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TARGETING ADENOVIRAL GENE THERAPY VECTORS TO HEAD AND NECK SQUAMOUS CELL CARCINOMA AND HEART

by

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To my family

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Targeting adenoviral gene therapy vectors to HNSCC and heart

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Abstract

Gene therapy aims to treat diseases by introducing genetic material to the diseased tissue. For cancer treatment it is important to destroy cancerous cells; this can be achieved by introducing a gene, which induces cell death or by allowing viral vectors to replicate, which also results in destruction of cancerous cells. For cardiac diseases the approach is more like the former, except the gene produces beneficial effects, like angiogenesis.

Adenoviruses have many beneficial qualities, which make the virus an interesting gene therapy vector; it can be produced relatively easily, its manipulation is quite easy and it has naturally broad tropism. By removing or replacing certain genes in the adenoviral genome, it can be made non-replicative.

In this study, adenoviral receptor expression patterns were characterized in both head and neck squamous cell carcinoma and the human heart. Adenovirus serotype 5 receptor expression in head and neck cancer cell lines was found to be highly variable between cell lines and overall at lower levels, while Ad35 receptor expression was more uniform and at higher levels in all analyzed cell lines. It was also shown that a hybrid virus Ad5/35 is able to infect cells refractory to Ad5, which correlates with receptor expression in these cells. Furthermore, this difference in infection properties extends to cell killing efficiency in case of conditionally replicative viruses. Expression levels of adenoviral receptors CAR, CD46, CD86 and α_v-integrins were found to be high both in normal and dilated cardiomyopathy heart tissue. The receptor levels also correlate with transduction efficiency after intracardiac injection. Ad5 showed superior transduction ability compared with Ad5/35, but evoked also a more profound immune reaction when administered this way.

Adenoviral gene therapy vectors are the most used delivery vehicles in clinical trials to date. These vectors have proven to be well tolerated and positive results have been obtained when combined with traditional treatments, although poor transduction efficiency has often been reported due to low-level expression of viral receptors on target cells. In spite of this, the results are encouraging and merit for further research.

Key words: gene therapy, adenovirus, targeting

Raine Toivonen
Adenovirus geeniterapia vektoreiden kohdentaminen pään ja kaulan alueen syöpään sekä sydämeen

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Tiivistelmä

Geeniterapialla pyritään hoitamaan sairauksia lisäämällä geneettistä materiaalia sairastuneeseen kudokseen. Syövän hoidossa on tärkeää tuhota syöpäsolut; tämä toteutetaan ”itsemurhageenin” avulla, joka aikaansaa solukuoleman, tai sallimalla virusvektorin replikoitua, jolloin solukko tuhoutuu viruksen toiminnan myötä. Sydänsairauksien hoidossa ei pyritä tuhoamaan kudosta, vaan käytettävällä geenillä on myönteisiä vaikutuksia sydämen toimintaan, kuten verisuonien uudismuodostuksen aikaansaaminen.
Adenoviruksillla on monia hyviä ominaisuuksia, joita geeniterapiavektorilta vaaditaan: helppo tuotettavuus, yksinkertainen manipulointi ja laaja tropismi. Poistamalla tiettyjä geenejä adenoviruksen genomista sen replikaatiokykyä pystytään säätelemään.
Tässä tutkimuksessa tarkasteltiin adenovirusreseptoreiden ekspressiota sekä pään ja kaulan alueen syövässä että sydänkudoksessa. Ad5-reseptorin eli coxsackie-adenovirus-reseptorin ilmentyminen pään ja kaulan alueen syövässä todettiin olevan hyvin vaihtelevaa solulinjojen välillä ja kaiken kaikkiaan alentunut normaaliin verrattuna. Päinvastaisesti Ad35-reseptorin, CD46:n, ilmentymisen todettiin olevan yhdenmukaisempaa solulinjojen välillä ja korkeaa kaikissa analysoiduissa solulinjoissa. Tutkimuksessa osoitettiin myös, että hybridivirus Ad5/35 pystyy infektoimaan soluja, joita Ad5 ei pysty. Lisäksi tämä ero ulottuu myös solujen tuhoamiskykyyn ehdollisesti lisääntyvillä adenoviruksilla. Eri adenovirusreseptoreiden ilmentymistasojen todettiin olevan yhdenmukaisempaa ihmisen sydänkudoksessa kuin syöpäsolulinjojen välillä. Lisäksi coxsackie-adenovirus-reseptorin ilmentymisen todettiin olevan voimakkainta sydänkudoksessa muihin adenovirusreseptoreihin verrattuna. Kudoksen reseptoritasot vaikuttavat adenovirusten infektiivisyyteen myös sydänkudoksessa, mutta Ad5 todettiin hyvin toksiseksi suoran sydänlihasinjektion jälkeen.
Adenovirus-geeniterapiavektorit ovat eniten käytettyjä vektoreita kliinisissä kokeissa. Niiden on osoitettu olevan hyvin siedettyjä ja ne kykenevät parantamaan nykyisten hoitomuotojen tehoa. Tosin heikko infektiivisyys kliinisissä olosuhteissa kertoo lisätutkimusten tarpeesta.

Avainsanat: geeniterapia, adenovirus, kohdentaminen

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Abbreviations

AAV	Adeno-associated Virus
Ad	Adenovirus
ADP	Adenovirus Death Protein
CAR	Coxsackie and Adenovirus Receptor
CPE	Cytopathic Effect
CRAd	Conditionally Replicating Adenovirus
CMV	Cytomegalovirus
CVD	Cardiovascular Disease
DCM	Dilated Cardiomyopathy
E1A	Adenovirus Early gene 1 transcriptional unit A
E1B	Adenovirus Early gene 1 transcriptional unit B
E2A	Adenovirus Early gene 2 transcriptional unit A
E2B	Adenovirus Early gene 2 transcriptional unit B
E3	Adenovirus Early gene 3
E4	Adenovirus Early gene 4
EGF	Endothelial Growth Factor
EGFR	Endothelial Growth Factor Receptor
fX	Blood Coaculation Factor X
HF	Heart Failure
HGF	Hepatocyte Growth Factor
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
Hsc70	70 kDa Heat Shock Cognate protein
hTERTp	Human Telomerase Promoter
hTRp	Human Telomerase RNA subunit Promoter
i.v.	Intravenous
L1	Adenovirus Late gene 1
L2	Adenovirus Late gene 2
L3	Adenovirus Late gene 3

L4	Adenovirus Late gene 4
L5	Adenovirus Late gene 5
LacZ	β-galactosidase encoding gene from *Escherichia coli*
LITR	Left Inverted Terminal Repeat
miRNA	Micro RiboNucleic Acid
MLP	Adenovirus Major Late Protein gene
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAb	Neutralizing Antibody
pRb	Retinoblastoma protein
RITR	Right Inverted Terminal Repeat
scFv	Single-chain Fv antibody Fragment
SR	Sarcoplasmic Reticulum
UT-SCC	Squamous cell carcinoma cell line established in Turku University Hospital
VEGF	Vascular Endothelial Growth Factor

List of Original Publications

This thesis is based on the following publications, referred to in the text by their roman numerals. The original publications have been reproduced with permission from the copyright holders. Unpublished data is also included.

I Suominen Elina, Toivonen Raine, Grenman Reidar and Savontaus Mikko (2006). Head and Neck Cancer Cells are Efficiently Infected by Ad5/35 Hybrid Virus. *J. Gene Med.* **8(10):** 1223-1231.

II Toivonen Raine, Suominen Elina, Grenman Reidar and Savontaus Mikko (2008). Retargeting Improves the Efficacy of a Telomerase-Dependent Oncolytic Adenovirus for Head and Neck Cancer. *Oncology Reports* **21:** 165-171.

III Toivonen Raine, Mäyränpää Mikko, Kovanen Petri and Savontaus Mikko (2010). Dilated Cardiomyopathy Alters the Expression Patterns of CAR and Other Adenoviral Receptors in Human Heart. *Histochem. Cell Biol.* **133(3):** 349-357. Epub 2009 Dec 2.

IV Toivonen Raine, Merentie Mari, Söderström Mirva, Ylä-Herttuala Seppo and Savontaus Mikko (2010). Intracardiac injection of a capsid-modified Ad5/35 results in decreased heart toxicity when compared to standard Ad5. (manuscript)

1. Introduction

Gene therapy holds great potential and can be used for treatment of both cancer and cardiovascular disease. Despite the recent advances in treatment modalities, cancer and cardiovascular diseases are the leading causes of death in the western world. The mortality of both diseases has been steadily declining in the past decades, but recently the decrease has significantly slowed down and a plateau has been reached. Currently available treatment methods are invasive and lack in efficacy, especially for cancer. Unfortunately both diseases comprise of a plethora of variable conditions and gene therapy must be designed for each case individually, from the vector to the transgene used.

Two facts make head and neck squamous cell carcinoma (HNSCC) one of the most promising targets for cancer gene therapy; HNSCC tumors are usually relatively easily accessible for intratumoral injection and local treatment (Fuller, C. D., *et al.* 2007, Khuri, F. R., *et al.* 2000), and metastasis is a late-stage occurrence and could be avoided by local treatment of early tumors (Goebel, E. A., *et al.* 1996, Blackwell, J. L., *et al.* 1999). About 500 000 new cases of HNSCC are diagnosed annually worldwide (Parkin, D. M., *et al.* 2005). Diagnosed patients usually have already local advanced tumors and are in need of surgery and/or radiotherapy. Even after treatment tumors recur in over 30 % of patients. Recurring tumors respond very poorly on current treatment methods and are considered incurable (Vokes, E. E., *et al.* 1993, van Dongen, G. A. and Snow. 1997).

Cardiovascular diseases are one of the leading causes of death in the western world. Available treatment modalities for end-stage heart failure caused by either ischemic or dilated cardiomyopathy (DCM) are limited and include lifestyle changes, medicines, surgery and implantation of electronic devices. These treatments deal mainly with symptoms and prevent disease progression, but will not cure the disease. In some cases DCM is an inherited condition in which case the only treatment available is medication to alleviate the symptoms and prevention of disease progression. Gene therapy could present a way to introduce long lasting effects and more efficient disease control. Most gene therapy applications aimed at treating cardiac diseases involve the promotion of angiogenesis in the damaged area of the heart. Many preclinical studies, including both viral and non-viral methods, have shown the importance of efficient gene delivery to the target tissue, in order to achieve reasonable therapeutic efficacy (Asaoka, K., *et al.* 2000, Douglas, J. T., *et al.* 2001, Dirven, C. M., *et al.* 2002, Joung, I., *et al.* 2005).

Adenoviruses (Ads) are natural human pathogens and can induce from mild to severe side effects. Also, the fact that most people have suffered an Ad infection and thus have neutralizing antibodies (NAbs) in their antibody repertoire, contributes to Ad toxicity and the effects after Ad vector administration vary greatly in different individuals. Adenovirus vector toxicity has been discussed ever since the first preclinical experiments were conducted. Although Ads can potentially cause even systemic inflammatory response, the most common side effects reported in clinical

studies are flu-like symptoms, such as fever, nausea and pain in the injected area (Nemunaitis, J., *et al.* 2001, Hamid, O., *et al.* 2003, Small, E. J., *et al.* 2006). A more severe side effect is the inflammation of the liver or spleen, which can occur when liver cells are infected by Ad or the Ad vector is actively taken up by liver cells (Huard, J., *et al.* 1995, Kalyuzhniy, O., *et al.* 2008). Retargeting Ad vectors effectively reduce the risk of liver infection, but since there are other mechanisms that result in liver transduction than specific receptor recognition, it may be necessary to "mask" or hide the viral vector from the host immune system and blood factors that bind the vector. The toxicity and infection of unwanted organs can be reduced by using modified Ad vectors (Shayakhmetov, D. M., *et al.* 2004), choosing carefully the administration strategy and keeping the vector doses within safe limits (Raper, S. E., *et al.* 2003).

Retargeting of adenoviruses has been shown to be of utmost importance for achieving safer and more efficient gene therapy vectors. The most commonly used adenovirus serotype 5 (Ad5) can readily infect liver cells, but in many cases it infects poorly the target tissue (Smith, T. A., *et al.* 1993, Hemmi, S., *et al.* 1998, Li, D., *et al.* 1999). Ad5 has been used extensively and has many advantages over other viral vectors and non-viral gene delivery methods. Ad5 can be readily produced in high titers, manufacturing processes have been well established, Ads readily infect both dividing and quiescent cell types, gene expression is transient and Ad genome does not integrate into the host genome. Broad tropism, however, is also a problem; too many unwanted organs and cell types can be infected leading to detrimental side effects. Thus, many strategies for retargeting the Ad vector have been developed. Three main retargeting systems are discussed here: 1) fiber exchange, part of a different serotype fiber protein is cloned to replace the corresponding native part; 2) fiber engineering, parts of fiber protein are mutated or sequences are added, and 3) two-component approach, where a separate molecule is used as a bridge between the vector and target receptor. All three retargeting systems have advantages and limitations and depending on the application one might be better suited than another. It is very unlikely that we will see a "universal" vector construction in the future but instead, many tailor-made constructs for each disease or patient group.

2. Review of the Literature

2.1. Adenoviral gene therapy vectors

Adenovirus serotype 5 is an interesting vector for gene therapy applications. Ad5 naturally infects a broad range of host cells, including both dividing and quiescent cells. This natural human pathogen is known to cause from mild to severe diseases depending on the site of infection and the serotype involved. Major syndromes caused by Ads are: 1) acute respiratory syndrome, 2) pharyngoconjunctival fever, 3) epidemic keratoconjunctivitis, 4) acute hemorrhagic cystis, and 5) gastroenteritis. Transmission of Ad can be via droplet inhalation, fecal-oral route, or exposure to infected blood or tissue. Even though Ad can survive prolonged periods outside the host severe morbidity is rare in immunocompetent adults. Ad is a non-enveloped icosahedral virus. It has been shown to tolerate modifications quite well and these modifications to the capsid, which consists mainly of three major capsid proteins hexon, penton and fiber, can be done relatively easily. Also the production and purification methods of Ad5 are well established. Unfortunately, there are also some major drawbacks in using native Ad5 as a gene therapy vector. Major problems and some solutions are discussed below.

2.1.1. Adenovirus life cycle

To date there are 52 different serotypes of Ads, divided in six different subgroups from A to G (Jones, M. S.,2nd, *et al.* 2007). Each subgroup has natural tropism for specific tissue and the physiological symptoms depend on the site of infection. Adenoviruses attach to cell-surface receptors when starting their infection cycle (Figure 1). These receptors vary between different Ad serotypes. The most common Ad receptor is coxsackie and adenovirus receptor (CAR). Ads from all subgroups other than subgroup B have been found to utilize this receptor for primary attachment. Subgroup B has been further divided into two groups; B1 and B2 and CD46 has been shown to be a common receptor for these groups (Segerman, A., *et al.* 2003b, Gaggar, A., *et al.* 2003, Sirena, D., *et al.* 2004). Group B1 has been shown to use the cell-surface molecules CD80 and CD86 as attachment receptors (Short, J. J., *et al.* 2004). In addition, various Ad serotypes utilize sialic acid, and heparan sulfate glycosaminoglycans for cellular attachment. After initial attachment the virus binding triggers clustering of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins. These integrins take part in the internalization of virus particles as viral protein penton base binds to these integrins (Chiu, C. Y., *et al.* 1999). Activation of α_v-integrins induces endocytosis. Once the virus is inside the cell, in a vesicle, it needs to escape. The insides of the vesicle are destroyed by low pH. The Ad particle resists complete destruction and instead, it starts to disassemble and eventually escapes the vesicle into the cytosol. Ad remains attached to the microtubules and is transported to the nuclear membrane where the viral capsid is further disassembled and the viral DNA is released into the nucleus (Leopold, P. L., *et al.* 2000, Suomalainen, M., *et al.* 2001). Within a few hours after infection the first Ad genes are transcribed.

After infecting a cell the virus must protect the cell from host responses. Normally, when a cell is infected by a virus it undergoes apoptosis to prevent the virus from spreading into other parts of the organism. This is normally done by activation of a controlled cell death program. One major signaling molecule in this program is p53, which acts as a promoter of cell death. Adenovirus prevents apoptosis by interfering with this cell death program. The first expressed Ad genes are E1A, E1B, and E3. E1A codes for a protein which binds to human retinoblastoma protein (pRb). pRb prevents the replication of damaged DNA (and foreign DNA) by halting the cell cycle to the G1 phase and the cell eventually undergoes p53-mediated apoptosis, if DNA damage cannot be repaired (Figure 2). When the E1A gene product binds pRb, it cannot function properly and the cell cycle is allowed to continue regardless of the presence of foreign DNA.

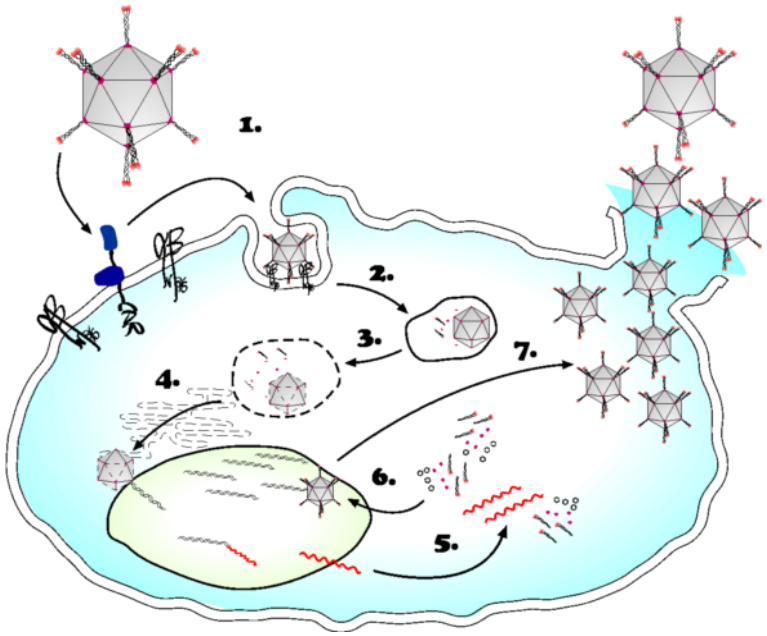


Figure 1. Adenovirus replication cycle. 1.) A virus particle attaches to a cell-surface receptor (CAR in case of Ad5). After attachment, signaling from the receptor leads to clustering of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. These integrins recognize penton base on the Ad and start the internalization of the virus. 2.) Ad is transported into the cell in a vesicle. Low pH destabilizes the viral coat and Ad is partially degraded. Partial degradation allows the virus to escape the vesicle. 3.) The vesicle is disrupted and Ad is able to escape into the cytosol. 4.) In the cytosol Ad is transported to the nuclear membrane via microtubules. At the nuclear membrane viral DNA is released into the nucleus. In the nucleus viral DNA is copied to make more viral genomes and viral genes are transcribed into mRNA (red). 5.) Viral mRNA acts in the same way as host mRNA and is recognized by cellular protein synthesis machinery, which will produce viral proteins. 6.) Viral structural proteins are transported back to nucleus, where assembly of virions takes place and viral DNA is packaged into virions. 7.) Viral particles are transported to the cellular membrane and after either overload of viruses or active disruption of cell membrane by adenoviral death protein, the cell bursts and new a batch of viral particles are released into extracellular space.

The gene product of E1B binds to the aforementioned cellular p53 and effectively prevents the infected cell from undergoing apoptosis, allowing the virus time to replicate and complete its life cycle (Liu, Y., *et al.* 2000). Ad gene E3 has similar functions as E1B. The gene product of E3, 10.4K/14.5K complex, removes the CD95 receptors from the cell surface. This prevents the cell from undergoing Fas-induced TNF-dependent apoptosis (Shisler, J., *et al.* 1997). These three early genes inhibit cell death during the virus replication cycle and can be exploited in gene therapy applications. In addition, the product of the fourth early gene, E4, has two different splice variants: the first one inhibits the destruction of infected cells by targeting cellular p53 for destruction thus augmenting the effects of E1B (Querido, E., *et al.* 2001). The second splice variant interferes with the host INF-α and INF-γ signaling which inhibits host antiviral responses (Ullman, A. J., *et al.* 2007). Other Ad genes produce all the necessary proteins needed for replication of the Ad genome and production and assembly of new virions. Ad capsid proteins are synthesized in the cytoplasm by the host translation machinery, after which these structural proteins are transferred to the nucleus by viral protein VI, where the assembly of new virions takes place. Copies of Ad genomes are packaged into the virions and immature viruses mature while being transported through the cytosol to the plasma membrane. New viruses are released to the extracellular space when the adenovirus death protein (ADP) facilitates the lysis of the host cell. ADP is also coded by gene E3, but as a different splice variant and under MLP promoter at a late stage of the infection cycle (Tollefson, A. E., *et al.* 1996).

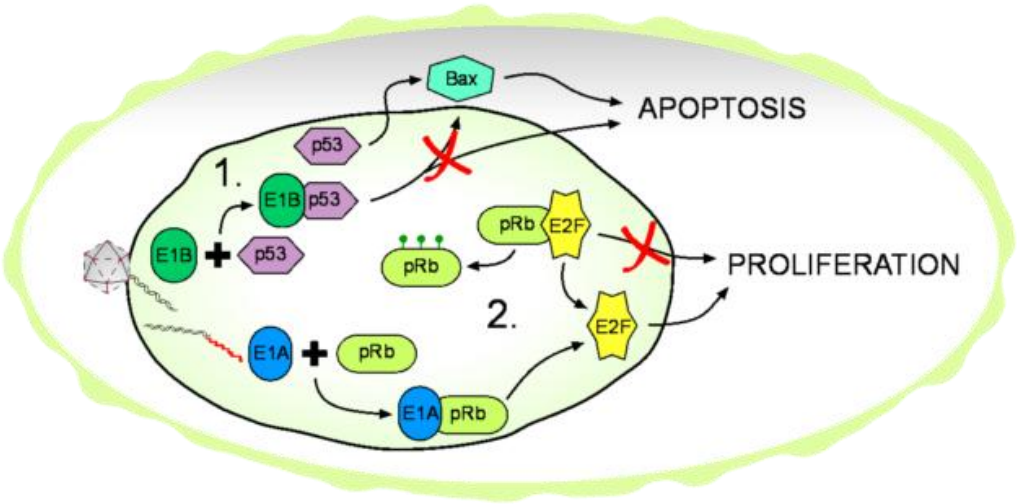


Figure 2. Simplified schematic description of E1A and E1B function. 1) Normally, when p53 is active it induces apoptosis by activating Bax. When adenoviral E1B binds to p53 it cannot activate the apoptotic pathway. 2) When DNA is damaged pRb stays bound to transcription factor E2F and the cell cycle is arrested in G1 phase. If no DNA damage is detected pRb is phosphorylated and E2F is released and the cell cycle is allowed to proceed. When pRb is bound by E1A it cannot associate with E2F and the cell cycle cannot be arrested.

2.1.2. Adenoviral receptors

Adenoviruses of different serotypes utilize various cellular receptors for primary attachment, when starting the infection of a host cell (Table 1). These receptors are specifically recognized by the fiber proteins protruding from the Ad capsids (Levine, A. J. and Ginsberg. 1967, Philipson, L., *et al.* 1968).

Table 1. Adenoviral receptors.

Receptor	Native function	Reference
CAR	Tight junction structural protein	(Cohen, C. J., *et al.* 2001)
CD46	Complement regulatory protein	(Liszewski, M. K., *et al.* 1991)
CD80	T-cell activation costimulatory molecule	(Freeman, G. J., *et al.* 1989)
CD86	T-cell activation costimulatory molecule	(Caux, C., *et al.* 1994)
Sialic acid	Ubiquitous cell surface receptor	
Heparan sulfate glycosaminoglycans	Varies from structural to cell-to-cell signaling	(Kolset, S. O., *et al.* 2004)
$\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins	Cell adhesion molecule and endocytosis activation signaling	(Smith, J. W., *et al.* 1990)

2.1.2.1. Coxsackie and Adenovirus Receptor

Coxsackie and adenovirus receptor is a common receptor for group B coxsackie viruses and various Ad serotypes (Bergelson, J. M., *et al.* 1997). CAR is a 46 kDa cell adhesion molecule belonging to immunoglobulin superfamily. After being identified as a virus attachment receptor CAR has been shown to be located in tight junctions and takes part in the regulation of junction formation (Cohen, C. J., *et al.* 2001, Coyne, C. B., *et al.* 2004, Raschperger, E., *et al.* 2006). It has also been hypothesized that CAR acts as a pathfinder molecule during fetal development. Interestingly, for example, after cardiac damage CAR is up-regulated. In contrast, many cancer types express CAR at lower than normal levels (Hemmi, S., *et al.* 1998, Li, D., *et al.* 1999, Matsumoto, K., *et al.* 2005). Since reduced adhesion is a major factor for tumor to become invasive and metastatic, the loss of CAR may play a significant role in this occurrence. CAR expression would thus also have a tumor suppressive role.

2.1.2.2. CD46

Before identification of this receptor, it was observed that group B Ads do not compete with commonly used serotypes Ad2 and Ad5 for the cellular attachment sites during infection, which led to the conclusion that group B Ads do not interact with CAR. The group B Ad receptor has been identified as CD46 (Segerman, A., *et al.* 2003b, Gaggar, A., *et al.* 2003, Sirena, D., *et al.* 2004). CD46 is a membrane cofactor protein and a regulator of complement activation. This receptor is expressed by all nucleated cells in the human body. Interestingly, CD46 has been identified as a receptor for many other viruses as well, including measles and some herpes viruses (Manchester, M., *et al.* 2000, Santoro, F., *et al.* 1999).

2.1.2.3. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$

Integrins are cell adhesion molecules, which take part also in cell motility. These cell-surface receptors are heterodimers composed of alpha and beta subunits. While $\alpha_v\beta_3$ and $\alpha_v\beta_5$ may act as attachment receptors, especially with engineered viral vectors, their main function is the internalization of attached viruses. The virus attachment to the primary receptor on the cell surface leads to clustering of integrins. The integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ recognize a RGD motif in the viral penton base protein and this signal starts the internalization process (Wickham, T. J., *et al.* 1993). Integrin signaling leads eventually to phagocytosis of the attached virus. In the endosome $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins also help the virus to escape from the vesicle into the cytoplasm (Wickham, T. J., *et al.* 1994). Interestingly, although the integrins have important roles to play in the infection cycle of Ad they are not a prerequisite for cell entry. It has been shown that when the RGD motif in the penton is destroyed by mutation the virus is still able to enter the cell, although at a reduced efficiency (Bai, M., *et al.* 1993).

2.1.2.4. CD80 and CD86

It has been shown that all group B Ads can utilize CD46 as attachment receptor. Early receptor studies revealed that there are at least two different receptors for group B Ads (Segerman, A., *et al.* 2003a). Since then it has been shown that subgroup B Ads can also use CD80 and CD86 receptors for primary attachment. This was first demonstrated for group B1 member Ad3 (Short, J. J., *et al.* 2004) and later for both group B1 and B2 serotypes as well (Short, J. J., *et al.* 2006). Receptors CD80 (B7-1) and CD86 (B7-2) are commonly expressed by mature B cells and dendritic cells. Their function is to activate T-cells by recognizing ligands CD28 and CTLA-4, respectively (Freeman, G. J., *et al.* 1989, Caux, C., *et al.* 1994, Lanier, L. L., *et al.* 1995).

2.1.2.5. Sialic acid

Commonly found on cell-surface glycoproteins and glycolipids, sialic acid has been shown to act as an attachment receptor for Ad serotypes 8, 19a and 37 (Arnberg, N., *et al.* 2000a, Arnberg, N., *et al.* 2000b). Crystallographic studies show conservation of amino acid residues involved in sialic acid binding in many group D serotypes (Burmeister, W. P., *et al.* 2004). Since sialic acid can be found in various animal tissues of different species and to some extent in plants, fungi and bacteria, it seems very unlikely that a human Ad would have evolved to attach primarily on these cell-surface receptors.

2.1.2.6. Heparan sulfate glycosaminoglycans

Long heavily sulfated carbohydrate chains which are found in the extracellular matrix are called heparan sulfate glycosaminoglycans (HS-GAGs). Ads 2 and 5 have been found to use HS-GAG as attachment receptors in addition to CAR (Dechecchi, M. C., *et al.* 2001). As HS-GAGs are similar to sialic acid in abundance it is more likely that HS-GAGs can be used as secondary receptors to gain cell entry in case primary

receptor CAR is not readily available. The amino acid sequence KKTK constitutes an HS-GAG binding motif. Interestingly, KKTK is found in shafts of the serotypes in subgroup C, but not on the shafts of serotypes in any other subgroup. When this motif is mutated in the Ad5 fiber shaft the virus tropism changes and importantly, liver transduction is impaired (Smith, T. A., *et al.* 2003). Because liver transduction has been recognized as one of the major problems in using Ad vectors, abolishing HS-GAG binding is an important aspect to consider when retargeting Ad-based gene therapy vectors.

2.1.3. Transductional retargeting of adenoviral vectors

Adenoviruses have specific receptors to which they attach via virus capsid fiber protein when infecting a target cell (Levine, A. J. and Ginsberg. 1967, Philipson, L., *et al.* 1968). These receptors are usually expressed in a broad range of cells and tissues in the human body. While this can be advantageous in some gene therapy applications, it can also lead to unwanted side effects. Also, the expression of native virus receptor may be decreased in the target tissue. Decreased receptor expression has especially been reported for Ad5 receptor CAR in various cancers (Hemmi, S., *et al.* 1998, Li, D., *et al.* 1999).

Liver transduction is also a major problem with Ad based vectors. The liver has been reported to express CAR abundantly and is susceptible to adenovirus infection and virus-mediated tissue damage (Everett, R. S., *et al.* 2003). Furthermore, it was recently reported that Ads are taken up by liver cells in a CAR-independent way. Blood coagulation factors bind to the major Ad capsid protein, hexon, which leads to the uptake of Ad particles by liver cells (Kalyuzhniy, O., *et al.* 2008, Waddington, S. N., *et al.* 2008). This finding suggests that not only must Ad vectors be transductionally targeted but also "masked" from other Ad-binding mechanisms in the host body to avoid the transduction of unwanted tissues. Without engineering the hexon Diaconu *et al.* (2009) successfully decreased liver transduction in a renal carcinoma model by swapping the fiber of Ad5 to that of Ad19p and inserting kidney targeting motif into the swapped fibers (Diaconu, I., *et al.* 2009). Interestingly, without affecting the hexon, and swapping the fiber to another CAR binding fiber (Table 2), but with different natural tropism, the liver transduction ability of the novel vector was almost completely abolished. This indicates that insertions to the Ad fiber may be sufficient to hide the vector from mechanisms involving liver uptake. There are three major systems which can be used to alter virus tropism to be better suited for gene therapy applications 1) Fiber replacement or fiber swapping, 2) Fiber engineering, and 3) Two-component systems.

2.1.3.1. Fiber replacement technology

This method of adenovirus retargeting makes use of different tropism of different serotypes of adenoviruses. There are more than 50 different serotypes of Ads, divided into six different subgroups (A-G), with varying target tissues and associated pathological conditions (Table 1). Viruses in each group prefer a different set of primary receptors and thus are naturally targeted to different target tissues. Fiber

replacement technology takes advantage of this fact and engineering viruses to contain parts of different serotypes results in novel hybrid viruses.

When a hybrid virus is produced successfully it has the binding characteristics of the fiber serotype, while the rest of the viral particle is derived from another serotype, usually Ad5. Hybrid viruses can still be produced and purified similarly to Ad5. This is a relatively straightforward method to achieve two goals in virus retargeting; first, the tropism of virus is changed and second, the native receptor recognition is abolished. Also, the hybrid virus usually has high affinity to cell receptors because the binding capability has been tested by Mother Nature in the course of evolution and no engineering is done to the fiber *per se*. Several different hybrid viruses have been manufactured to date. These hybrids are usually based on Ad5 with fibers introduced from other serotypes. The most commonly used "fiber donor" serotypes are subgroup B members 3, 7, 11, 16, and 35 (Krasnykh, V. N., *et al.* 1996, Gall, J., *et al.* 1996, Stone, D., *et al.* 2005, Goossens, P. H., *et al.* 2001, Shayakhmetov, D. M., *et al.* 2000). The common primary receptor for subgroup B serotypes has been identified as CD46 (Gaggar, A., *et al.* 2003). CD46 is a membrane cofactor protein which is expressed by all nucleated cells in the human body and functions as a regulator of complement activation (Liszewski, M. K., *et al.* 1991).

Table 2. Adenovirus groups.

Group	Serotypes	Primary receptor	Tropism	Reference
A	12, 18, 31	CAR	Gastrointestinal mucosa	(Roelvink, P. W., *et al.* 1998)
B B1	3, 7, 16, 21, 50	CD46, CD80, CD86	Respiratory mucosa / Epithelium	(Gaggar, A., *et al.* 2003, Sirena, D., *et al.* 2004, Short, J. J., *et al.* 2004, Marttila, M., *et al.* 2005)
B2	11, 14, 35, 36	CD46	Respiratory mucosa / Epithelium / Kidney* / Urinary tract*	(Gaggar, A., *et al.* 2003, Marttila, M., *et al.* 2005, Segerman, A., *et al.* 2000)
C	1, 2, 5, 6	CAR	Respiratory mucosa	(Roelvink, P. W., *et al.* 1998)
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	CAR, sialic acid	Ocular mucosa	(Arnberg, N., *et al.* 2000b, Roelvink, P. W., *et al.* 1998)
E	4	CAR	Respiratory mucosa	(Roelvink, P. W., *et al.* 1998)
F	40L, 40S, 41L, 41S	CAR**	Gastrointestinal mucosa	(Roelvink, P. W., *et al.* 1998)
G	52	unknown	Gastrointestinal mucosa	(Jones, M. S.,2nd, *et al.* 2007)

* Ad14 has not been found in these tissues

** CAR is the receptor for 40L and 41L. 40S and 41S receptors unknown (non-CAR)

Although fiber replacement is a straightforward and relatively easy way to retarget Ad vectors, there are also a few limitations to this method. As the table 2 shows, the number of different Ad receptors is limited. Even though there are 52 different serotypes of Ads, most of them bind CAR. Interchanging fibers of these serotypes does not produce an Ad vector with novel binding characteristics. Another issue is the naturally broad tropism of Ads. This may be beneficial for some applications, but also detrimental to others.

While hybrid Ads have been retargeted in regard to the native virus, the novel attachment receptor is usually expressed by a broad range of cells and tissues in the human body as well. Although, one vector might decrease the infection of liver, which is a major concern with Ad5 vectors, the novel vector might induce a severe infection of another tissue as a side effect. Replacing only the Ad fibers is not sufficient to prevent liver transduction. It has been shown that many, but not all, serotypes bind blood coaculation factor X (fX) via hexon and this leads to liver uptake of Ad (Waddington, S. N., *et al.* 2008). Furthermore, the shaft length has been associated with liver transduction efficiency. Hybrid viruses with fibers from serotypes with shorter fiber shafts have been shown to infect liver cells with reduced efficacy both *in vitro* and *in vivo*, when compared with Ad5 (Shayakhmetov, D. M., *et al.* 2004, Vigne, E., *et al.* 2003, Nakamura, T., *et al.* 2003). Fiber replacement combined with hexon swapping might further lessen the hepatotoxicity of Ad vectors and yield less toxic, better tolerated and more effective Ad vectors.

2.1.3.2. Fiber engineering

More specific retargeting may be done by genetically engineering the fiber to alter the binding properties. There are two distinct sites which have been proven to tolerate insertions well without the loss of fiber trimerization; the C-terminus and HI-loop. Both sites are located in the fiber knob. Early experiments included the insertion of a polylysine chain to the C-terminus and insertion of an RGD motif to the HI-loop (Yoshida, Y., *et al.* 1998, Wickham, T. J., *et al.* 1997). The polylysine modification resulted in redirecting of the vector from CAR to heparin sulfates on various cell types. The RGD insertion redirected the vector from CAR to $\alpha_v\beta$ integrins and was observed to allow the infection of cell types which had been previously refractory to native Ad5 infection. Recently the RGD insertion showed some more success when Li *et. al.* (2010) successfully targeted tumor vascular endothelial cells (Li, P., *et al.* 2010). The fiber knob was first mutated to ablate native CAR binding activity and an RGD peptide was inserted to the HI-loop to redirect the vector to vascular endothelium expressing α_v-integrins. The resulting vector was effective against both breast cancer and melanoma in animal models without observed toxicity in the normal tissues.

The major problem with this method of retargeting is the size limitation of insertions and the fact that the inserted molecule must be able to fold correctly in the cell cytoplasm. The early reported maximal size for insertion to the C-terminus was only up to 25 amino acids. Larger insertions prevent the correct trimerization of the fiber protein which is necessary for the production of functional viral particles (Hong,

J. S. and Engler. 1996). The HI-loop tends to allow insertions of larger size than the C-terminus. Insertions within the size limitations have been shown to be effective in targeting various cancer types including glioma, pancreatic, meningioma and ovarian cancer (Dirven, C. M., *et al.* 2002, Grill, J., *et al.* 2001, Wesseling, J. G., *et al.* 2001, Hemminki, A., *et al.* 2001).

In order to target cardiac vasculature Nicol *et. al*. (2009) combined fiber replacement and fiber engineering techniques (Nicol, C. G., *et al.* 2009). The Ad5 fiber was first swapped for Ad19p fiber. Hybrid Ad5/19p displays as efficient transduction properties as Ad5 on vascular endothelial and smooth muscle cells, but significantly lower transduction levels on liver cells (Denby, L., *et al.* 2004). A cardiac specific peptide was inserted into the HI-loop of this hybrid fiber knob. The resulting engineered hybrid vector was analyzed in both *in vitro* and *in vivo* models and was shown to be more specific for endothelial cells and cardiac tissue than either Ad5 or Ad5/19p without peptide (Nicol, C. G., *et al.* 2009). Similarly Ad5/19p with insertion of kidney targeting peptide showed transduction levels comparable to wild type on renal cancer cells, but only minimal transduction of the liver cells (Diaconu, I., *et al.* 2009). The works of Nicol *et al.* and Diaconu *et al.* show that it is feasible to use more than one method simultaneously to modify an Ad vector. This is probably even mandatory for achieving vectors which have the best possible safety and efficacy profiles.

Recently the fiber protein has been engineered more extensively. Myhre *et. al.* (2009) have inserted different affibodies (58 amino acid molecules) to the fiber HI-loop separately and in tandem without loss of virus function (Myhre, S., *et al.* 2009). The ability of different sites in the Ad capsid to tolerate insertions has been evaluated, and the HI-loop seems to be the best site in this sense when compared with fiber C-terminus, hexon or pIX (Campos, S. K. and Barry. 2006, Kurachi, S., *et al.* 2007). Studies have also shown the importance of the fiber knob. It seems that, in addition to being in a key role in receptor recognition, the knob coding nucleotide sequence is needed for efficient fiber mRNA synthesis and subsequent protein synthesis. The correct folding and trimerization of the fiber protein is also impaired in virions with knobless fibers (Henning, P., *et al.* 2006).

As discussed above, when manipulating the virus capsid it is paramount to retain the ability of capsid proteins to fold correctly. It would be possible to genetically delete parts of capsid proteins at the nucleotide level and thus add room for bigger insertions, but this would most likely interfere with correct protein folding and be deleterious to vector production. The other methods of targeting Ad vectors, discussed previously in chapter 2.1.3.1. and below in chapter 2.1.3.3., do not usually suffer from misfolding or loss of functionality problems since no engineering is done to the fiber *per se*. One might also try to manufacture "synthetic fibers" based on any suitable protein frame and incorporate these to the Ad vector genome in place of the native fiber. A single-chain Fv fragment (scFv) has already been used as this kind of a "synthetic fiber". Since antibodies and antibody fragments are not compatible with Ad capsids an

interesting specific complexing system was developed. Instead of the fiber gene, the vector genome codes for a secreted form of scFv. These secreted scFvs are then specifically associated with virions after cell lysis in the extracellular space (Glasgow, J. N., *et al.* 2009). This system allows the screening and affinity maturation of specific scFv fragments by powerful *in vitro* or *in vivo* screening methods separately, as with two-component systems. After finding a target specific scFv, it can be introduced into the viral genome to make a complete retargeted Ad vector system. It must be noted that Glasgow's system could also be categorized under section *2.1.3.3.* as it is much like a two-component system, with an important "upgrade": the bridging component is genetically incorporated into the vector genome and its association with virions is specific. Importantly, one of the major drawbacks associated with two-component systems (discussed below) has been removed in Glasgow's system.

2.1.3.3. Two-component adenoviral systems

One important aspect of retargeting Ad vectors is the ablation of native binding specificity. This can be done quite easily by blocking the receptor binding site on the Ad surface. For example, by binding an antibody against the fiber knob the virus can no longer bind to its target receptor. Targeting to a new set of host cells can be achieved if the antibody is conjugated with a target specific receptor ligand. This was in fact the first method used for targeting Ad gene therapy vectors.

Watkins *et. al.* (1997) constructed a bispecific ligand containing an Ad fiber binding scFv and endothelial growth factor (EGF) for retargeting the Ad vector to EGF receptors (EGFR). The scFv, named S11, alone has been shown to inhibit Ad infection in cells otherwise readily infected by Ad (Watkins, S. J., *et al.* 1997, Haisma, H. J., *et al.* 2010). S11 has been fused with many peptides and ligands to target Ad vectors to different receptors on various cell types, including EGFR on squamous carcinoma cells, CD105 on epithelial cells, carcinoembryonic antigen on breast cancer and melanoma cells and VEGFR2 and Tie2 on endothelial cells (Watkins, S. J., *et al.* 1997, Haisma, H. J., *et al.* 2010, Nettelbeck, D. M., *et al.* 2001, Korn, T., *et al.* 2004).

This method differs from the ones described previously mainly in the fact that this is not a genetically targeted system. No genetic manipulations are done to the Ad vector in order to retarget it. This fact means that one does not need to worry about disrupting a complex multiprotein structure while engineering the retargeting system. Single chain Fv–ligand or double scFv -molecules are much smaller and easier to work with than the Ad as a whole. Furthermore, scFvs can be quite easily produced in bacterial cultures in high quantities. However, this system has some drawbacks. Adding another molecule between the vector and the target increases the complexity of the whole system. The bridging molecule must be thoroughly purified and analyzed again from each produced batch, which adds steps to the manufacturing process. In contrast, if the retargeting information were genetically incorporated to the Ad vector these steps would be unnecessary. Importantly, if the two-component retargeting system is used for transductional targeting the vector cannot be made conditionally replicative, because the redirecting component is not coded in the vector genome or the

binding reaction between vector and directing component does not usually happen effectively *in vivo*.

2.1.4. Transcriptional retargeting of adenoviral vectors

Wild type Ads use a wide range of host cells for replication. This presents a problem when only a certain types of cells need to be addressed, e.g. only cancer cells are targeted for termination or only cells with a dysfunctional gene need to be introduced with a functional copy. As discussed previously, Ad vectors can be transductionally targeted to the target tissue, but especially if the end result is the destruction of infected tissue, like in cancer therapy, it is paramount to have a second level of control for the vector replication or transgene expression. Usually, even if the vector is retargeted to infect only target cells, there is some "leakiness" in the system and unwanted cell types are infected to some degree. This may or may not be a severe issue, depending on the vector function. If the vector aims to introduce a functional copy of a gene to a cell population with a non-functional copy, the infection of neighboring normal cells would probably not lead to any severe side effects in the patient. On the contrary, if the intended vector function is to target a cell population and destroy it, infection of normal cells could have severely detrimental side effects. Thus, the vector replication, and in some cases the expression of a transgene, must also be targeted to the target cells with its own control elements.

2.1.4.1. Oncolytic adenoviruses

Deletion of non-essential adenoviral genes or subjecting these genes under the control of a tissue-specific promoter system (discussed below) produces conditionally replicating adenoviruses (CRAds). As discussed in chapter 2.1.1. adenovirus infection results in the destruction of the host cell. This has been exploited in gene therapy of cancer. Viral vectors used this way are generally called oncolytic viruses. The principle of oncolytic viruses relies on controlling the viral replication in the host and aiming it specifically to the target tissue (Figure 3). Transductional targeting blocks the efficient transduction of normal cells. A small proportion of normal cells may become infected, but the virus is not able to replicate. On the other hand, the targeted cancer cells are effectively transduced and viral replication takes place. The released viruses infect neighboring cells and tumor tissue is gradually destroyed by virus action. Ideally, when the patient is void of target cells the oncolytic virus is flushed from the body by host immune system.

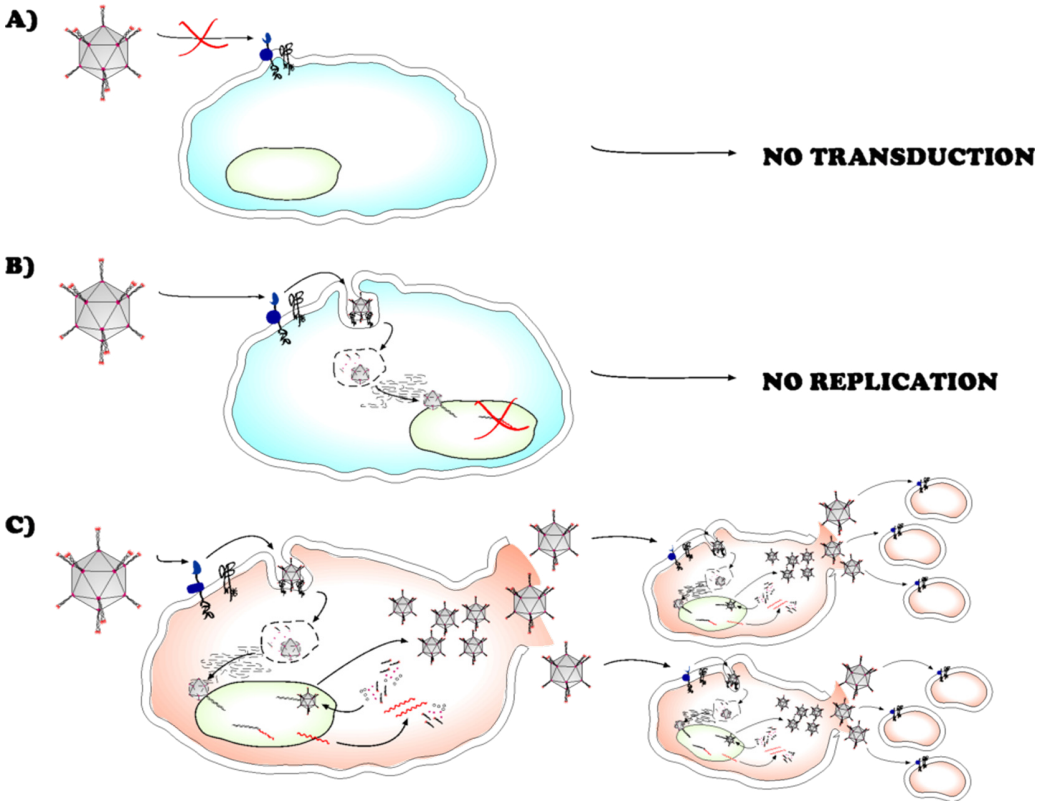
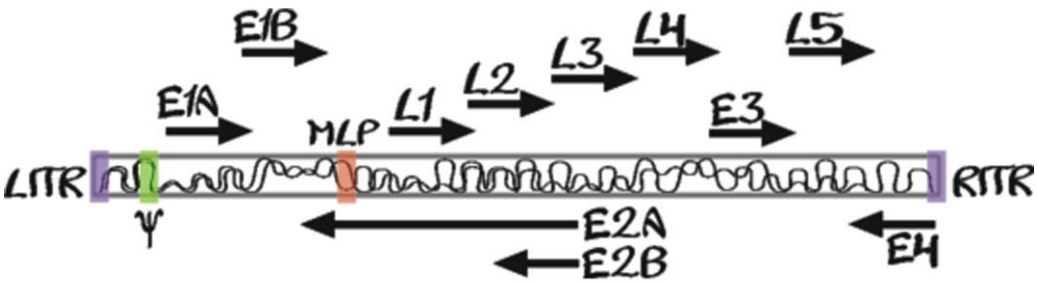


Figure 3. Principle of oncolytic viruses. A) Normal cells are not transduced by oncolytic viruses due to modification to the viral capsid. B) The virus is not able to replicate in normal cells due to transcriptional control elements even if transduction occurs. C) Cancer cells are efficiently transduced and destroyed by viral replication. The released viruses infect neighboring cells and the effect is amplified each cycle.

2.1.4.2. Deletion of essential viral genes

As discussed in section 2.1.1., there are a few viral genes which are necessary for the continuation of the virus life cycle after infection of a cell (Figure 4). These genes include E1A, E1B, and E3, which are discussed here. If the E1B gene is deleted from the viral genome, the virus cannot prevent the host immune system from reacting to the infection and infected cells will undergo apoptosis before the viral replication is complete. This fact has been exploited to design safer Ad vectors for cancer treatment. Many cancer types have been reported to have a non-functional p53 gene (White, E. 1994). If E1B is removed from the virus genome, the virus cannot complete the replication cycle in normal cells with functional p53. In cells with non-functional p53 the E1B gene is not needed, and the virus can replicate in these cells. Similarly, the interaction of gene product E1A and cellular pRb protein is a prerequisite for viral replication in normal cells. E1A inhibits pRb function (discussed in 2.1.1.), which allows the replication of viral DNA. E1A areas are usually replaced by the transgene in non-replicating Ad vectors. These vectors can be used to introduce a therapeutic gene

into tissues that are not marked for destruction. Like p53, pRb has been shown to be nonfunctional in many cancers (Sherr, C. J. 1996). Thus, removal of the E1A gene from the viral genome renders the vector selective for cancerous tissue with pRb mutation and unable to replicate in normal cells.



Gene	Product Function	Gene	Product Function	Gene	Product Function
E1A	Binds human pRb and prevents cell cycle arrest	L3	Viral protease, cleaves various viral precursor proteins	L5	pVI, fiber, a major capsid protein
E1B	Binds human p53 and prevents p53 meadiated apoptosis	L4	pXIII, hexon associated protein	LITR	Left inverted terminal repeat, contains origin of replication
E2A	Replication of viral DNA	E4	Various transcripts, involved in viral DNA replication	RITR	Right inverted terminal repeat, contains origin of replication
E2B	Viral polymerase	L1	polypeptide IIIa, a minor capsid protein, takes part in capsid assembly	ψ	psi-sequence, the packaging signal of viral DNA
E3	Removes CD95 from cell surface and prevents TNF dependent apoptosis	L2	pV and pVII, DNA packing, condensation of viral DNA	MLP	Major late promoter, promoter for all late-genes

Figure 4. Organization of adenovirus genome and tasks involving early and late expressing adenoviral genes.

A groundbreaking viral vector in the field of gene therapy was *dl*1520 (better known as ONYX-015) (Heise, C., *et al.* 1997). In the year 2000 ONYX-015 was the first CRAd to ever enter clinical trials (Ganly, I., *et al.* 2000). ONYX-015 has a 55 kDa deletion in the E1B gene, which alters the viral replication properties as described above. In 2005 China approved the use of CRAd H101 as treatment for head and neck cancer. The H101 virus is similar to ONYX-015, as it also has the 55 kDa E1B deletion, but in addition, it also has a second deletion in the E3 gene, which further limits the virus function in normal cells and thus increases safety. As described in section 2.1.1., the viral ligand for cellular pRb is gene product E1A. Gene E1A was partially deleted to create CRAd Ad5Δ24. Ad5Δ24 was shown to be effective in killing glioma cells both *in vitro* and *in vivo* while being unable to replicate in quiescent normal cells with functional pRb (Fueyo, J., *et al.* 2000). This vector has been developed further by adding also transductional targeting to the virus via RGD insertion to the fiber knob (Suzuki, K., *et al.* 2001). More recently Ad5Δ24RGD, with combined transcriptional and transductional control elements, has been shown to be very effective in killing glioma tumor cells when combined with chemotherapy in a mouse model (Alonso, M. M., *et al.* 2008).

2.1.4.3. Promoter-controlled virus replication

A different kind of replication control can be achieved by incorporating tissue-specific promoters into the Ad vector genome. The first reported CRAd with its replication regulated by a tissue-specific promoter was CN706 that has a prostate-specific promoter-enhancer element controlling the adenoviral E1A gene. This CRAd was shown to replicate preferentially in PSA-producing cell lines and to be able to inhibit tumor growth in a mouse xenograft model (Rodriguez, R., *et al.* 1997). Since then the virus CN706 has been in phase I clinical trials and shown to be well tolerated and to decrease PSA levels in patients (DeWeese, T. L., *et al.* 2001).

While PSAp is specific for prostate cancer, another promoter, human telomerase reverse transcriptase promoter (hTERTp), has activity in various different cancer types. The telomerase reverse transcriptase gene has been found to be reactivated in many cancers (Ito, H., *et al.* 1998, Kawakami, Y., *et al.* 2000, Saito, K., *et al.* 2002) and it has been hypothesized that this occurrence might be one of the prerequisites for cancer cells to become immortalized (Shay, J. W., *et al.* 2001). Normally hTERTp is active only at fetal development and after birth it remains active only in stem cells (Ulaner, G. A., *et al.* 1998). Cancer cells with reactivated hTERTp can be targeted by inserting hTERTp into the Ad genome to control the expression of essential genes like E1A. An hTERTp-controlled CRAd, Adv-TERTp-E1A, was one of the first created. It has been tested both *in vitro* and *in vivo* models (Huang, T. G., *et al.* 2003). Adv-TERTp-E1A has been found to be as effective in killing hTERTp active hepatocellular carcinoma cells as wt Ad5, but unable to replicate in hTERTp inactive fibroblasts, which are effectively killed by the wild-type virus. Importantly, it was also demonstrated that the Adv-TERTp-E1A virus did not induce severe inflammation in the liver; even though it is an accepted fact that wt Ad5 is severely hepatotoxic. The telomerase promoter system has been developed further with more efficient and specific control elements to restrict the viral replication into the target cells. The human telomerase is a ribonucleoprotein composed of RNA and reverse transcriptase subunits (Weinrich, S. L., *et al.* 1997). The subunit transcripts are controlled by separate promoters, hTRp and hTERTp, respectively. Both promoters have been evaluated with Ads expressing a suicide gene or as a control element for CRAd replication. It has been reported that hTRp is more efficient than hTERTp in producing oncolytic effects or sensitizing cancer cells through transgene expression in non-replicating vector systems (Abdul-Ghani, R., *et al.* 2000, Plumb, J. A., *et al.* 2001, Dufes, C., *et al.* 2005). Interestingly, comparisons between CRAds controlled by these promoters showed no deifferences in cytopathic effects or viral E1A expression (Bilsland, A. E., *et al.* 2007). Recently an interesting report was published by Onimaru *et. al.* (2010), where they used in combination CRAd and non-replicative vectors to treat pancreatic cancer in a mouse model (Onimaru, M., *et al.* 2010). When an hTERTp-controlled CRAd was administered in combination with non-targeted vector bearing a therapeutic gene, the transgene expression was observed to be enhanced. The investigators hypothesize that this enhancement is due to the partial restoration of replicative properties of the transgene vector by CRAd-expressed E1A gene in co-infected target cells.

Many other CRAds have also been created with different promoter systems targeting different tissues. These promoters include CMV (not tissue-selective), survivin, COX2, heparanase, SLPI, CXCR4, EPG-2, mesothelin and midkine promoters (Krasnykh, V. N., *et al.* 1996, Zhu, Z. B., *et al.* 2004b, Yamamoto, M., *et al.* 2001, Breidenbach, M., *et al.* 2006, Barker, S. D., *et al.* 2003, Zhu, Z. B., *et al.* 2004a, Lu, B., *et al.* 2005, Breidenbach, M., *et al.* 2005, Adachi, Y., *et al.* 2000). Each promoter has its own properties and can be chosen according to target tissue or application.

2.1.4.4. MicroRNA based transcriptional targeting

Gene expression in eukaryotes is partly under the control of microRNAs (miRNAs) and are one of the key regulators of gene expression also in humans (Ambros, V., *et al.* 2003, John, B., *et al.* 2004). It has been shown that miRNAs can be tissue-specific and are expressed differentially in cancers (Chang, J., *et al.* 2004, Jay, C., *et al.* 2007). This presents a possibility to control Ad replication using miRNAs. MicroRNA122 has been identified as liver-specific (Chang, J., *et al.* 2004). This allows for specific down-regulation of genes in the liver. The miRNA122 target sequence was incorporated into the Ad E1A gene in non-coding region by Ylösmäki *et al.* (2008). A novel CRAd Ad5/3K-122 was tested in tissue culture analyses and about 10,000 fold attenuation of viral replication in liver cells was observed (Ylosmaki, E., *et al.* 2008). More recently this control mechanism was used in combination with a tissue-specific promoter. CRAd Ad[CgA-E1A-miR122] is targeted to neuroendocrine tumor cells and simultaneously detargeted from the liver. This CRAd was shown to replicate in endocrine pancreatic tumor cells at levels similar to wild type Ad5. In hepatic cells the replication was attenuated over 1000 fold when compared with wild type Ad5, and over 50 fold when compared with promoter-controlled CRAd (Leja, J., *et al.* 2010). The work of Leja *et al.* shows the feasibility of combining two control mechanisms in the same Ad vector. Importantly, utilization of miRNAs does not prevent the use of tissue-specific promoters but, on the contrary, adds another level of control. With addition of capsid modification, the resulting CRAd would have a total of three control levels: 1) transductional control, 2) transcriptional control, and post-transcriptional control, which all cumulatively add to the safety profile of the Ad vector in question.

2.1.5. Adenoviral vectors in clinical trials

Adenoviral vectors are the most commonly used vectors in clinical trials. By the end of the year 2009, adenoviral vectors were used in 392 (24%) out of 1644 clinical trials. Two of the most studied diseases involving adenoviral vectors are cancers (n = 1060) and cardiovascular diseases (n = 143). Apart from Ads, other viral vectors have been used in clinical trials as well. Retroviruses, Vaccinia virus, Poxvirus, Adeno-associated virus, Herpes simplex virus and Lentiviruses have all entered clinical trials mainly in cancer research. (www.wiley.co.uk/genmed/clinical, accessed October 26, 2010) All viral vectors have different properties with their own pros and cons. Ideally the application would dictate the viral vector to be used, but to date Ads and retroviruses

are significantly most utilized and researched, although lentiviruses and especially adeno-associated viruses have been used more and more in pre-clinical settings.

Adenoviruses have been proven to be well tolerated in clinical trials. Usually mild flu-like symptoms (fever, fatigue, chills, nausea and vomiting) have been reported as adverse effects (with doses up to 2×10^{13} vp) (Nemunaitis, J., *et al.* 2001, Hamid, O., *et al.* 2003, Small, E. J., *et al.* 2006). Most adverse effects, including serious ones, have been reported as transient and dose-related. An extreme case occurred in 1999 when a patient in clinical trials died after systemic administration of Ad vectors intravenously (Raper, S. E., *et al.* 2003). This unfortunate case teaches valuable lessons: 1) pre-existing levels of NAbs must be measured for each patient, 2) dosage must be carefully selected, 3) delivery route must be selected to minimize vector exposure to unwanted organs. For example, in case of HNSCC intratumoral injection should be preferred over intravenous injection. However, it must be noted that cancer metastases are hard to reach without systemic administration. And 4) a more advanced vector with inserted control elements should be used.

Neutralizing antibodies against Ad vectors rise after administration of the vector, and usually within four weeks all patients have detectable levels of NAbs. No correlations between NAb levels and toxicity have been observed in clinical trials (Nemunaitis, J., *et al.* 2001). However, vector-induced immunity can reduce transgene expression to less than 10 % of the original levels after systemic delivery (Barcia, C., *et al.* 2006).

Partial and complete responses are frequently observed in Ad clinical trials for various cancers (Ganly, I., *et al.* 2000, DeWeese, T. L., *et al.* 2001, Khorana, A. A., *et al.* 2003, Lu, W., *et al.* 2004, Dummer, R., *et al.* 2004, Dummer, R., *et al.* 2010). Freytag *et. al.* (2007) observed good tolerance and promising results from phase I studies with oncolytic Ad for treatment of prostate cancer (Freytag, S. O., *et al.* 2007). Doses of 10^{11}, 10^{12} or $10^{12} \times 2$ viral particles were well tolerated, with grade 4 adverse event reported only in 1 out of 9 patients (hypoglycemia of unknown reason in a patient suffering from diabetes). All patients had decreased PSA levels and 7 patients had prostate biopsies free of cancer after one year. More recently Nokisalmi *et. al.* (2010) published a report describing treatment of solid progressive metastatic tumors of various origin and refractory to conventional treatments (Nokisalmi, P., *et al.* 2010). Patients were injected with CRAd doses ranging from 0.4×10^{11} to 1×10^{12} viral particles. Most patients developed NAbs over time, but these antibody levels did not correlate with toxicity. The observed adverse effects were of levels 1-3 and similar to what has been published previously, no level 4 or more serious adverse effects were observed. Five out of 12 patients showed decreased tumor size or tumor density. In another recent study Cerullo *et. al.* (2010) showed for the first time virus-induced antitumor immunity being raised in humans after treatment with non-replicating Ad vectors in addition to the expected antitumoral effects due to the viral vector carrying granulocyte macrophage colony-stimulating factor (GMCSF) gene. Fifty percent of evaluable patients showed positive responses (12% complete, 6% partial, and 31%

stable disease) in both injected and non-injected tumors (Cerullo, V., *et al.* 2010). This finding supports the theory that viral gene therapy vectors have potential to induce beneficial bystander effects, which further enhance the therapeutic potential of this kind of treatment.

There is a clear similarity in results observed in Ad-vector-based gene therapy clinical trials. These results indicate rather clearly, what must be taken into account when Ad vectors are considered as a treatment method: Ads are well tolerated vectors, but the administered dose must be selected carefully. The immune system will recognize the vector and arising NAbs can reduce the therapeutic effect of the administered vector dose. The Ad vector must be targeted to infect the target tissue and natural tropism towards the liver must be reduced or ablated. To avoid side effects related to Ad and transgene function, the transgene expression and / or viral replication must be restricted to the target tissue as strictly as possible. Overall, the clinical trials done thus far using Ad vectors, show varying efficacy between trials and often the differences between control and vector groups remain quite low. On the other hand, results from clinical trials encourage for further vector development as the bullseye has not been hit yet.

2.2. Head and neck squamous cell carcinoma and gene therapy

About 940 new cancer cases per 100,000 people and over 500,000 cancer-related deaths are reported annually in the United States alone. These numbers translate to 1500 deaths per day and over $228 billion used for cancer treatment and care annually (www.cancer.org, accessed June 11, 2010). In Finland in the year 2008 over 530 new cancer cases per 100,000 residents were diagnosed and over 11,000 cancer-related deaths were reported. The incidence rate has more than doubled over the last 30 years (www.cancerregistry.fi/tilastot, accessed July 20,2010).

2.2.1. Head and neck squamous cell carcinoma

Over 35,000 new cases and 7600 deaths due to HNSCC are reported annually in the U.S. Incidences are two times more common in men than in women. HNSCC has been associated with smoking and alcohol use, with combined use raising the risk factor over 30 fold. A different type of HNSCC can develop also due to human papilloma virus (HPV) infection. HPV-induced HNSCC usually has a better prognosis, less genetic alterations and less cell differentiation. Relative survival rates for diagnosed HNSCC patients are 83% for 1-year, 60 % for 5-year, and 49 % for 10-year survival. However, with metastatic HNSCC the 5-year survival drops to 28 % (www.cancer.org, accessed 11.6.2010). This shows that patients with metastases have severely worse prognoses and early detection and treatment is paramount. Unfortunately, the majority of patients diagnosed with HNSCC already have late-stage disease. Furthermore, HNSCC has a high recurrence rate and up to 40% of tumors treated with surgery and chemotherapy recur (Argiris, A., *et al.* 2008).

HNSCC is histologically characterized by nests of squamous cells and intercellular bridges within stromal fibrosis. Progressing carcinoma breaks through basal membrane and infiltrates subepithelial connective tissue. Invasive HNSCC can further infiltrate skeletal muscle, craniofacial bones and facial skin. The histology of HNSCC changes as the disease progresses and cells differentiate. Although HNSCC comprises of a heterogeneous group of tumors, depending on the origin, the vast majority has nullifying mutations in genes involved in p53 and / or pRb function. It has been shown that over 50% of HNSCC tumors have mutations in the p53 gene and these mutations contribute to reduced survival after surgery (Olshan, A. F., *et al.* 1997, Poeta, M. L., *et al.* 2007). Disruption of the pRb pathway in HNSCC occurs by inactivation of p16 due to mutation, promoter hypermethylation or loss of heterozygosity at chromosome region 9p21 (Olshan, A. F., *et al.* 1997). Loss of heterozygosity at chromosome region 9p21 has been reported in 75% of HNSCC cases (Gonzalez, M. V., *et al.* 1995). The uniform loss of gene function represents a target for gene therapy applications and an Ad vector targeting the p53 function has been extensively tested in clinical settings (discussed in 2.2.2.). Even though therapy with Ad vectors targeting the p53 function has been successful, the disease progression is not as simple. There are at least three recognized specific mutations which are required for development of HNSCC: 1) inactivating mutation of pRb pathway (loss of p16), 2) inactivating mutation of p53 gene or pathway, and 3) amplification of cyclin D. The first two mutations can be absent in HPV-induced HNSCC and function of these pathways are inhibited by HPV-coded oncoproteins (Scheffner, M., *et al.* 1990, Hafkamp, H. C., *et al.* 2003).

2.2.2. Gene therapy of squamous cell carcinoma of the head and neck

HNSCC is an excellent target for Ad-mediated gene therapy. As previously discussed, Ad vectors induce immune reactions more efficiently when the vector is injected intravenously. HNSCC tumors are usually quite easily accessible for intratumoral injection to allow for local treatment. Clinical studies with Ad vectors for treatment of HNSCC have mainly centered on Ads expressing p53 cDNA (Ad5-p53). It has been shown that p53 is mutated and inactivated in 40-60 % of HNSCC patients. Also, active p53 has been shown to sensitize tumor tissue to chemo- and radiotherapy (Brennan, J. A., *et al.* 1995). Ad5-p53 has been tested in phase I-III clinical trials (Clayman, G. L., *et al.* 1998, Clayman, G. L., *et al.* 1999, Han, D. M., *et al.* 2003, Zhang, S. W., *et al.* 2003, Zhang, S. W., *et al.* 2005, Pan, J. J., *et al.* 2009). Ad5-p53 has been well tolerated with most common side effects being fever and pain at the injection site. Clinical efficacy of this vector has been shown for various patient groups, recurrent tumors, resectable, non-resectable, higher grades (III and IV) and in combination with radiotherapy. The overall response rate for Ad5-p53 has been up to 93 %. Patients treated with Ad5-p53 also had higher rates of complete response. Two separate studies show that about two thirds of the patients with any response have complete response, while only one fourth of the patients with any response have complete response in groups receiving only radiation therapy. In follow-ups the patients who received Ad5-p53 showed increased time being disease-free, although overall survival time was not always prolonged. Since then, the Chinese government has approved the use of Ad5-

p53 (Gendicine) in combination with radiation therapy for treatment of solid tumors. (Rewieved in (Peng, Z. 2005))

2.3. Cardiovascular disease and gene therapy

Cardiovascular disease (CVD) includes all diseases of the heart and the vasculature. Although, the occurrence of the most common CVD, the coronary heart disease, has decreased in the last 40 years, cases of other CVDs have steadily increased. Highest mortality from coronary heart disease in Finland was in 1967: 508 deaths for every 100,000 people. Since then the mortality has decreased 64% and in the year 2005 the corresponding number was 109. In spite of this significant decrease over the past years Finland is still a country with one of the highest rates of incidence in the western world, along with the UK. In 2007 in the UK CVD was the number one cause of death with over 190 000 cases (313 cases per 100,000 people). In comparison, all cancers combined were responsible of just below 160,000 deaths. Additionally, the treatment costs for CVD were over 3.2 billion pounds (www.ktl.fi/attachments/suomi/julkaisut/julkaisusarja_b/2008/ 2008b02_2.pdf accessed July 6, 2010, www.bhf.org.uk/publications accessed June 8, 2010).

2.3.1. Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is generally recognized as a hereditary condition and can show autosomal dominant, autosomal recessive or X-linked inheritance pattern. Instead of being inherited DCM can also be acquired; for example extensive alcohol use can lead to DCM and mothers can develop DCM during pregnancy or shortly after delivery. In DCM the heart muscle becomes weak and chamber walls thinner, which results in impaired heart function. Usually the left ventricle is dilated and its contractile ability has become weak. Symptoms of DCM range from none to those described for heart failure, which include shortness of breath, tiredness, palpitations and swelling of ankles. Currently there is no curative treatment for DCM. Patients can suppress the symptoms with correct medication, but the condition remains throughout life.

Over 30 DCM-related genes have been discovered to date, showing vast genetic heterogeneity in this disease. Mutations in these associated genes can cause the development of DCM, but the highest frequency for a single gene (lamin A/C) is only 6%. Lamin A/C mutations also show autosomal dominant DCM, progressive disease, poor prognosis and many times prevalence of other muscular dystrophies, making it one of the most important genes in DCM (Taylor, M. R., *et al.* 2003). Other important genes associated with DCM include MYH7, AnkRD1, LDB3, MYBPC3, SCN5A, and TNNT2 (Villard, E., *et al.* 2005, Duboscq-Bidot, L., *et al.* 2009, Daehmlow, S., *et al.* 2002, Hershberger, Ray E., *et al.* 2008).

2.3.2. Gene therapy of the heart

Gene therapy presents a novel treatment method for DCM by allowing the introduction of correcting or therapeutic genes into the heart. Although no clinical trials targeting

heart failure specifically caused by DCM have been completed to date, there are a number of gene therapy trials for heart failure (HF). Adenylylcyclase type VI (AC_{VI}) is one promising therapeutic gene, the function of which could improve cardiac function after damage by heart disease. It has been shown that intracoronary injection of Ad-AC_{VI} can improve left ventricular function in an animal model with dilating hearts (Rebolledo, B., *et al.* 2006). This work is important in the fact that Rebolledo *et. al.* were able to actually improve the heart functionality, as others have reported only reduction or arrest of heart failure progression (Hoshijima, M., *et al.* 2002, Lai, N. C., *et al.* 2004). Another interesting gene which has potential in improving the functionality of a failing heart is SERCA2a. One commonly recognized defect in failing hearts is abnormal function of the sarcoplastic reticulum (SR). Defect in SR leads to problems in intracellular Ca^{2+} handling. SERCA2a is a key regulator of Ca^{2+} fluctuations in muscle contraction in the heart and many studies have shown decreased SERCA2a expression in late stage HF (de la Bastie, D., *et al.* 1990, Arai, M., *et al.* 1993, Hasenfuss, G., *et al.* 1994, Schwinger, R. H., *et al.* 1995). Thus, introduction of active SERCA2a into heart muscle could reinstate appropriate contractile function of the heart. An adeno-associate virus (AAV) carrying SERCA2a cDNA is being currently tested in clinical trials for treatment of HF (Jaski, B. E., *et al.* 2009). In preclinical studies the AAV-SERCA2a has been shown to be well tolerated and has shown promise in improving the function of failing hearts by introducing functional SERCA2a levels.

Clinical trials have mostly concentrated on coronary heart disease, where blood flow is impaired to parts of the myocardium causing ischemic areas. Many genes can be introduced to damaged myocardium to improve the functionality of a failing heart. This may be done either by introducing a working copy of the malfunctioning gene that causes the disease or by using a gene that otherwise improves functionality of the heart. The ischemic areas could be rescued by re-establishing blood flow. Therefore, one of the most studied applications in gene therapy of the heart is improvement of cardiac function by angiogenesis in damaged areas. Neovascularization has been induced by both hepatocyte growth factor (HGF) and vascular endothelial growth factors (VEGFs).

Adenovirus bearing HGF (Ad-HGF) has been tested on human patients with coronary heart disease in phase I safety assessment studies (Yuan, B., *et al.* 2008). The virus was well tolerated after intracoronary administration and 78% of patients showed improved cardiac function after treatment. The response rate was also observed to be dose-dependent, with the group receiving the highest dose showing positive response. In a similar study with the same virus, patients were given Ad-HGF and stent into the coronary artery. Compared with the control group the treatment group had significantly higher HGF, VEGF and monocyte attractant protein-1 serum levels (Yang, Z. J., *et al.* 2009). Both studies show good tolerance for Ad vectors when administered via intracoronary injection and beneficial properties of HGF for patients with coronary heart disease. Effects of Ad-VEGF have been evaluated in phase II clinical trials with encouraging results. The viral vector was well tolerated after administration during

percutaneous coronary intervention. Compared with the control group, the only significant treatment-related side effects were fever, elevated serum C-reactive protein, and rise of anti-Ad antibody levels. One Ad-VEGF-treated patient showed significantly improved myocardial perfusion. Functional capacity and exercise time improved in all groups with no significant differences between groups (Hedman, M., *et al.* 2003).

Recently Huusko *et. al*. (2009) compared the angiogenic properties of different VEGFs and evaluated their effects in an animal model (Huusko, J., *et al.* 2010). As in our work (discussed in 5.4.), after intracardiac injection all Ad groups showed moderate tissue damage and inflammation, with one group showing progressive toxicity, even though in contrast with our experiments all vectors were of Ad serotype 5 with varying transgenes. This may have been caused by the transgene or as a combined effect as VEGF enlarged the capillaries and induced endothelial cell proliferation resulting in more inflammatory cells migrating to the site of the vector. There has been only limited amount of reports addressing the Ad-induced toxicity in heart tissue as most toxicity experiments have focused on tissues like liver, spleen, kidney and lungs. A number of biodistribution studies have been reported which include an analysis of the heart; these studies have mainly concentrated on the transduction efficiency and report the transgene expression levels or viral genomes detected in various tissues (Wright, M. J., *et al.* 2001, Ni, S., *et al.* 2005). It has been shown that the Ad5/35 hybrid virus is able to transduce efficiently the heart, lungs and ovaries of the tgCD46 mouse. Also, in this mouse strain the hybrid Ad is less toxic than Ad5 as measured by increase in IL-6, TNF-α and liver enzyme levels. Interestingly, for liver enzymes, the situation is opposite in wt mice (Ganesh, S., *et al.* 2009). The liver infection by Ad5/35 observed in wt mice, is likely to be due to lack of receptors in mouse tissues, which results in virus accumulation in the liver. As it has been shown Ad5 is scavenged by liver cells by interaction with the Ad5 hexon (Waddington, S. N., *et al.* 2008). The presence of appropriate receptor in transgenic mouse tissues presents targets for Ad5/35 attachment and fewer viral particles are available for liver uptake.

One important aspect concerning the use of Ad vectors is the transient expression of transgene of about 2-3 weeks due to the fact that Ad genome does not integrate into the host genome. This can be viewed both as an advantage and as a weakness of the vector. Transient expression is an advantage because there is no fear of onset of oncogenes by vector integration. On the other hand, it is a weakness because it may take a significant amount of time before physiological effects are seen after initiation of transgene expression and in certain applications continuous expression is sought but is usually not gained with Ad vectors. With non-replicating Ad vectors carrying therapeutic genes, such as Serca2a or VEGF, the transient expression may present limitations for effective gene therapy of the heart. There might be several strategies that could be employed to increase Ad-mediated transgene expression time: 1) The Ad vector could be engineered to integrate into the host genome. This is probably not feasible due to increased risk of setting off oncogenes. 2) Making the Ad less immunogenic or

masking infected cells from the host immune system would extend the vector's expression time. 3) Engineering the Ad genome more stable as an episomal element in the host cell would also prolong the vector-mediated transgene expression time. Transient expression is usually not an issue with CRAds, because the initial viral load is multiplied within the target tissue.

3. Aims of the Study

The overall aim of this study was to analyze the transduction efficiency of capsid-modified adenovirus vector in comparison with non-modified vector both in cancer and cardiovascular models. The specific aims were:

1. To evaluate the differences in transduction efficiencies between non-modified Ad5 and capsid-modified Ad5/35 gene therapy vectors in HNSCC cells **(I)**.
2. To evaluate the HNSCC cell killing properties of CRAds Ad5-TERT and Ad5/35-TERT in both *in vitro* and *in vivo* models **(II)**.
3. To study the expression patterns of various Ad receptors in normal and diseased human heart tissue **(III)**.
4. To evaluate the differences between Ad5 and Ad5/35 vectors in transgene expression efficiency and vector toxicity in heart after intramyocardial administration **(IV)**.

4. Materials and Methods

More detailed descriptions of the methodologies used in this study can be found in the original publications (**I-IV**).

The human heart tissue samples were a generous gift from Dr. Mikko Mäyränpää and Dr. Petri Kovanen, Wihuri Institute, Helsinki. Left ventricle samples of failing hearts were harvested at the time of transplantation surgery from seven patients with end-stage heart failure due to idiopathic dilated cardiomyopathy (DCM). Normal heart samples were collected from donor heart left ventricles from patients whose hearts could not be used for transplantation. All heart tissue samples were frozen at the time of harvesting. More detailed description can be found in publication **III**.

Adenovirus construction was done using a two plasmid system. Transgene is inserted into a shuttle plasmid which is co-transformed together with backbone plasmid into a high-recombinase strain of *E. coli*. Homologous sequences in the plasmids allow homologous recombination to take place. Recombined plasmid, containing an intact Ad genome (except the E1A region) is isolated and propagated in low-recombinase *E. coli* strain. The bacterial control elements are removed and linearized Ad genome is transformed into HEK293 cells, which provide the missing E1A region transcripts and allow virus production. Viral vectors are produced and collected from HEK293 cultures. Finally Ad vectors are purified by CsCl gradient centrifugation and dialysis. Figure 5 shows the schematic presentation of key modification to the Ad genome of each vector used in this study.

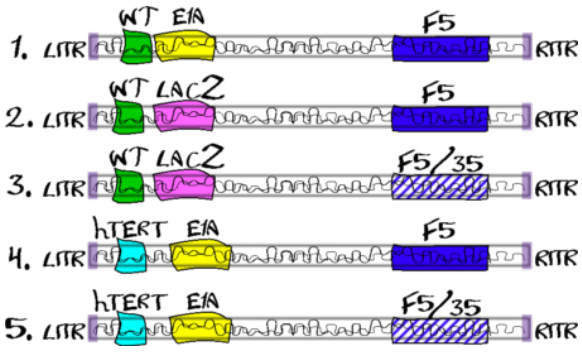


Figure 5. Schematic presentation of the genomes of Ad vectors used in this study. 1) Wild type Ad5, 2) Ad5-lacZ, E1A region has been replaced with *E. coli* β-galactosidase gene, 3) Ad5/35-lacZ, E1A region has been replaced with *E. coli* β-galactosidase gene and the fiber is a hybrid with that of serotype 35, 4) CRAd5, wt promoter of E1A has been replaced with hTERTp, 5) CRAd5, wt promoter of E1A has been replaced with hTERTp and the fiber is a hybrid with that of serotype 35.

Viruses and viral vectors

Vector	Capsid	Promoter	Transgene	Replication	Used in
Ad5-lacZ	wt	RSV	β-galactosidase[1]	non-replicative	I, II
Ad5/35-lacZ	Ad5 w/ serotype 35 fiber	RSV	β-galactosidase[1]	non-replicative	I
Ad5-TERT	wt	hTERTp	none	conditional	II
Ad5/35-TERT	Ad5 w/ serotype 35 fiber	hTERTp	none	conditional	II
Ad5 wt	wt	wt	none	replicative	II

[1]Transgene cloned to Ad gene E1A area

Methodology

Method	Used in
Cell culture	I, II
DNA cloning	I, IV
Flow cytometry	I, II
Immunofluorescence microscopy	III
Immunohistochemistry	I, II, III, IV
In vivo animal models	I, II, IV
Live cell microscopy	I, II
Marker gene transfer assays	I, IV
MTT assay	II
Q-RT-PCR	III
Statistical analysis	I, II, III, IV
Trypan Blue exclusion assay	II
Western blot	III

Reagents and compounds

Reagent	Description	Used in
DAB	Substrate for HRP, (IHC staining)	I, II, III, IV
DAPI	Nuclei staining	III
Eosin	Cytosol staining	IV
Hematoxylin	Nuclei staining	I, II, III, IV
Hoechst	Nuclei staining	III
MTT reagent	Cell dye reacting with live cells	II
Trypan Blue	Cell dye specific for dead cells	II
X-gal	Substrate for β-galactosidase	I, IV

Animals

Strain	Description	Used in
scid/scid	A homozygous mouse strain with severe combined immune deficiency, lacks T and B lymphocytes and immunoglobulins	I, II
nod/scid	A heterozygous mouse strain, non-obese diabetic with severe combined immune deficiency	II
huCD46tg*	A homozygous transgenic mouse strain which expresses human CD46 receptor	IV

*huCD46tg mouse strain was a generous gift from Dr. Ann-Beth Jonsson, Uppsala University, Sweden

Cell lines

Cell line	Description	Source	Used in
HEK293	Transformed human embryonic kidney cells	ATCC[1]	II, IV
UT-SCC-5	Human squamous cell carcinoma cells, tongue	Turku University Central Hospital[2]	I
UT-SCC-7	Human squamous cell carcinoma cells, temporal skin	Turku University Central Hospital[2]	II
UT-SCC-8	Human squamous cell carcinoma cells, epiglottis and hypopharynx	Turku University Central Hospital[2]	I
UT-SCC-9	Human squamous cell carcinoma cells, glottis larynx	Turku University Central Hospital[2]	I
UT-SCC-10	Human squamous cell carcinoma cells, tongue	Turku University Central Hospital[2]	I, II
UT-SCC-12A	Human squamous cell carcinoma cells, skin of nose	Turku University Central Hospital[2]	I
UT-SCC-13	Human squamous cell carcinoma cells, glottis larynx	Turku University Central Hospital[2]	I
UT-SCC-16B	Human squamous cell carcinoma cells, neck, metastasis	Turku University Central Hospital[2]	I
UT-SCC-18	Human squamous cell carcinoma cells, gingiva	Turku University Central Hospital[2]	I
UT-SCC-29	Human squamous cell carcinoma cells, glottis larynx	Turku University Central Hospital[2]	I

[1]Purchased from American Type Culture Collection (ATCC), Manassas, VA, USA
[2]Established at the time of operation

Antibodies

Epitope	Clone	Manufacturer	Application	Used in
CAR	E1-1	Abcam, UK	WB, IHC	III
CAR	RmcB	Upstate, USA	FC	I, II
CD46		Sigma-Aldrich, USA	WB	III
CD46	MEM-258	Biolegend, USA	FC, IHC	I, II
CD80	2D10	Abcam, UK	WB, FC, IHC	III
CD86	2F7	Abcam, UK	WB, FC, IHC	III
α_v-integrin	L230	N/A[1]	WB, FC, IHC	I, II, III
β-actin	AC-74	Sigma-Aldrich, USA	WB	III
Hsc70	SPA-815	StressGen Biotechnologies, Canada	WB	III

[1]Conditioned medium collected from ATCC HB-8448 hybridoma cell line

Oligonucleotides

Name	Gene	Direction	Sequence (5'-3')	Used in
Ad_CMV	CMV promoter	forward	CGTTACATAACTTACGGTAAATGGC	IV
Ad_tg_seul	human Serca2a	reverse	CGGATATCTTATCTAGAAGCTTAGGC	IV
MS-173	CD46	forward	TGACAATTCAGTGTGGAGTCG	III
MS-174	CD46	reverse	TGGAAATCGACATTTGACCA	III
MS-175	CAR	forward	ATGAAAAGGAAGTTCATCACGATA	III
MS-176	CAR	reverse	AATGATTACTGCCGATGTAGCTT	III
MS-177	α_v-integrin	forward	TTATACAATTTTACTGGCGAGCAG	III
MS-178	α_v-integrin	reverse	ACACATCTGCATAATCATCTCCA	III
MS-181	CD80	forward	TGGGCCATTACCTTAATCTCA	III
MS-182	CD80	reverse	CATCTTGGGGCAAAGCAG	III
MS-183	CD86	forward	CAAGACGCGGCTTTTATCTT	III
MS-184	CD86	reverse	ATCCAAGGAATGTGGTCTGG	III
MS-185	CD80	forward	AAGCAAGGGGCTGAAAAGAT	III
MS-186	CD80	reverse	TGGGGTAATCTTGTCCATCTG	III
MS-187	CD80	forward	GCTGTTCATGTTACTCATGACTCC	III
MS-188	CD80	reverse	GTTGCGTCCACTTCTGGTCT	III
UPL8	CD80	forward	CTGCCTTC	III
UPL10	CD80	forward	CCACCTCC	III
UPL27	CD80	forward	GCTGCCTG	III
UPL30	CD86	forward	CCTCAGCC	III
UPL50	CD46	forward	GCTCCAGA	III
UPL59	α_v-integrin	forward	TGCCACTG	III
UPL86	CAR	forward	CCACCTCC	III

UPL = Universal probe library

5. Results and Discussion

5.1. Expression of Adenovirus Receptors

Adenovirus infection begins with the recognition of a cellular receptor. Different Ad serotypes use various receptors for primary attachment, many of which have been identified. Two of the most studied Ad receptors are CAR (group C) and CD46 (group B) and these receptors will also be the main focus here. Efficient gene delivery to the target tissue has been shown to be of great importance in order to achieve the desired level of therapeutic effect with gene therapy applications. When using Ad vectors that harbor native or chimeric fiber proteins it is possible to identify the level of Ad receptors expressed on the target tissue. Several primary Ad receptors have been identified for different serotypes, and antibodies against these receptors are usually readily available for use in tissue characterization experiments. It has been previously shown that potential Ad target tissues have reduced CAR expression levels leading to poor transduction efficiency in these tissues (Hemmi, S., *et al.* 1998, Li, D., *et al.* 1999). Determining Ad primary receptor levels on target tissues would give an estimate on the transduction efficiency of the vector and guide in selecting the best Ad construct to be used on a specific target.

5.1.1. Head and Neck Squamous Cell Cancer (I)

Head and neck cancer cell lines used in this study have been established in Turku University Hospital during surgery of primary and metastatic tumors. These cell lines are named UT-SCC with running numbering distinguishing each histologically different cell line. These cells have not been previously characterized for their Ad receptor expression profile. In this work we analyzed various Ad primary receptor levels on several HNSCC cell lines (n = 9) with different clinical and histopathological characteristics (I: Table 1). Similarly, as reported earlier for different cell types, also our analysis showed greatly varying CAR levels between cell lines (Table 3). Two out of nine cell lines presented extremely low CAR expression and would most likely be refractory to Ad5 infection. Also, the highest detected CAR expression was only 61.5 %, but as seen from our transduction studies, this level already allows quite efficient Ad infection. In addition to CAR we also analyzed CD46 and α_v-integrin levels on these cells. Contrary to CAR, we found that both CD46 and α_v-integrin levels are expressed more uniformly and at a higher level. CD46 was expressed between 84.5–99.8 %. This consistent and high-level receptor expression should make these cells readily susceptible to Ad5/35 hybrid virus infection. α_v-integrin was expressed mostly at high levels with two exceptions showing only 39.4 % and 50.5 %. These levels should allow efficient internalization of all attached Ads. These results indicate vast heterogeneity in receptor profiles in HNSCC cancer. Similar results have been previously reported with other cancer types, such as osteosarcoma, ovarian cancer, and bladder cancer (Matsumoto, K., *et al.* 2005, Witlox, M. A., *et al.* 2002, Zeimet, A. G., *et al.* 2002). Our receptor analysis involved only a fraction of the different UT-SCC cell lines available. The observed difference in CAR expression in our sample group

suggests extremely high variance of CAR between patients, which makes unmodified Ad5 capsid-based vectors a very poor choice when designing a gene therapy vector for HNSCC. On the contrary, Ad5/35 hybrid vectors seem more suitable for HNSCC targeting. As stated above, the high level and low variance in CD46 expression pattern should allow for efficient transduction for vectors harboring group B fibers that recognize this receptor.

Table 3. Adenoviral receptor expression levels on head and neck cancer cell lines. HEK 293 cell line was included as control. Values indicate percentage of positive cells as measured using FACS.

	UT-SCC-5	**UT-SCC-7**	**UT-SCC-8**	**UT-SCC-9**	**UT-SCC-10**	**UT-SCC-12A**
CAR	47,4	55,2	37,9	41,1	3,2	28,8
CD46	84,5	90,0	96,3	99,7	94,0	99,8
α_v-integrin	39,4	91,3	89,5	96,3	76,6	94,7
	UT-SCC-13	**UT-SCC-16B**	**UT-SCC-18**	**UT-SCC-29**	**HEK-293**	
CAR	61,5	28,6	18,2	1,6	99,8	
CD46	97,7	99,4	99,4	96,3	99,5	
α_v-integrin	50,5	98,0	98,3	91,3	99,7	

5.1.2. Human Cardiac Tissue (III)

Human cardiac tissue has been scarcely studied for expression of Ad receptors other than CAR. Following the same line of thought as with HNSCC, we analyzed various Ad receptor expression levels on 14 human cardiac samples (7 normal, 7 DCM) (III: table 1). Contrary to HNSCC studies, we found that CAR was significantly up-regulated in the diseased tissue when compared to normal samples (Figure 6). CD46 was expressed slightly less in DCM (non-significant) and α_v-integrin levels were lower in the diseased than in the normal samples. We also analyzed two other Ad receptors, CD80 and CD86. CD86 was up-regulated in DCM according to receptor staining studies on tissue sections, but down-regulated according to gene expression studies by detection of mRNA, although both results were statistically non-significant, which suggests that CD86 expression is not affected by onset of DCM. The lack of difference between healthy and diseased tissue makes CD86 less than an optimal receptor for targeted gene therapy. Observed discrepancy between assay results also suggests that CD86 is quite stable at the cell surface and is not continuously transcribed, but further studies are needed to corroborate this hypothesis. CD80 levels seemed to be up-regulated in the DCM, but unfortunately overall expression of this receptor was barely detectable and the difference was non-significant. Extremely low expression levels do not make CD80 an interesting candidate for targeting Ads to the heart, even if the receptor is differentially expressed in normal heart tissue and in DCM. As discussed earlier, efficient transduction of target cells is paramount for achieving therapeutic efficacy when using Ad vectors in gene therapy applications. Very low receptor expression levels would most likely not allow for efficient transduction as shown in study I for Ad5 with cell lines UT-SCC-10 and -29.

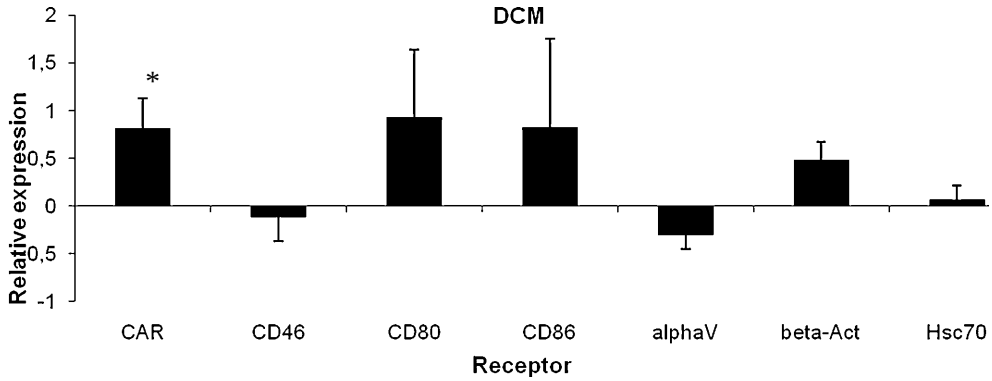


Figure 6. Relative receptor expression quantified from western blot analysis of normal and DCM human heart samples (n = 7, statistical significance *$p < 0.05$). Value for healthy tissue set as 0.

It is interesting that CAR expression was found up-regulated in DCM samples but not in non-DCM samples and further investigations might shed more light on CAR's specific function. CAR has been identified as part of the tight junction and is also involved in formation of the junction by recruiting other macromolecules to the site of the junction formation (Cohen, C. J., *et al.* 2001, Coyne, C. B., *et al.* 2004). More specific role of CAR as part of the tight junction or other functions are still unknown. It has been proposed that CAR would regulate tissue permeability and homeostasis (Raschperger, E., *et al.* 2006). If CAR has a signaling property which guides for correct organization of specific cells in tissues, increasing the expression of CAR would be reasonable in DCM. In DCM, heart tissue is abnormal, which could trigger the need for cellular reorganization as heart tissue tries to regain the normal state. Cell-to-cell recognition properties of CAR has been reported previously in the context of embryogenesis, where CAR is strongly expressed (Ito, M., *et al.* 2000). It has also been shown in a rat model that CAR expression decreases up to 190-fold after birth (Fechner, H., *et al.* 2003).

Our immunofluorescence staining of CAR and CD46 in human heart tissue revealed differences between CAR and CD46 expression localization in the cardiac tissue. CD46 was found to be more ubiquitously expressed in the heart and CAR more localized in blood vessels. Interestingly, both receptors are localized seven times more frequently to vessel wall endothelium in DCM hearts than in non-DCM hearts. In normal hearts CAR was localized to subendothelial layer over 16 times and CD46 over 24 times more frequently than in DCM tissue (**III**: Figure 7). CAR has previously been reported to be expressed also in sarcolemma and intercalated disk in DCM heart tissue and with very low expression on normal heart tissue. This differential CAR expression has also direct impact on heart susceptibility for Ad infection; normally a healthy heart is quite refractory to Ad infection, but DCM hearts have been shown to be 15 times more vulnerable for Ad5 infection (Noutsias, M., *et al.* 2001).

The difference between both CAR and CD46 expression pattern in non-DCM and DCM presents interesting implications for gene therapy. The heart can be targeted with engineered viruses carrying a therapeutic gene. Our results show that both CAR and CD46 expression shift from subendothelial layer to endothelial cells in DCM, but only CAR expression is up-regulated. These results indicate that it would be more feasible to infect DCM hearts via intravascular administration. The choice of the therapeutic gene can be evaluated taking into account the expression sites of these receptors, e.g. tight junctions (CAR) and vein endothelial cells (both CAR and CD46). For example, using either Ad5-VEGF or Ad5/35-VEGF vectors one could target the VEGF expression directly to the vein endothelium, where the angiogenesis takes place. This should result in vascularization of surrounding areas of the heart and blood flow would be reinstated into oxygen depleted areas. Unfortunately, there is a number of other issues yet to be resolved before this kind of straightforward model is feasible, starting with choosing the correct therapeutic gene.

5.2. Adenovirus mediated transgene expression in HNSCC (I)

Ad receptor analysis on HNSCC cells suggested that Ad5/35 hybrid vector would be more efficient in transducing these cells than non-targeted Ad5. Four cell lines were chosen for the transduction studies. Cells were grown *in vitro* and infected with either Ad5-lacZ or Ad5/35-lacZ reporter viruses. Transgene expression was observed in cell cultures and was found to correlate with Ad receptor levels, higher receptor level cells were transduced more efficiently than low receptor level cells (quantified in Figure 7 and visualized in **I**: Figure 2).

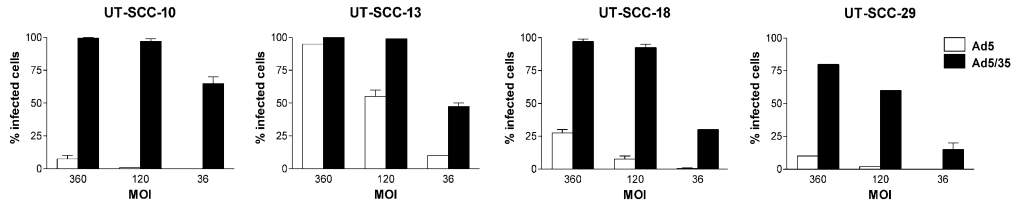


Figure 7. *In vitro* transduction efficiency of Ad5-lacZ and Ad5/35-lacZ in selected UT-SCC cell lines.

As expected, cell line UT-SCC-13, with the highest detected CAR expression (61.5%), was fairly efficiently infected with Ad5-lacZ virus. The observed correlation between receptor levels and Ad transfection efficiency in UT-SCC *in vitro* studies led to the hypothesis of a receptor threshold level, since UT-SCC-13 was transduced quite efficiently, about half of the cells were infected, and yet UT-SCC-18 with 18.2% CAR expression was transduced poorly, showing only a few individual infected cells. Overall Ad5-lacZ was very poor in infecting the UT-SCC-10, -18 and -29 cell lines, with the highest infection percentage less than 30 with the highest virus concentration. On the contrary, Ad5/35-lacZ was able to infect 60-100% of the cells with the virus concentration dropped to only one third. These findings were further investigated *in*

vivo. UT-SCC tumors grown in the flanks of scid mice were injected with Ad5-lacZ or Ad5/35-lacZ. Sections of tumors were immunostained and evaluated for transgene expression. The results corroborated those obtained from *in vitro* experiments. Ad5/35-lacz induced β-galactosidase expression was observed throughout the sections, while Ad5-lacZ transduced only a few separate areas within the tumor section (**I**: Figure 6).

The Ad5 versus Ad5/35 setting was taken through experiments from *in vitro* to *in vivo* and all studies show the superior transduction ability of the hybrid vector when compared to Ad5. Further experiments, for example with larger study groups or with more cell lines, could further provide more insight to questions like: what is the receptor level allowing for efficient infection and what is the exact minimum amount of virus needed for efficient transgene expression. It should be possible to change the transgene of vectors Ad5-lacZ and Ad5/35-lacZ to a therapeutic gene with no significant change to the expression patterns observed here for the reporter vectors.

Receptor analysis and transduction studies suggest that hybrid Ad5/35 capsid construction is better suited for targeting HNSCC than Ad5. From this perspective, the performance of vector Ad5-p53 (discussed in part 2.2.2.), which has met success in clinical trials and has even entered pharmaceutical markets in China (Zhang, S. W., *et al.* 2005), would benefit from fiber swapping technology. By changing fibers to those of Ad35 and creating Ad5/35-p53, the safety profile of the vector should improve, since smaller doses of the hybrid vector could be used to achieve the same level of therapeutic effect. On the other hand, capsid structure is also the main determinant for how immunogenic the vector is. Thus, the immunogenic properties will change when the fiber is swapped and new safety studies would be mandatory.

5.3. Cytotoxic effects (II)

To evaluate the cytotoxic properties of CRAds Ad5-TERT and Ad5/35-TERT, we selected two UT-SCC cell lines, which represent high and low CAR expression, but similar levels of CD46 expression. UT-SCC-7 and UT-SCC-10 cell lines were used both *in vitro* and *in vivo* experiments. We hypothesized that the hybrid virus would be able to lyse both cell lines more efficiently than Ad5-TERT and that Ad5-TERT-induced cytopathic effect (CPE) would be greater on UT-SCC-7 cell line than on UT-SCC-10. Our hypothesis was proven true both *in vitro* cell culture (Figure 8) and *in vivo* xenograft model (Figure 9). As shown previously for transduction studies, primary receptor expression levels on target cells correlated with CRAd mediated cell killing efficacy. Also the viruses were able to replicate in HNSCC cells even with their replication restricted due to tissue-specific promoter. The human telomerase promoter system can be used to target Ad replication to the HNSCC tumors.

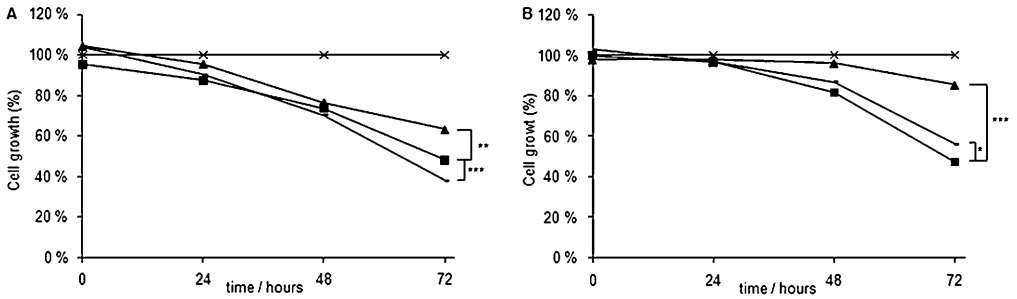


Figure 8. Cytotoxic effects of Ad5-TERT (solid triangle) and Ad5/35-TERT (solid box) viruses on UT-SCC-7 (A) and UT-SCC-10 (B) cell lines. Wild type Ad5 (minus sign) was included to assess maximal cell death at given time points. PBS control (x) was included to represent the maximal non-hindered cell growth. Statistical significance**$p < 0.01$, ***$p < 0.005$ (**II**: Figure 4 C and F).

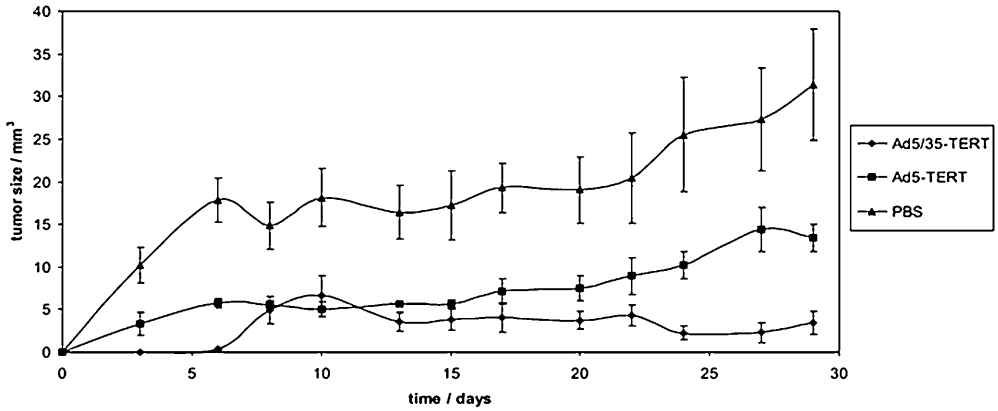


Figure 9. Tumor growth in an *in vivo* mouse model. Ad5/35-TERT, Ad5-TERT or PBS mock infected tumor cells were injected into mice subcutaneously.

We analyzed the correlation between cytopathic effect (CPE) formation and primary receptor level expression. Four UT-SCC cell lines representing varying levels of CAR expression were analyzed in a simple MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell survival assay using both Ad5-TERT and Ad5/35-TERT viruses. Ad5/35-TERT is clearly more efficient in reducing cell growth on low-CAR cells. On the contrary, with mid-CAR cell lines, UT-SCC-7 and UT-SCC-13, both viruses are almost equally effective (Table 4). Since CD46 is expressed at a high level by all four cell lines, survival after Ad5/35-TERT treatment can be used as the "maximal viral effect" with these hTERTp controlled CRADs for each cell line. Looking at the survival differences between viruses in cell lines UT-SCC-7 and UT-SCC-13 we can make the following cautious statement: the threshold of primary receptor expression needed for efficient transduction is higher than 60%. More UT-SCC cell lines should be analyzed and ideally a high-CAR (over 90% expression) cell

line would be found and subjected to the same experiments. The results would allow for more specific statements of receptor threshold needed for efficient adenoviral infection. It must also be noted that different UT-SCC cell lines have vastly different growth rates even without any treatment. The cellular growth rate also influences the survival after viral treatments. A higher growth rate allows the viruses to work faster and slow-growing cells show a more modest effect at the same time point, as long as the cell growth does not counteract the cell killing effect of the virus.

Table 4. Percentage of cells surviving after virus treatment compared with PBS control in UT-SCC cell lines expressing different levels of CAR. Growth rate: +++ robust, ++ average, + slow and +/- very slow.

	UT-SCC-10	**UT-SCC-18**	**UT-SCC-7**	**UT-SCC-13**
Survival w/Ad5-TERT	85 %	76 %	63 %	83 %
Survival w/ Ad5/35-TERT	47 %	45 %	48 %	70 %
Growth rate	+++	+	++	+/-
CAR expression	3 %	18 %	55 %	62 %
CD46 expression	94 %	99 %	90 %	98 %

5.4. Adenoviral transduction and toxicity in the heart (IV)

As discussed in section 5.1.2., we analyzed Ad receptors in human cardiac tissues and observed CAR to be up-regulated in DCM. Next, we wanted to study the effects of Ad5-lacZ and hybrid Ad5/35-lacZ reporter viruses on a mouse model after intracardiac injection. A transgenic mouse strain was used, which expresses human CD46 gene in a similar pattern as observed for humans. Details on intracardiac injection can be found in study IV.

As expected after receptor studies, we observed significantly more profound transgene expression with Ad5-lacZ (Figure 10). The vector with native capsid was able to induce transgene expression in over 3 times larger area than Ad5/35-lacZ hybrid virus ($p = 0.01$). Surprisingly though, we also observed more severe tissue damage due to immune reactions with Ad5-lacZ when compared with the hybrid virus (Figure 10).

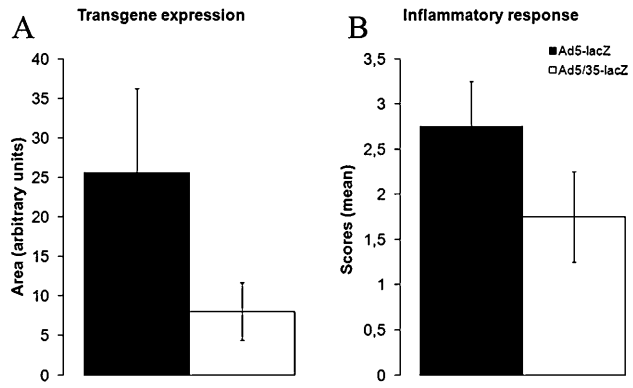


Figure 10. Ad5-lacZ transduces significantly more effectively heart tissue than Ad5/35-lacZ (A) $p < 0.01$, but also induces more severe immune reactions (B) p = ns.

This Ad5-lacZ-provoked toxicity can undermine the benefits gained for efficient gene expression. Most likely this level of immunoreactions would lead to severe complications and impaired heart function. However, it must be mentioned that our results for the difference in cytotoxicity are statistically non-significant and the groups were very small (n = 4). To corroborate the suggested difference in cytotoxicity between Ad5 and Ad5/35, a larger study must be carried out. In addition to direct analysis of heart sections, other inflammatory markers should also be analyzed, such as IL-6, IL-12 and IFN-γ. This more thorough study would answer the question of cytotoxicity decisively, either confirming our suggested results or indicating no difference between the two viruses. Whichever the case, these results shown here implicate severe toxicity of Ad5 when administered intramyocardially. Combined with already compromised cardiac function in DCM, the Ad5 treatment may even be more harmful than helpful with some patients. If Ad5 vectors are to be used for treatment of heart disease, the vector must be made far less immunoreactive than the native serotype 5 adenovirus.

Most studies involving adenoviral activity in the heart compose of biodistribution studies after intravenous (i.v.) administration. It has previously been shown that both Ad5-lacZ and Ad5/35-lacZ genomes are present at relatively high concentrations in the hearts of hCD46Ge mice and baboons. For Ad5/35-lacZ, the heart is one of the most effectively transduced tissues in both models (Ni, S., *et al.* 2005, Ganesh, S., *et al.* 2009). Interestingly, with the primate model no transgene activity was reported in the heart tissue for any vector despite the presence of viral genomes. Analysis of genome copies in tissues does not discriminate between active or inactive viral particles or between intracellular genomes and viruses in extracellular space. Unfortunately, only this method of analysis was employed by Ganesh *et. al.* (2009). Here we show the expression and activity of viral transgene in hCD46Ge mice. The results indicate that both vectors are transcribed in this murine model. Ganesh *et. al.* (2009) also analyzed vector-mediated toxicity by measuring serum IL-6 and liver enzyme levels and showed

decreased overall toxicity with Ad5/35-lacZ vector after i.v. administration. Our results together with previous studies suggest that changing Ad5 fiber to those of Ad35 increases the safety profile of gene therapy vectors. Also, vector administration by i.v. instead of direct intramyocardial injection may be better suited for gene therapy targeting the heart.

6. Summary and Conclusions

The goals of this study were to characterize target-tissue Ad receptor levels and after selection of a proper vector to analyze the difference in efficacy of a targeted vector in both cancer and cardiovascular models.

It has been shown that Ad receptors may be down-regulated in various diseases leading to impaired therapeutic effects with Ad vectors. We have shown with different HNSCC cell lines, that CAR levels vary significantly even within a cancer type, while another Ad receptor, CD46, remains at higher and more constant levels. It may also be that there is an as-yet-unknown threshold level of Ad receptors which allows for effective infection of target tissue. On the other hand, it is clear that tissues not expressing the correct Ad receptor are refractory to Ad transfection. These tissues can be targeted and effectively transduced by a hybrid Ad vector with changed tropism.

In cancer treatment the basic goal is to rid the patient from the cancer cells without killing normal cells. One way of doing this with viral vectors is to utilize the natural ability of viruses to kill their host cells. We used an hTERT promoter to control and target the replication of the Ad vector. By combining the transductional targeting of a hybrid virus and the transcriptional control implemented by the insertion hTERT, we constructed a CRAd and characterized its tumor-killing properties both *in vitro* and *in vivo* model systems. In comparison with non-targeted Ad5-TERT vector and wt Ad5 virus the Ad5/35-TERT hybrid virus was more efficient in killing HNSCC cells. The efficacy is largely dependent on expression of primary Ad receptors on target cells.

In case of cardiovascular diseases the approach is quite different. The goal obviously is not to kill target cells, but to transfect them and get them to start producing some beneficial peptide or protein which helps the functionality of heart tissue. The efficient transfection of target tissue is, again, of paramount importance. We investigated expression of various Ad receptors in human heart tissue. Contrary to previous studies with HNSCC, CAR was found to be expressed at a high level and up-regulated in DCM. A subsequent hypothesis based on these findings was that Ad5 is more efficient in transducing cardiac tissue than hybrid Ad5/35. This hypothesis was tested in a transgenic mouse model with ultrasound-guided intracardiac injection using reporter viruses of both capsid configurations. As expected, we observed more extensive transgene expression with Ad5 than with Ad5/35. Interestingly, we also observed more severe vector-induced inflammation and tissue damage in mice treated with the Ad5 virus. This suggests that native Ad5 is poorly tolerated when injected directly into the cardiac wall.

To date numerous clinical trials have been conducted using Ad vectors for many diseases. Ads have been shown to be well tolerated, but dosage and the delivery method must be carefully chosen. Most problems involve transduction efficiency as transgene expression or viral function tends to remain lower in clinical settings than in

the laboratory. Also the advantage offered by gene therapy has varied greatly between trials and individuals, and often the benefit gained is modest although definitive.

More work and research on viral vectors is needed before the breakthrough in gene therapy will be achieved. Research on diseases and growing understanding of disease mechanisms will help in the design and construction of the best vector configuration for each particular application. Based on our studies and work done by others, it is clear that the vector selection must be based on the intended application. Different possible combinations for viral vectors are innumerous; selection of the virus, capsid modifications, promoters, transgene / suicide gene, replicating / non-replicating characteristics, etc. Each combination will have its own pros and cons, which will be more pronounced in some ailments than others. The door is open and the tools are there, now it is time to choose the right tool for the right job.

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Raine Toivonen

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