as human adenoviruses do not replicate productively in

normal murine cells (Blair et al. 1989). However, recent study suggests that murine cancer tissue might support some level of replication (Hallden et al. 2003). Importantly, the mice treated with control virus did not display measurable levels of hCEA.

Oncolytic viruses expressing trackable marker proteins might represent a new generation of anti-tumor agents, which can be dynamically monitored. These would be highly useful tools for evaluating the replication, persistence and efficacy of the viruses.

SUMMARY AND CONCLUSIONS

Previous studies have suggested that CAR-deficiency on cancer cells hinders the efficacy of adenovirus mediated gene transfer. In order to circumvent this obstacle, we created a retargeted *E1*-deleted virus. Specifically, we explored substituting the receptor binding fiber knob domain of Ad5 with the serotype 3 knob (Ad5/3luc1). This resulted in CAR-independent transduction of ovarian cancer cells, as Ad3 has a distinct, but unidentified receptor. Our studies suggest, that in comparison to CAR, the Ad3 receptor is expressed at higher levels on ovarian cancer cells, and that 5/3 serotype chimeras bind to the Ad3 receptor but not to CAR. Importantly, we show that exploiting the different tropism of Ad3 leads to enhanced infectivity of ovarian cancer cell lines and human primary cancer cells.

In order to evaluate preclinical toxicity of the Ad5/3 chimera, we explored murine liver toxicity after i.v. injection of the virus, and the results were comparable among the groups. Further, we evaluated the murine biodistribution after i.p. administration of the viruses. Transgene expression was analyzed in the panel of tissues. Significant differences were found in peritoneum, where Ad5/3-mediated transgene expression was lower. As a final experiment for the murine safety profile, we analyzed virus blood clearance rates after i.v. injection. All the clearance curves were similar showing rapid elimination of the virus from the blood stream.

CRAds represent a novel approach for treating neoplastic diseases. However, the oncolytic potency of replicating agents is directly determined by their capability of infecting target cells. Therefore, we created the Ad3 receptor retargeted CRAd, *i.e.* Ad5/3- Δ 24. This novel oncolytic virus, in addition of having the chimeric fiber, expresses a mutant E1A protein unable to bind the Rb protein. This binding normally allows adenovirus to induce S-phase entry, needed for virus replication. Therefore, Ad5/3- Δ 24 replicates only in the cancer cells inactive in the Rb/p16 pathway, which may include most human cancers. We compared Ad5/3- Δ 24 to the non-fiber modified isogenic control virus. We demonstrated that retargeting a selectively oncolytic adenovirus to the Ad3 receptor results in improved infectivity of ovarian cancer cells, and overcomes the CAR-deficiency on primary cancer cells. Consequently, therapeutic efficacy was dramatically increased *in vitro* and *in vivo*. Further, we have demonstrated that Ad5/3- Δ 24 allows cell killing comparable or superior to previously described Ad5- Δ 24RGD.

However, the major problem in following CRAd efficacy in humans has been the lack of the method to repeatably measure CRAd spreading and elimination. To address this issue, we created a modified version of Ad5/3- Δ 24. Ad5/3- Δ 24-hCEA has an hCEA gene in the partially deleted *E3* region. We demonstrated that during virus replication, soluble hCEA expressed from a selectively

oncolytic adenovirus can be measured in growth medium or plasma. Further, this allowed us to follow the persistence and anti-tumor efficacy of the virus *in vivo*. Finally, we compared the replication kinetics to tumor eradication evaluated by *in vivo* imaging. These developments could be useful for monitoring CRAAd replication in humans, which might increase the quantity and quality of correlative data obtained in early phase cancer gene therapy trials.

Ad5/3- Δ 24 could be an effective agent for treatment of ovarian cancer and other tumors with an inactive Rb/p16 pathway and high expression of the Ad3 receptor. Of note, this approach might be further advanced when combined with TSP driving the *E1A* expression. Clinical trials will ultimately show if preclinical advances such as reported here can be translated into similar progress in the treatment of cancer patients.

Effective tumor transduction continues to be the limiting step for achieving clinical results with adenovirus vectors. Therefore, it is likely that replicating agents, CRAAds and others, will become increasingly popular. Another central realization in the adenovirus field has been that the primary receptor, CAR, is often expressed at a very low level on primary tumor tissue. Thus, it is likely that targeting strategies will significantly improve efficacy in clinical trials. Though other viral vectors might be more useful for treatment of hereditary diseases, adenoviruses are highly promising and safe agents for oncology, as suggested in number of phase I trials. Finally, considering the synergism and a lack of cross resistance of CRAAds with chemotherapy and radiation therapy, the combination treatment with existing modalities could be the future of CRAAds in oncology.

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