Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

Presented by

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born in:	**Munich**
Oral-examination:	

Genetic Modification of Oncolytic Adenoviruses for Anti-Cancer-Therapy

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ZUSAMMENFASSUNG

Typische Merkmale von Krebserkrankungen sind unkontrolliertes Wachstum, Streuung der Tumorzellen in umliegende Gewebe und letztlich auch die Resistenz gegen verschiedene Therapieansätze. Besonders letzteres zeigt den Bedarf an neuen, innovativen Behandlungsmöglichkeiten wie die adenovirale Onkolyse. Dabei werden Tumorzellen mit tumorspezifisch replikationsfähigen oder onkolytischen Adenoviren (Ad) infiziert und durch deren lytischen Replikationszyklus zerstört.
Die Expression therapeutischer Gene mit Hilfe von onkolytischen Ad ist ein neues und vielversprechendes Konzept zur Behandlung von Krebs. Neben der Art des Transgens ist die Effizienz und Kinetik der Transgenexpression entscheidend. Das Ziel des Projektes war es das Transgen so in das virale Genom zu inserieren, dass seine Expression nach der viralen Replikation erfolgt. Diesbezüglich habe ich das Reportergen Luziferase mit Hilfe einer Internen Ribosomen Bindungssequenz, einer selbst spaltenden 2A-Sequenz oder einer zusätzlichen Spleiß-Akzeptor Sequenz in die späte Transkriptionseinheit des adenoviralen Genoms eingefügt. Für eine Melanom-spezifische, virale Replikation ersetzte ich den Promotor des essentiellen viralen Gens E1A durch den Melanom-spezifischen Tyrosinase Promotor. Während alle onkolytischen Ad dieselbe Zytotoxizität in Melanomzellen zeigten, war der lytische Effekt der Melanom-spezifischen Viren in nicht melanotischem Gewebe um den Faktor 10 – 1000 reduziert. Meine Ergebnisse zeigten, dass die Spezifität der Transgenexpression von der Strategie der Transgeninsertion im Virusgenom abhängt. Die höchste Spezifität der Luziferasexpression konnte für die Kombination von transkriptionellem Targeting und Transgenexpression mittels alternativen Spleißens gezeigt werden. Zur Verknüpfung von Virustherapie und molekularer Chemotherapie inserierte ich ein Prodrug-aktivierendes Gen mittels optimierter Spleiß-Akzeptor Sequenz in das virale Genom. Dieser Ansatz resultierte in indirektem, transkriptionellem Targeting der genetischen Prodrug-Aktivierung und zeigte eine verbesserte Effizienz der Onkolyse in der Kombinationstherapie.
Die therapeutische Anwendung von onkolytischen Ad würde deutlich von einem gerichteten Tumorzelleintritt profitieren, der durch den natürlichen Ad Tropismus nicht gegeben ist. Dies erfordert die Ablation des nativen Ad Tropismus und den Einbau Zell-bindender Liganden. Die kurzen Fiberproteine des Ad41 (Ad41s) Kapsids konnten bereits aufgrund ihrer reduzierten Lebertransduktion erfolgreich als Ausgangsformat für die Tropismusablation eingesetzt werden. Basierend auf Studien bezüglich Insertionspositionen für Peptidliganden im Ad41s Fiberprotein war mein Ziel den Replikationszyklus von Ad mit derartigen Kapsidmodifikationen zu untersuchen. Mittels des Modellpeptides RGD konnte die Produktion der onkolytischen Ad, welche eine interne RGD Insertion in unterschiedlichen Loops des Fiberproteins aufweisen, gezeigt werden. Abhängig von der Insertionsposition hat sich die Genexpression der frühe und späte viralen Gene der Fiber-Chimären Ad deutlich unterschieden. Die Viren wiesen im Vergleich zum Wildtyp Ad5 eine reduzierte Zytotoxizität auf, welche unabhängig von der Insertionsposition des Peptids war. Die Ergebnisse deuten auf eine reduzierte oder verspätete Viruszusammensetzung hin. Für den gerichteten Krebszelleintritt wurde ein kürzlich beschriebenes, EphA2-bindendes Peptid in eine definierte Position des Ad41s Fiberproteins eingebaut. Das resultierende Virus konnte spezifisch EphA2-positive Zellen infizieren, aber es wies im Vergleich zur entsprechenden Kontrolle ein deutlich geringeres lytisches Potential auf.
Die Kombination von effektiver Onkolyse mit einer gerichteten Gentherapie konnte in dieser Arbeit eindrucksvoll gezeigt werden. Des Weiteren liefert diese Studie wichtige Grundlagen für die Entwicklung von Peptid-abhängigem Zelleintritt onkolytischer Ad.

1. SUMMARY

Cancer is characterized by growing incidence, early metastasis, and resistance against effective treatment for advanced disease, suggesting a pressing need for novel therapeutic approaches. Recombinant adenoviruses (Ad) have emerged as promising agents in therapeutic gene transfer, genetic vaccination and virotherapy. Virotherapy is defined as killing of cancer cells by specific virus infection, replication, cell lysis and virus spread by so called oncolytic viruses.

"Armed" oncolytic Ad in which a therapeutic gene is genetically engineered into the virus and dependent on the tumour-selective replication of the virus for expression, represent a new and promising strategy for cancer treatment. This was achieved by using either an internal ribosomal entry site, a 'self-cleaving' 2A peptide or by an additional splice acceptor site to insert the reporter gene luciferase into the late transcription unit. In addition, I engineered novel melanoma-targeted, conditionally replicative Ad by replacing the promoter of the essential viral gene E1A with a cassette containing a human tyrosinase enhancer/promoter construct. While all oncolytic adenoviruses showed nearly similar cytotoxicity in melanoma cells, the melanoma specific Ads had a 10 – 1000 fold lower cytopathic effect in cell lines derived from various nonmelanocytic tissues. I showed that the mode of transgene expression and the locale of transgene insertion into the virus genome critically determine the efficacy of this approach. The most specific transgene expression (up to 1500 fold) was observed for an Ad which combines the tyrosinase promoter and the transgene expression by alternative splicing. In summary, I could show that transcriptional targeting combined with splice acceptor site mediated transgene expression is feasible and results in the highest levels of selectivity for replication and transgene expression. To combine the benefit of viral oncolysis with molecular chemotherapy, I inserted a suicide gene for gene directed enzyme prodrug therapy via optimized splice acceptor site into the virus genome. This approach resulted in indirect transcriptional targeting of genetic prodrug activation by the "armed", melanoma specific Ad as well as in increased therapeutic effect of the oncolytic Ad.

Therapeutic applications of Ad would benefit form a targeted virus cell entry into cancer cells. Such tropism-modification of Ad requires the ablation of their natural cell binding properties and the incorporation of cell-binding peptides. The short capsid fiber proteins of Ad subgroup F has recently been suggested as a tool for genetic Ad detargeting based on the reduced liver infectivity of corresponding fiber chimeric Ad-vectors *in vitro* and *in vivo*. Based on previous studies to determine functional insertion sites for peptide ligands into the Ad41 short fiber of Ad-vectors, I investigated the lytic potency of oncolytic Ad engineered with peptide ligands inserted in the Ad41 fiber. With the RGD model peptide I could demonstrate that the production of oncolytic Ad with ligands inserted into different loops of the fiber is feasible. Depending on the insertion site, viral replication, early and late viral gene expressions of the fiber-chimeric Ad differs in comparison to matching control viruses, carrying the same ligand in the HI loop of Ad5 fiber. The cytotoxicity of fiber-chimeric viruses is reduced irrespective of the insertion site. The results indicate that a decreased or delayed virus assembly could be a reason for my observation. Furthermore, I used fiber-chimeric virus as a novel platform for genetic targeting of Ad cell entry. I generated an ablated virus which targets EphA2 receptor expressing melanoma cells via inserted EphA2 peptide ligand. However, the lytic potency of the chimeric fiber Ad was significantly reduced compared to the matching control virus.

Summarized, I developed an oncolytic Ad which combined effective oncolysis with targeted prodrug activation therapy of melanoma and I generated an oncolytic Ad showing peptide-dependent cell entry for targeted oncolysis of melanoma.

2. INTRODUCTION

2.1. Cancer and cancer therapies

Cancer is a disease of chaos, a breakdown of existing biological order within the body. More specifically, the disorder seen in cancer appears to derive from a population of cells, which display uncontrolled growth, invasion of adjacent tissues and metastasis (spread to other location in the body via lymph or blood). Most of the 10^{13} cells in our body can cross over the border from normalcy to malignancy and transform into a cancer cell. This explains why cancer can affect all tissues. Depending on their origin they are classified as carcinoma (malignant tumours derived from epithelia), sarcoma (tumours derived from a variety of mesenchymal cell types), lymphoma (malignancies derived from hematopoietic tissues, including the cells of the immune system), blastoma (tumours caused by malignancies in precursor cells) and melanoma (tumours of melanocytes). These observations about the origin of cancer force us to consider our thinking about how cancers are formed or what can trigger a cell to transform into a cancer cell. Is it an inherent risk of incessant cell divisions during normal biological processes or are there some factors, which increase the risk of cancer?

In 1915, Katsusaburo Yamagiwa published the first experimental induction of tumours by treatment of rabbit skin with a chemical carcinogen (coal tar condensate). Together with Peyton Rous's observation, that tumour formation can be induced by a chicken sarcoma virus, the first evidences that some types of cancer are associated with specific exposures, virus infection or lifestyle, were given.

These days, much more is known about the cause of cancer and the state of knowledge is that cancer is a genetic disease. Heredity and environment (chemical carcinogens, ionizing radiation, viral or bacterial infection) are the two factors, which mainly determine the risk of cancer. The avoidance of certain cancer causing factors in diet and lifestyle is associated by up to 50% reduction in the risk of dying from cancer in the West. For example, tobacco smoking is associated with many types of cancers, and causes 90% of all lung cancers. Hepatitis B and C viruses and human papillomaviruses play key roles in development of different cancer types such as cervical cancer, as well. Furthermore, laboratory research supported that only 1 in 10^{17} cell divisions lead to a clinically detectable cancer, so that the risk to develop cancer caused on an error during the cell cycle is less than the epidemiologically

observed risk. However, there are still open questions in the process of cancer pathogenesis, which need to be explained and understood.

In the eighteenth century, the first cancer therapy was described by John Hunter (1728-1793). He suggested that surgery could be a method to remove the tumour and cure cancer. The first non-surgical treatment came up with the discovery of X-rays by Wilhelm Conrad Roentgen and with the discovery of natural radioactivity by Henry Becquerel. Most of the anticancer treatments used today were developed in the period before 1975. In this time the knowledge about the genetic and biochemical mechanisms of cancer pathogenesis was marginal. This could be one explanation why most approaches for cancer therapy have been not so successful. For example, in 1970 in the United States, the 5 years survival rate of patients diagnosed with lung cancer was 7%. Three decades later, this number had risen to 14%, a relatively minor enhancement. However, a few types of cancer could be already cured such as testicular cancer.

Hence, one of the most difficult questions often posed to cancer researchers still is: How is cancer going to be cured? In regard of the today's knowledge it doesn't exist a simple answer, which advises a single therapy that will cure all cancers, because cancer is not a single disease. Instead, there will several individual anti-cancer therapies, which are targeted specific ally to one or a small group of cancer types. In 2001 the first company came out with a targeted cancer therapy: Trastuzumab (Herceptin).It blocks human epidermal growth receptor 2, which is overexpressed in 25% of all breast cancers and in almost half of glioblastomas. Furthermore Gleevec, Avastin and Rituxan come to mind here. Gleevec was developed to block the tyrosine kinase activity of Bcr-Abl fusion protein, which is constantly active in chronic myelogenous leukaemia (CML). Rituxan can be used for the treatment of B-cell tumours. But there are still a high number of research projects on going to develop new anti-cancer therapies. These therapeutic agents included small-molecular-weight drugs, proteins, monoclonal antibodies, immunotherapy, gene therapy and virotherapy.

2.2. Gene therapy for cancer treatment

The term gene therapy originally encompasses a range of treatments that use correction of a defective gene function as the solution to cure genetic disorders. Defective genes, which are the reason for the disease, are mostly replaced with the intact genes for restoring the lost gene function in the patient. This should result into

an elimination of the disease. For that purpose, DNA segments of the gene of interest are cloned into so called vectors, which are able to deliver the healthy gene into the target cells. A wide range of these vectors were investigated either *ex vivo* or *in vivo* and shown remarkable efficacy. A variety of different methods has been developed for transferring the therapeutic gene into the cell such as microinjection, electroporation, liposome formulations, gene gun and the use of viral vectors. Meanwhile, viral vectors have evolved to become the most common gene delivery system based on their high efficiency. Several types of viral vectors, including retrovirus, adenovirus, adeno-associated virus (AAV), and herpes virus, have been used for gene therapeutic applications.

In 1990, the first person was successfully treated with viral gene therapy. W.F. Anderson could cure *severe combined immune deficiency* (SCID) by delivering the adenosine-deaminase (ADA) gene via a retrovirus into the stem cells of a four year old girl (Beardsley et al. 1990; Culver, Anderson et al. 1991). In the mean time, only one mutation which causes SCID is curable by gene therapy. Furthermore, gene therapies are still aimed to hereditary genetic diseases, as cystic fibrosis, duchenne muscular dystrophy, arthritis and lysosomal storage diseases. However, the most clinical treatments involving gene therapy are directed against cancer. Many approaches for cancer gene therapy try to compensate mutations of either tumour suppressor genes or oncogenes. The tumour suppressor genes retinoblastoma (RB), p53, adenomatous polyposis coli (APC), Breast Cancer 1 (BRCA 1) and $p16^{INK4a}$ are often mutated and become inactive in many tumour types. A promising viral vector is a replication-incompetent adenovirus transferring the p53 gene to cancer cells resulting in inhibition of angiogenesis and tumour cell invasion as well as apoptosis through induction of Fas/CD95-mediated cell death (Merritt, Roth et al. 2001; Swisher, Roth et al. 2003).

An additional and innovative approach for cancer gene therapy is the concept of boosting the immune system in order to target and destroy cancer cells. Therefore, the anti-tumour immune response is augmented by delivering immunostimulatory genes into cancer cells. These transgenes are often cytokines that produce further pro-inflammatory immune stimulating molecules and encourage the development of an anti-tumour immune response, mainly cytotoxic T-lymphocytes (CTLs), which are able to kill the cancer cells (Nawrocki, Wysocki et al. 2001; Handerson, Mossman et al. 2005). For example, in a phase I trial an adenovirus was used to transfer the cytokine IL-2 into high-risk localized prostate cancer to augment an inflammatory

response (Trudel, Trachtenberg et al. 2003). Another very promising immunotherapy based on viral vectors resulted in the successfully treatment of metastatic melanoma in 2006. A retrovirus-mediated delivery of a melanoma-specific T-cell receptor for an adoptive T-cell therapy demonstrated to cause tumour recognition by autologous lymphocytes from peripheral blood (Morgan, Dudley, Wunderlich et al. 2006).

Gene-directed enzyme-prodrug therapy (GDEPT) is also another promising approach for gene therapy. This prodrug based cancer therapy consists of two components: a nontoxic prodrug, which can be applicated systemically at high doses, and a prodrug activating enzyme. Whereas the dose dependent side effect limited the traditional chemotherapy, the prodrug therapy tries to minimize this toxicity by selectively converting the chemotherapeutic agent by the enzyme at the target tumour site. Ideally, surrounding tumour cells are also killed by the activated chemotherapeutic drug (local bystander effect). Predominantly adenoviral and retroviral vectors have been used in GDEPT clinical trials. Thymidine kinase (TK) and cytosine deaminase (CD) and their respective prodrugs ganciclovir (GCV) and 5-flourocytosine (5-FC) are the most advanced prodrug based therapies, which have shown increased therapeutic potency (Kirn, Niculescu-Duvaz et al. 2002; van Dillen, Mulder et al. 2002). In this regard, Immonen et al. engineered an adenoviral vector coding for the thymidine kinase (HSV-TK), which improves in combination with the prodrug ganciclovir survival of patients with malignant glioma (Immonen et al. 2004).

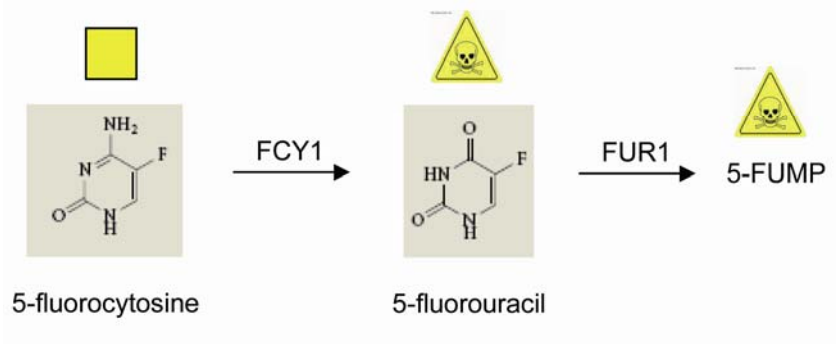


Fig.1: **Prodrug activation system FCU-1 / 5-FC.** Schematic outline of the mechanism of prodrug 5-fluorocytosine (5-FC) activation by the suicide gene *FCU-1*, which encodes a fusion protein derived from the *yeast cytosine deaminase* (*FCY-1*) and *uracil phosphoribosyltransferase genes* (*FUR-1*) (Erbs et al. 2000). *5-FUMP*, *5-fluoro-UMP*

Of note, a new and highly potent suicide gene is *FCU-1*, which encodes a fusion protein derived from the *yeast cytosine deaminase* (*FCY-1*) and *uracil phosphoribosyltransferase genes* (*FUR-1*) (Erbs et al. 2000; Fig. 1). The FCY-1 domain deaminates the nontoxic prodrug 5-fluorocytosine (5-FC) to its highly toxic

derivative 5-fluorouracil (5-FU). Its active metabolite is 5-FUTP, which can either be incorporated into RNA resulting in the inhibition of the mRNA processing or can be converted to 5-FUMP by FUR1. The toxic 5-FUMP inhibits the cellular enzyme thymidylate synthases which is required for DNA synthesis. Erbs et al. have suggested that the insertion of the fusion gene *FCU-1* in cancer cell significantly increases cancer cell death after 5-FC application. Interestingly, and in contrast to GCV and its toxic metabolites, 5-FC and 5-FU can penetrate cells by passive diffusion and expand the local toxic effects to neighbouring, uninfected cells, irrespective of the presence of specific cellular connexins. The second advantage of the FCU-1/5-FC system is the more effective prodrug activation *in vitro* and *in vivo* in comparison to TK/GCV and CD/5-FC (Erbs et al. 2000). Other enzyme/pro-drug combinations are Nitroreductase and CB1954 (Braybrooke et al. 2005) as well as CYP2B6 and Cyclophosphamide (Barzon et al. 2004; Singh et al. 2001).

Meanwhile, viral vectors have been evaluated as a promising new tool for the development of novel cancer gene therapies. The key requirement of this approach is the stringent delivery of the appropriate gene into tumour cells without harming healthy cells. Therefore, the therapeutic genes have to be expressed only in cancer cells in order to act in a specific and safe manner. The main approaches by which viral vectors and thus transgene expression can be made tumour selective are by modification of viral cell binding and entry (= transductional targeting), by specific regulation of gene expression (= transcriptional targeting) and especially by the combination of both targeting strategies. Transductional targeting has been realised by modification of the viral tropism from the native receptor to a surface protein preferentially expressed on cancer cells through genetic engineering of the virus capsid (Glasgow, Everts et al. 2006) or by conjunction with bispecific adapters (Haisma et al. 2000). Transcriptional targeting has been achieved by insertion of a tissue/tumour-selective promoter driving the expression of therapeutic genes (Nettelbeck, Jerome et al. 2000). For cancer therapy, a few promoters have already been used, such as the ErbB-2 promoter (Pandha et al. 1999) and human alpha-lactalbumin promoter (Anderson et al. 1999) for breast cancer and the prostate-specific-antigen (PSA) promoter for prostate cancer (Pang, Taneja et al. 1995; Lee,Liu et al. 1996; Martiniello-Wilks, Garcia-Aragon et al. 1998). In this regard, the tyrosinase promoter (Vile and Hart 1993; Hughes, Wells et al. 1995) is a promising candidate for targeting malignant melanoma. Tyrosinase is a pigment cell-restricted enzyme that catalyzes the rate limiting step in melanin synthesis. Mechanisms of

tissue-specific transcriptional control were intensively explored beginning in the 1980s, resulting in promoter studies as exemplified for genes associated with pigmentation pathways in melanocytes (Kluppel et al. 1991; Shibata et al. 1992; Lowings et al. 1992; Yavuzer and Goding, 1993; Ganss et al. 1994; Bentley et al. 1994). Subsequently, tissue specific promoters were optimized for gene therapy applications, thus as the tyrosinase promoter (Vile and Hart 1993; Siders et al. 1996). The study of Siders et al. describes optimized regulatory sequences of enhancer and promoter derived from human tyrosinase promoter region that is sufficient for high-level, tissue-specific transcription in pigment lineage cells. However, these targeting strategies imply that the expression of the therapeutic gene is highly specific and efficient expressed at high level in the tumour cells.

2.3. Virotherapy for cancer treatment

In the quest to develop novel cancer therapies, virotherapy, or viral oncolysis, has recently emerged as a promising new modality to specifically kill tumour cells. It is defined as killing of cancer cells by tumour-selective virus infection, replication and concomitant cell lysis. The newly synthesized oncolytic viruses are able to spread through tumour mass and start a new therapeutic cycle by virtue of viral replication and cell lysis. Key advantage of viral oncolysis is the amplification of the therapeutic agent in the patients' tumour.

Already in the beginning of the last century, a first case of viral anti-tumour activity was reported where a patient with cervical carcinoma showed significant tumour regression after rabbies vaccination (de Pace et al. 1912). More recent clinical reports have described the remission of Burkitt's and Hodgkin' lymphomas following natural infection with measles virus (Bluming and Ziegler et al. 1971; Taqi, Abdurrahman et al. 1981). The mechanisms of naturally occurring oncotropismus of some viruses are poorly understood but are likely to be found in the genetic and cellular changes acquired during carcinogenesis. For example, mouse fibroblasts are normally resistant to reovirus infection but become susceptible to virus infection following transformation of the ras-pathway, which is often constitutively activated in tumours (Coffey, Strong et al. 1998; Strong and Lee, 1996). Additional strategies are used for genetic engineering that facilitates targeting oncolytic viruses to tumour cells. On the one hand, tumour selectivity can be achieved by deleting of viral gene that are essential for viral replication in healthy tissue but are dispensable upon infection of transformed cells, or on the other hand by placement of a tumour-specific

promoter upstream of a viral gene, which is a key player of the viral replication (see chapter 2.4.3.1). The first viral gene deletion mutant was reported in 1991. In this herpes simplex virus I (HSV-I) the thymidine kinase gene was deleted, which is needed for nucleic acid metabolism and only produced at high concentration in proliferating cells, thus as tumour cells (Martuza et al. 1991).

This knowledge provides the basis for clinical trials with potentially oncolytic viruses. In most studies oncolytic viruses have not been very successful in destroying tumours, yet. Several factors have been identified that could explain the marginal therapeutic benefit of previously investigated oncolytic viruses. In most cases the virus is neutralized by the host immune system and failed to impact tumour growth. Furthermore, efficient viral cell entry and spread within the tumour mass as well as potential cell killing are also an important requirement for a successful tumour remission. To overcome these hurdles, intensive research has aimed to genetically engineer viruses in order to optimize tumour specificity without harming their oncolytic potency. These days, optimized adenovirus (Hecht, Bedford, Abbruzzese et al. 2003), herpes simplex virus I (Markert, Medlock, Rabkin et al. 2000), Vaccinia virus (Wallack, Sivanandham, Balch et al. 1998) and Newcastle disease virus (Batliwalla, Bateman, Serrano et al. 1998) are tested in phase II and phase III trials.

2.4. Adenovirus and their use as gene therapy vector or oncolytic virus

Several biological features have made adenovirus (Ad) a promising tool for anti-cancer therapy. It is endemic in human population and its natural pathogenicity is related only with mild respiratory infection (Shenk et al. 1996). Also the adenoviral double stranded DNA genome exists only in episomal form in infected cells, thus avoiding the risk of insertion mutagenesis. Furthermore, Ads are highly stable and have the ability to infect a wide variety of dividing and non-dividing cells, as well as possessing established procedures for manufacturing and production of high viral titers, which is essential for clinical utility. Beside these features, Ads own the capacity of transgene insertion (up to 8kb) without loss of replication potential and importantly, the technology and genetic knowledge to manipulate the viral genome is also already available so that Ads can be modified to become tumour selective. Consequently, Ads have already been used in clinical trails and in regard to these studies Ads offer a powerful, attractive therapeutic for cancer.

2.4.1. Adenoviruses: Virion Structure, Cell Entry and Genome Organization

2.4.1.1. Serotypes and Virus Structure

Since the first isolation of Ad from adenoid tissue by Rowe and colleagues (Rowe, Huebner et al. 1953) a large number of different species, and more than 100 different serotypes have been reported, among of these 52 in humans. The human Ad serotypes have been sub grouped from A to F based on genome size, organization, homology, nucleotide composition, oncogenicity and hemagglutination properties (Fields, Knipe et al. 1996). The most well studied serotypes are the group C viruses, which consist of the Ad serotypes 1, 2, 5, and 6. So far, the most gene therapy and virotherapy studies have been carried out using the serotypes 2 and 5 (Ad2 and Ad5). Since the discovery of Adenovirus (Ad) rapid advances have been made in the understanding of molecular virology. The genome structure and organization, capsid configuration and lytic replication cycle of adenoviruses are exceptionally well described in detail (Zhang 1999; Russel 2009)

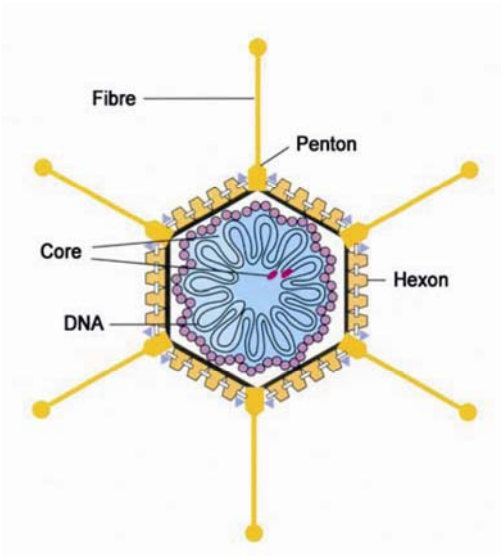


Fig.2: **Schematic diagram of the Ad5 virion.** Adenoviruses are non-enveloped particles of 70-90nm in diameter with an inner nucleoprotein core. The double stranded DNA is packaged within an icosahedral protein capsid. The major protein of the capsid is the trimeric hexon that constitutes the 20 triangular faces of the icosahedron. The penton base and fiber proteins are localized at each of the 12 vertices of the Ad capsid. (origin: http://biomarker.cdc.go.kr:8080/index.jsp)

Adenoviruses are non-enveloped, icosahedra particles of 70-90nm in diameter with an inner nucleoprotein core containing the Ad genome, which is encased in a protein shell, the so-called capsid (Fig. 2). The capsid is comprised of three major protein components: hexon, penton, and fiber. Hexon, a stable trimer, is the most abundant structural component and constitutes the 20 triangular faces of the icosahedron. The pentons contain a pentameric penton base, which associate with trimeric fiber proteins to form penton capsomer complexes. The penton acts as an anchor for the fiber protein, which extends outward from the virion like an antenna (Philipson 1984; Rux and Burnett 2004).

The fiber is a homotrimer in which three identical polypeptides are arranged in parallel orientation (Stouten, Sander et al. 1992). Each of these polypeptides consists of three distinct domains: tail, shaft, and knob. The tail domain non-covalently binds fiber to the penton base, whereas the shaft domain extends away from the virion surface. The C-terminus of the trimer forms a propeller-like knob (Xia, Henry et al. 1994), which is responsible for binding to the host's primary cellular virus receptor. Furthermore, the knob domain is essential for trimerization and stabilizing the trimeric configuration of the fiber, which has been shown to be crucial for association of the fiber with the penton base in the assembly of native virions (Novelli and Boulanger 1991).

2.4.1.2. Cell Binding and Entry

The primary receptor for many human Ad serotypes, including the subgroup C serotype 2 and 5 viruses, is the coxsackie- and adenovirus-receptor (CAR) (Bergelson, Cunningham et al. 1997; Tomko, Xu et al. 1997; Roelvink, Mi Lee et al. 1999). CAR is a transmembrane protein and is present in many human tissues including heart, lung, liver, and brain (Howitt, Anderson et al. 2003). Recent research has shown that besides CAR Ads can use other molecules as receptors as well. The coagulation factor X is discussed to mediate liver transduction via the viral hexon protein *in vivo* (Waddington et al. 2006).

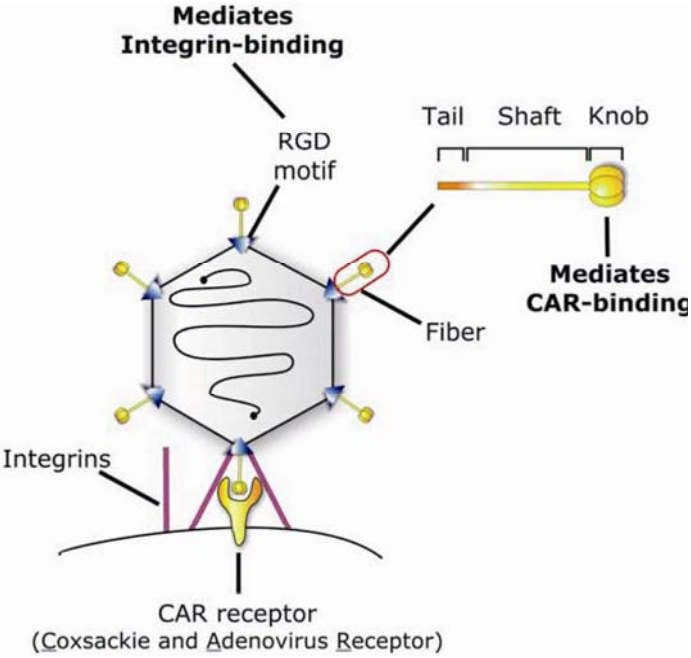


Fig.3: **Cell binding mechanism of Ad5 virion**. Ad5 binds to its receptor CAR through the fiber. The Fiber protein consists of a tail, shaft and knob domain. Subsequently, integrins interact with the RGD peptide motif in the penton base and facilitate cell entry by endocytosis.

The best known are major histocompatibility complex I (MHCI) and heparan sulfate glycosaminoglycans (Hong, Karayan et al. 1997; Dechecchi, Melotti et al. 2001).
In contrast, most subgroup B viruses (particularly serotypes 3, 11, 14, 16, 21, 35, 50) seem to bind to the plasma membrane protein CD46 ubiquitously expressed (Zhang and Bergelson 2005).
Cell infection for most non-group B viruses starts with high-affinity binding to CAR via the fiber knob domain (Fig. 3). Following attachment, the Arg-Gly-Asp (RGD) motifs of the penton base interact with cellular integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which initiates viral endocytosis within a clathrin-coated vesicle (Wickham, Mathias et al. 1993). The acidic environment of the endosome alters the topology of the virus as capsid components dissociate, which ultimately induces escape of the virions into the cytoplasm. With the help of microtubules the virions translocate toward the nucleus pore complex. Meanwhile, the viral particle disassembles and viral DNA enters the nucleus via the nuclear pore where it associates with histone molecules (Meier and Greber 2003) and activates viral gene expression and genome replication.

2.4.1.3. Genome Organization and Viral Replication

The Ad genome itself is a linear, double-stranded DNA that is approximately 36kb in length with inverted terminal repeats (ITRs) at both ends. Both ITRs in company with the encapsidation signal on the left end, act as cis elements, which are necessary for viral DNA replication and packaging. The genome is subdivided in five early (E1A, E1B, E2, E3 and E4), four intermediate (IVa2, IX, VAI, and VAII) and one late transcription units (Fig. 4).

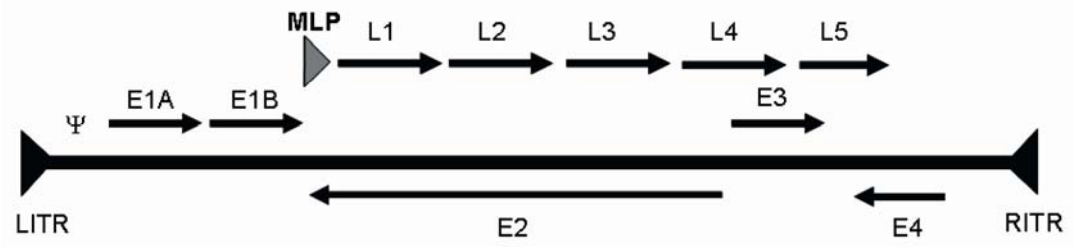


Fig.4: **Schematic representation of adenovirus genome and transcription units.** The central solid line represents the viral genome. Positions of the left and right ITR (inverted terminal repeats), the packaging sequence (ψ), the early transcription units (E1A, E1B, E2, E3 and E4), and the major late transcription unit (major late promoter (MLP), L1-L5) are shown. Arrows indicate the direction of transcription (McConnell and Imperiale 2004).

The first viral protein to be expressed is E1A, which activates transcription of the early transcription units and induce the host cell to enter S phase by interfering with proteins of the retinoblastoma (Rb) pathway, thereby providing an optimal environment for virus replication. Rb acts as a tumour suppressor by inhibiting cell cycle progression via binding to E2F, a transcriptional activator that promotes expression of genes necessary for driving cells into S phase. E1A proteins are able to sequester Rb, which releases E2F, allowing it to drive the expression of cellular genes involved in cell cycle entry and DNA replication. Such a cell cycle deregulation would normally lead to the accumulation of the tumour suppressor p53, followed by apoptosis. Expression of the adenoviral E1B genes prevents premature cell death, thus allowing viral replication to occur unimpeded. The E1B55K protein directly binds to p53, induces its degradation and inhibits its ability to induce expression of pro-apoptotic genes (Sarnow, Ho et al. 1982; Ben-Israel and Kleinberger 2002). However, adenovirus infection also stimulates apoptosis by a p53-independent mechanism, which is blocked by the E1B19K protein. The E1B19K protein shares structural and sequence homology with cellular Bcl-2 proteins. Proteasomal degradation of the critical anti-apoptotic Bcl-2 family member Mcl-1 by E1A expression results in the release and activation of the pro-apoptotic Bcl-2 family member BAK, normally bound by Mcl-1 in uninfected cells. E1B19K directly binds to BAK and another pro-apoptotic protein, BAX, inhibiting their co-oligomerization and forming pores in the outer mitochondrial membrane, allowing survival of infected cells (White, Cipriani et al. 1991; Han, Sabbatini et al. 1996; Han, Modha et al. 1998; Sundararajan, Cuconati et al. 2001; McNees and Gooding 2002). In Ad-*E1B19K* mutants, formation of such BAK-BAX heterocomplexes correlate with the release of cytochrome c and Smac/DIABOLO from the intermembrane space of mitochondria, resulting in activation of the downstream caspases 9 and 3 and the ensuing apoptosis program (Cuconati, Degenhardt et al. 2002).

Further, the E2 region encodes for proteins needed for viral DNA replication: DNA polymerase, preterminal protein and single-stranded DNA binding protein (de Jong, van der Vliet et al. 2003). Even though adenovirus replication takes place in the nucleus, it requires its own enzymatic machinery because of its chromosomal structure. The viral genome does not have telomeres, so the integrity of the DNA ends is ensured by the preterminal protein (pTP), which is covalently linked to the 5´ end of each genome strand and acts as a primer for the viral DNA polymerase. Additional, the DNA-binding protein (DBP) is encoded in early region E2a. It is a

multifunctional protein involved in several aspects of the adenovirus life cycle, including an ability to modulate transcription.

The proteins of the E3 unit subvert the host immune response and allow persistence of infected cells by reducing the presentation of viral antigens of major histocompatibility complex (MHC) class I determinants (Bennett, Bennink et al. 1999). Furthermore these proteins protect the infected cell from lysis by TNF-α and Fas by clearing their receptors from the cell surface (Gooding, Ranheim et al. 1991; Shisler, Yang et al. 1997). In contrast to the immunomodulatory functions of all other E3 genes the E3 11.6kd protein, which is also referred to as the adenovirus death protein (ADP), increases the efficiency of lysis and release of progeny virus from the infected cell and accumulates mainly during the late stage of infection (Tollefson, Ryerse et al. 1996; Tollefson, Scaria et al. 1996). Of note, it is not necessary for adenoviral replication *in vitro*.

The E4 proteins are known to play a role in DNA replication, late gene expression, and splicing (Tauber and Dobner 2001); together with other early proteins they also mediate the inhibition of the production, maturation, or stability of host cellular DNA, RNA and/or proteins.

Among the intermediate transcription unit the IX gene acts as a transcriptional transactivator as well as a minor component of the viral capsid that increases virus stability (Sargent, Meulenbroek et al. 2004). The IVa2 protein is crucial for assembly of adenoviruses and packaging of viral DNA (Zhang and Imperiale 2003). The VA (VA I and VA II) genes encode for short stretches of RNA (approx. 200 bases), which form double-stranded hairpin-loop structures that are essential for maintaining translation of adenoviral genes by inhibiting the cellular protein kinase R (PKR) pathway, which otherwise would block protein translation and finally trigger apoptosis in order to prevent further viral spread.

The adenoviral late genes are divided into five subunits, L1-L5, and are transcribed from one promoter, the major late promoter (MLP) that is particularly efficient during late infection, which starts with replication of the virus genome. The major late transcription unit encodes up to 20 different mRNAs, all of which are derived from a long precursor transcript by differential splicing and polyadenylation. These transcripts primarily encode structural proteins of the virus and other proteins involved in virion assembly. Viral particle assembly in the nucleus starts about 8h post-infection (dependent on the cell type) and results in the production of

approximately 10^4 new viral particles per cell. At approximately 30 – 40 h after virus entry the new virus generation is released by cell lysis (Büchen-Osmond, 2003). One protein that facilitates the spread of progeny virus involves ADP that localizes to the nuclear membrane, endoplasmatic reticulum and golgi apparatus where it interacts with a protein called MAD2B. However, how this interaction leads to cell death and by which mechanism the cell dies still remains uncertain. Adenovirus-induced cell death was long presumed to be apoptotic (Hall et al., 1998) but recent studies either suggest a "necrosis-like programmed cell death" (Abou El Hassan, van der Meulen-Muileman et al. 2004) or autophagy to be involved in the death of adenovirus-infected cells (Ito, Aoki et al. 2006).

2.4.2. Adenoviral vectors

In the last two decades, adenoviral vectors have received much attention as gene transfer agents and offer a wide variety of gene therapeutic applications. In the so called first generation of adenoviral vectors, the viral early region 1 (E1), and less often the early region 3 (E3), were substituted by transgenes. The E1 region encodes proteins necessary for the activation of viral promoters and for the expression of further early and late genes. Hence the E1 proteins are key regulators of viral replication and the removal of this region renders the vectors replication-defective. For the viral growth of E1 deleted adenoviruses helper cell lines are necessary to complement the E1 functions *in trans*. By transforming a human embryonic kidney cell line with sheared fragments of adenovirus type 5 DNA, which also includes the E1 region, the Ad producer cell line 293 was established (Graham, Smiley et al. 1977; Fallaux, Bout et al. 1998; Schiedner, Hertel et al. 2000). The E3 region-encoded proteins are not essential for the viral replication *in vitro*, and therefore, no helper cell lines are needed for the production of an E3 deleted adenoviral vector (Wold, Tollefson, Hermiston et al. 1995). The benefit of E1 / E3 double deleted vectors is a higher capacity for foreign DNA. Since adenovirus can package approximately 38 kb without affecting growth rate and viral titer (Bett, Prevec, Graham et al. 1993), E1 deleted virus can accept insertion up to 5.1 kb, while E1 / E3 deleted adenoviruses can accommodate about 8.2 kb of transgene DNA.

First generation adenovirus causes strong systemic immune response *in vivo*, chiefly due to the *de novo* synthesis of viral proteins at very low levels (Gilgenkrantz et al. 1995; Yang, Su, Wilson et al. 1996). To avoid this significant immunogenicity, additional viral genes responsible for viral replication have been deleted, giving rise

to the second generation of adenoviral vectors. These are characterized by deletion of E1, E2 (Amalfitano, Hauser et al. 1998; Lusky, Christ et al. 1998) and E4 (Armentano, Sookdeo et al. 1995) genes. A positive effect in regard to a reduction of an inflammatory response could be demonstrated only in some studies, however, others failed (Gao, Yang et al. 1996; Lusky, Christ et al. 1998).

To further enhance the transgene capacity of adenoviruses, a third generation of vectors has been established. These so called “high capacity” or “helper-dependent” vectors (Kochanek et al. 1996; Parks et al. 1996; Fisher et al. 1996; Kumar-Singh) are deleted for all viral genes, but retain the two ITRs and the Ψ, as *cis*-acting signal required for viral replication and packaging, which allows up to 36kb transgene insertion. However, their viral replication, growth and purification turned out to be difficult due to complementation of viral protein functions by helper cell lines or helper viruses and need to be optimized.

2.4.3. Oncolytic adenoviruses

In the last two decades, genetically engineered oncolytic adenoviruses (OAds) that specifically target and destroy tumour cells by viral lysis, offer more and more the opportunity for the development of promising cancer therapies.

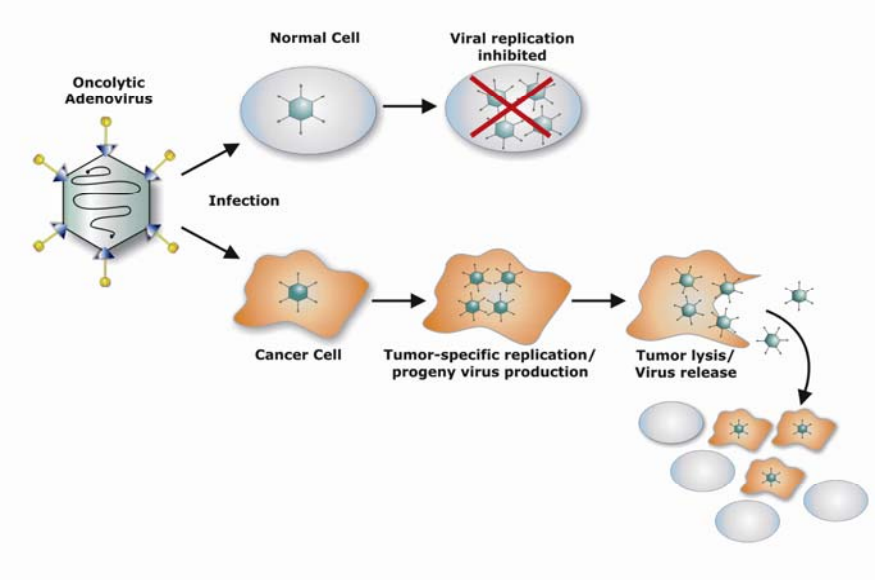


Fig.5: **Concept of adenoviral oncolysis.** Oncolytic adenoviruses are derived from human adenoviruses via genetic modifications. Such modifications include the mutation or deletion of viral genes, or the insertion of tumour-specific promoters. Oncolytic adenoviruses infect tumour cells, replicate their genome, assemble new viral particles and kill the host tumour cell by lysis, resulting in the release of the progeny viruses. This new virus generation spreads, and starts a new cycle of virus replication and tumour cell killing. Genetic modifications of adenoviruses allow for efficacy enhancement and/or tumour cell restriction at various steps of the viral life cycle, such as viral infection, viral replication and/or virus release/tumour cell lysis. As a consequence, infection of normal cells by oncolytic adenoviruses and/or replication within these cells is impaired. Thus, the ideal oncolytic adenovirus represents an efficient and specific anti-cancer agent.

This strategy exploits their highly evolved quality of efficient cell entry and lytic property during virus replication to destroy tumour cells. The viral mediated tumour regression is propagated through the specific infection of adjacent tumour cells by the newly released progeny virus particles (Fig. 5).

2.4.3.1. Strategies for restricting Ad replication to tumour cells

The key requirement for adenoviral oncolysis is safety. This can be achieved through restriction of viral replication to cancer cells. Therefore, different approaches have been established. On the one hand, deletion (fully or in part) of early viral genes which are critical for viral replication in healthy cells but dispensable upon infection in cancer cells has been one strategy. Otherwise, the replacement of viral promoters with tissue or tumour-specific promoters has also successfully targeted viral replication to tumour cells. Of note, both mechanisms of transcritptional control are cellular genetic tools, which operate post entry.

Most approaches for the development of oncolytic adenoviruses have focused on the genetic engineering of their early E1 genes. The E1 genes are the first genes expressed after viral cell entry and are the key regulator for viral replication. The two E1 genes E1A and E1B (E1B55K) have been mostly modified in order to achieve tumour selective replication. Normally, their proteins force the host cell to enter the S phase, an assumption for viral replication. A 24pb deletion in the E1A region avoids that the E1A protein is able to bind and inactivate the retinoblastoma protein (see chapter 2.4.1.3). In this regard, S phase induction by the OAds is ablated, which is dispensable in proliferating cells lacking a functional RB pathway. An example of an E1A mutated OAds is Ad5Δ24 (dl992-947), which has a deletion of 24bp in the pRb-binding domain of E1A (Fueyo et al. 2000, Heise et al. 2000). Efficiency of AdΔ24 was shown in several tumour types, such as lung adenocarcinoma, prostate cancer and ovarian cancer (Bauerschmitz et al. 2002, Fueyo et al. 2000, Suzuki et al. 2001) and it also has been demonstrated that AdΔ24 replicates in cancer cells as efficient as wild type adenovirus, because other E1A functions that are mandatory for viral replication are retained. Another OAd, called dl1520 or ONYX-015 was derived by deletion of E1B55K (Bischoff et al. 1996). Since the tumour suppressor protein p53 cannot be inactivated by binding E1B55K, apoptosis is induced in infected cells, aborting replication and spread of the virus (Bischoff, Kirn et al. 1996). Thus, the presumption was that ONYX-015 can only replicate in p53-deficient tumour cells. However, many approaches indicated that the proposed basis for selective replication is far more complex and not solely determined by p53 deficiency

(Geoerger et al. 2002, Kirn et al. 2001). In summary, these virus mutants revealed a proof of principle for tumour selectivity of adenoviral replication, a favourable safety profile in clinical trails and offer a basis for on-going research efforts.

To achieve specificity of adenoviral replication, investigators have also used a second strategy based on exploiting transcriptional control regions. This so called "transcriptional targeting" deals mostly with placing the essential viral gene E1A under the control of tissue or tumour-specific promoters, which are preferentially active in tumour cells and inactive in normal cells. The first approach of a tissue-specific OAd has been used for prostate cancer. The OAd Calydon CV706 was limited to prostate-specific antigen (PSA) -producing tissues, such as human prostate cancer cells, by insertion of the PSA gene promoter-enhancer element upstream of the E1A gene, which is needed to induce the viral replication cycle. This study showed, that CV706 was able to specifically destroy prostate tumours in xenograft mouse models (Rodriguez, Schuur et al. 1997) and demonstrated a high safety index in a phase I trail (Deweese, Vander, Li et al. 2001).

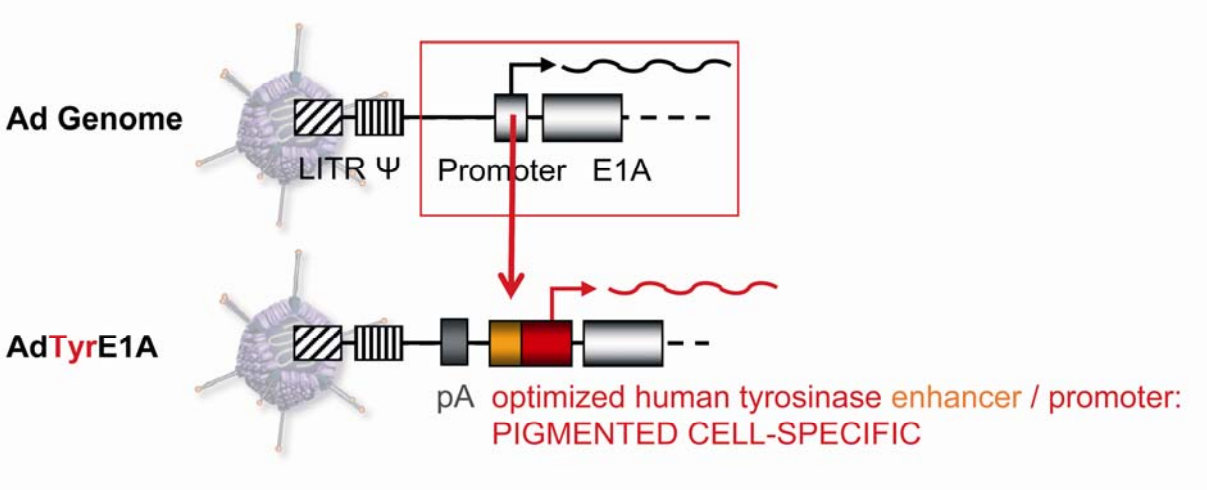


Fig.6: **Transcriptional targeting by an optimized human tyrosinase enhancer / promoter construct (Nettelbeck et al. 2002).** A schematic outline of modifications introduced into the adenovirus serotype 5 genome is shown. The optimized tyrosinase enhancer / promoter construct consists out of two tandem enhancers and the core promoter (-209/+51) of the humane tyrosinase genes; LITR, left inverted terminal repeat; Ψ, packaging signal; pA, polyadenylation signal.

Another promising OAd, which embodies this principle and is of special interest for malignant melanoma, use the optimized tyrosinase enhancer/promoter, TyrE/P (Nettelbeck et al. 1999), to drive expression of E1A (Fig. 6) or E4 gene (see also chapter 2.2). In this regard, a highly melanoma-selective expression of E1 or E4 was shown. Importantly, specific expression of E1 or E4 from TyrE/P resulted in reduced virus progeny production and cytotoxicity in non-melanoma cells, whereas replication and cytotoxicity were similar to wildtype adenovirus in melanoma cells (Nettelbeck et

al. 2002; Banerjee et al. 2004). Furthermore, the selectivity of TyrE/P was confirmed in a co-culture system and in three-dimensional organotypic cultures, a mixed population of melanoma cells and primary human keratinocytes (Nettelbeck et al. 2002; Banerjee et al. 2004). Based on these results, TyrE/P is a prominent candidate for tissue-selective targeting of OAds to malignant melanoma. However, this principle of tissue targeting implies that the OAds can also replicate in healthy tissues from which the tumours originate. In the case of OAds targeted by PSA promoter or TyrE/P, harming of prostate epithelium or pigmented cells of the skin might be tolerable.

In addition to tissue-specific regulatory elements, many other tumour selective promoters have been characterized and tested to target adenoviruses to hepatocellular carcinoma, using AFP promoter (Hallenbeck, Chang et al. 1999; Li, Yu et al. 2001; Ohashi, Kanai et al. 2001); to breast carcinomas, using DF3/MUCI gene promoter (Kurihara, Brough, Kovesdi et al. 2000); to ovarian carcinoma, using a secretory leukoprotease inhibitor promoter (Barker, Dmitriev, Nettelbeck et al. 2003); and neuroblastoma, using the midkine differentiation factor promoter (Adachi, Reynolds, Yamamoto et al. 2001).

2.4.3.2. Strategies for tropism modification of adenoviruses

Currently, ONYX-015 has been tested in phase I and II clinical trials for head and neck-, pancreatic-, ovarian-, colorectal-, lung-cancer and oral carcinoma. Despite of a high titer application systemically or locally, no statistic significant tumour regression was observed. Toxicity and intratumoral spread of this first generation adenovirus seem to be the limitation *in vivo*. One explanation has been shown to result from a deficiency of the primary receptor CAR on tumour cells. Accordingly, most tumour cells freshly purified form tumour biopsies have been revealed to be resistant to adenovirus infection due to a lack of CAR expression (Hemmi, Geertsen et al. 1998; Miller, Buchsbaum et al. 1998; Li, Pong et al. 1999). As all present OAds are derived from serotype 2 or 5, which both infect cells via binding to CAR, tropism modification of these agents is critically required for efficient oncolysis. Moreover, tumour targeting could also lessen side effects, such as hepatocellular cytotoxicity associated with systemic adenovirus application.

To overcome this obstacle, the cell binding domain of Ad5 was switched between different adenovirus serotypes. In this regard, substitution of the Ad5 fiber knob domain with the corresponding domain of subgroup B Ad3 implements potent cell

entry of the tropism-modified OAd into primary melanoma cells (Rivera et al. 2004B), ovarian cancer cells (Kanerva, Mikheeva et al. 2002), renal cancer cells (Haviv, Blackwell et al.2002), and squamous cell carcinoma (Kawakami, Li et al. 2003), which are all resistant to Ad5 infection. However, this strategy results rather in enhancing of unselective cell entry than in increased tumour specificity, potency and safety. To achieve tumour cell-selective entry of OAds, the ablation of the natural tropism and the retargeting for example by incorporation of targeting ligands into the virus capsid are required. Notably, all oncolytic adenoviruses described to date are based on post-entry restriction of the adenoviral life cycle.

First efforts focused on insertion of candidate ligands into the HI loop or at the C-terminus of the adenoviral fiber. The incorporation of few peptides was feasible without the loss of fiber stability and folding properties and furthermore, the ligands could retain their binding capacity. Note that the insertion of recombinant antibody fragments as targeting motifs into the adenoviral capsid is hampered by the incomplete folding of most antibodies in the cytosol, where adenoviral proteins are produced. Dmitriev and co-workers inserted the targeting peptide RGD-4C into the HI loop to broaden the viral tropism to the αv integrin-mediated entry pathways without harming the fiber firmness (Dmitriev, Krasnykh et al. 1998). In an Ad context, the placement of RGD motif in the HI loop showed increased transduction efficacy for primary and established cancer cells, such as squamous cell carcinoma of the head and neck (Kasono, Blackwell et al. 1999) and pancreatic cancer cells (Wesseling, Bosma et al. 2001) as well as dendritic cells (Asada-Mikami, Heike et al. 2001). However, only one study by Baker and colleagues could demonstrate selective adenoviral transduction of endothelial cells by insertion of a targeting peptide into the Ad5 fiber (Nicklin et al. 2001). Besides, previous reported tumour-specific peptides and nanobodies consisting of a single monomeric variable antibody domain (Cortez-Retamozo et al. 2004) represent ligands with high potential for targeting of OAds cell binding and entry. However, efficient and selective as well as successful anti-cancer therapy mediated by targeting viral cell entry is not shown yet.

A prerequisite for a successful Ad tumour targeting is still the ablation of the native tropism to avoid infection of healthy tissues and liver sequestration. Several point mutations of the AB (S408E) (Einfeld et al. 2001; Jakubczak et al. 2001b), DE (Y477A) (Alemany et al. 2001) and FG loop (Mizuguchi et al. 2002) of the Ad5 fiber knob domain have been analysed that successfully abolish CAR binding resulting in

strongly reduced viral transduction *in vitro*. Surprisingly, *in vivo* the CAR binding ablated vectors showed no or only minimal reduction on hepatocyte transduction in mice, rats and nonhuman primates (Alemany et al. 2001; Leissner et al. 2001; Nicol et al. 2004; Roelvink et al. 1999; Smith et al. 2002; Smith et al 2003a).

The penton base RGD motif mediates binding to membrane integrins and stimulation of endocytosis. Hence, integrins can normally serve as secondary receptor of the entry pathway, in the absence of CAR binding, this penton base integrin interaction alone can also result in virus transduction (Huang et al. 1996). As the penton base RGD motif plays a significant role in liver transduction, the deletion of the RGD sequence in combination with CAR ablation leads to a detargeted Ad5 with minimal liver uptake and reduced macrophage stimulatory activity in some, but not in all, studies (Schoggins, Falck-Pederson et al. 2006).

Although the canonical two step entry pathway via CAR and integrins is the dominant mechanism contributing to Ad5 transduction *in vitro*, interactions of the viral capsid with major histocompatibility complex molecules and heparin sulphate glycos-aminoglycans (HSG) also play an important role of cell entry (Dechecchi, Melotti, Bonizzato et al. 2001; Hong, Karayan, Tournier, Curiel and Boulanger et al. 1997). In this regard, mutation of the KKTK motif, a putative HSG binding domain, in the Ad5 shaft showed reduced liver transduction *in vitro* and *in vivo* (Nicol, Graham, Miller, White, Smith, Nicklin and Baker et al. 2004; Smith and Stevenson et al. 2003). Furthermore, replacement of the Ad5 fiber shaft with a short shaft derived from the Ad35 or short Ad40 fiber mediated reduced transduction of hepatic tissue (Koizumi et al. 2003; Nakamura et al. 2003).

Recently, the short fiber of subgroup F adenovirus serotypes 40 and 41 has been suggested as a tool for genetic adenovirus detargeting. These serotypes possess two different fiber molecules, a long fiber with the cell binding domain and a short fiber. As no cellular receptor has been reported for the short fiber, it seem not to be involved in virus attachment (Roelvink et al. 1999; Favier et al. 2002; Nakamura et al. 2003), but might be essential for stability of the virus capsid. Furthermore, Ad40 and Ad41 lack the RGD motif in their penton bases or fibers (Albinsson and Kidd 1999). In this regard, several studies observed that Ads with chimeric Ad40/Ad41s-derived fibers did not mediate efficient adenoviral gene transfer *in vitro* and *in vivo* (Nakamura et al. 2003; Nicol et al. 2004; Schoggins et al. 2003; Schoggins et al. 2005). Based on these observations, the short fibers of subgroup F constitute a scaffold of interest for target ligand insertion into adenovirus capsid. Different

functional insertion sites for the model peptide RGD-4C into the Ad41 short fiber knob were determined (Hesse et al. 2007). In conclusion, the feasibility of ligand insertion into the Ad41 short fiber knob domain resulting in molecules that retain both fiber/capsid integrity and the potency of the inserted peptide to bind its cellular receptor has been shown.

2.4.3.3. Strategy for efficient transgene expression by oncolytic adenoviruses

Several OAds have entered the clinics. However, clinical experiences showed that these agents are safe, but not potent enough as monotherapy to effect complete tumour regressions or to generate sustained clinical response. A promising strategy to improve therapeutic efficacy of oncolytic adenoviruses is to combine viro- and gene therapy via “arming” of oncolytic viruses with therapeutic genes. In this regard, therapeutic genes could mediate killing of uninfected cancer cells by means of molecular chemotherapy/prodrug activation (Fig. 7), anticancer immune activation, apoptosis induction, angiogenesis, or other genetic effector mechanisms (bystander effect).

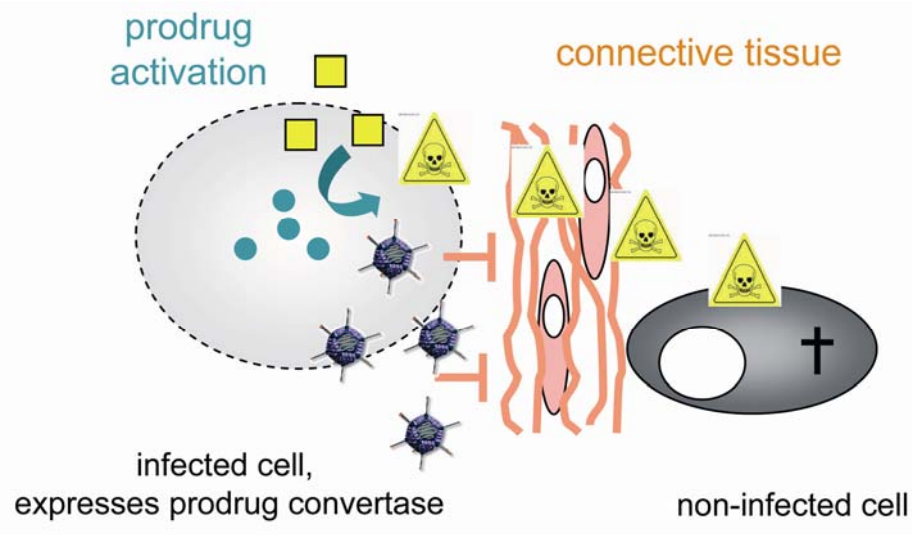


Fig.7: **Gene directed enzyme prodrug therapy in combination with viral oncolysis.** An attractive strategy to improve therapeutic efficacy of OAds is to develop “armed” viruses by insertion of suicide genes into the virus genome. After converting of the prodrug into highly toxic metabolites this chemotherapeutic can also cross connective tissue which poses a physical barrier to viruses.

Choosing the appropriate transgene, the mode of transgene expression and the locale of transgene insertion into the virus genome are the major questions for implementation of “armed” OAds. In this regard, the insertion of therapeutic genes into the genome of OAds has to consider the compact genome structure and the limited packaging capacity of the viruses in order to retain oncolytic potency. One strategy offered by the replicating virus is to use endogenous viral gene expression

control machinery (promoter/enhancer, polyadenylation, and splice signals) by replacing viral genes such as the non essential E3 gene. In this case, the transgene expression should follow the normal kinetic of the substituted endogenous gene (Freytag et al. 1998; Wildner et al. 1999; Van Beusechem et al. 2002; Geoerger et al. 2004; Lamfers et al. 2005; Oosterhoff et al. 2005). A second approach is the transgene insertion into adenoviral transcription units, thereby exploiting the viral gene expression mechanism and kinetics. This has been implemented by fusion of the transgene by an internal ribosome entry site (IRES) to the viral genes, which have been successfully employed in replicating viruses (Sauthoff et al. 2002; Rivera et al. 2004; Sova et al. 2004). Using of viral splice acceptor sites (Fuerer and Iggo, 2004; Jin et al. 2005; Carette et al. 2005) for splicing of transgene from viral mRNA could be also a possibility for transgene expression.

Specific cellular promoters have been utilized for indirect or direct transcriptional targeting of transgene expression by oncolytic adenoviruses. By selective amplification of the transgene template, transcriptional targeting of adenoviral replication indirectly confers specificity to the expression of the transgene inserted into the virus genome. The second mechanism of indirect control is achieved when transgene is expressed from the major late promoter, which should be only active after selective replication (promoter dependent) of the viral genome. Direct targeting can be implemented by expression of the transgene from a selective cellular promoter construct (Davis et al. 2006). One problem of the indirect or direct transcriptional targeting of transgene expression could be that the promoters might loose their selectivity during virus replication (Rohmer et al. 2008). Indeed, regulation of therapeutic gene expression by OAds remains challenge and a highly efficient and selective strategy for transgene expression for an anti-cancer therapy is still missing.

3. OBJECTIVES OF THE STUDY

The aim of my first project was the development of selective and efficient late therapeutic gene expression by transcriptionally targeted oncolytic adenoviruses. This study was based on previously developed adenoviruses with melanoma-restricted replication potential via the tight tyrosinase promoter (Nettelbeck et al. 2000). I used this strategy of transcriptional targeting of the viral replication to malignant melanoma cells should to indirectly control late transgene expression which should be replication-dependent. Specifically, it should be analyzed if the mode of transgene expression and the locale of transgene insertion into the virus genome critically determine the efficacy and specificity of the transgene expression. To address this issue I developed different strategies for transgene expression from the viral late transcription unit by either fusion the transgene to the late virus gene via internal ribosome entry side or a 'self-cleaving' 2A peptide or by inserting the transgene into the viral genome via an additional splice acceptor site. First, the selectivity and efficiency of late transgene expression should be analyzed. Especially, the influence of the different transgene insertion strategies on indirect transcriptional targeting of transgene expression was of interest, because so far it has not been analyzed. Importantly, viral gene expression and oncolytic potency should not been affected when the transgene is inserted into the late transcription unit downstream of the fiber gene. Finally, it should be investigated whether the therapeutic efficacy of oncolytic adenoviruses is improved by inserting a therapeutic gene via the optimized insertion strategy into the viral genome. Towards this goal, I planned to combine viral oncolysis with genetic prodrug activation (molecular chemotherapy).

The second project was designed to develop strategies for targeting oncolytic adenoviruses to cancer cells by tumour-selective cell entry. Notably, all oncolytic adenoviruses described to date are based on post-entry restriction of the adenoviral life cycle. My initial work in this project was focus on the engineering of the virus capsid for optimal ablation of native virus tropism and insertion of targeting ligands in context of oncolytic viruses. Therefore, I investigated efficiency of viral replication and the lytic potency of oncolytic adenoviruses engineered with the model peptide RGD inserted in the Ad41 fiber knob. Additionally, tumour cell-binding peptides should be analyzed after insertion into the best suited position of my fiber formats in the context of oncolytic adenoviruses.

4. Materials and Methods

4.1. Materials

4.1.1. Chemicals, filters and enzymes

The utilized chemicals such as salts, buffer substances, solvents and antibiotics were purchased from Roche (Mannheim), Dianova (Hamburg), Merck (Darmstadt), Invitrogen (Karlsruhe) and Sigma (Deisenhofen). The nutrient medium for bacteria derived from Roth (Karlsruhe). Filters were purchased from Schleicher and Schuell (Dassel), enzymes from New England Biolabs (Frankfurt/Main) and Invitrogen (Karlsruhe).

4.1.2. Buffers and solutions

4.1.2.1. Buffers and solutions for gel electrophoresis

4.1.2.1.1. Electrophoresis of nucleic acids

Agarose gel: 0.5-2% Agarose in 1x TAE buffer (addition of 10µl ethidium bromide in 100ml Agarose)

50x TAE buffer: 2M Tris-HCl; 1M sodium acetate; 62,5mM EDTA, pH 8.5

10x DNA loading buffer: 0.1% bromophenol blue; 50% glycerol; 0.1M EDTA pH 8.0

Ethidium bromide: 10mg/ml (Carl Roth, Karlsruhe)

DNA ladder: 1kb DNA ladder (Invitrogen, Karlsruhe)

4.1.2.1.2. Electrophoresis of proteins

4x separating buffer: 1.5mM Tris-HCl pH 8.8; 0.04% SDS

4x stacking buffer: 0.5M Tris-HCl pH 6.8; 0.4% SDS

Acrylamide: 30% (Carl Roth, Karlsruhe)

10% APS

Temed (Promega, Mannheim)

10x running buffer: 2M Glycine; 250mM Tris; 1% SDS

RiPa lysis buffer: 10mM Tris-HCl pH 7.5; 150mM NaCl; 1% NP40 (Igepal) 1% sodium desoxycholate; 0.1% SDS; 1mM PMSF; 20mM sodium fluoride; 2mM sodium orthovanadate

4x sample buffer: 200mM Tris-HCl pH 6.8; 400mM DTT; 8% SDS; 0.4% bromophenol blue; 40% glycerol; 10% β-Mercaptoethanol (β-Me)

Protein gel marker: Page Ruler Prestained protein ladder (Fermentas, St. Leon-Rot)

4.1.2.2. Buffers and solutions for western blot analysis

1x transfer buffer: 25mM Tris; 192mM Glycine; 20% methanol

10x TBS: 250mM Tris-HCl pH 7.4; 1.5M NaCl

PBS: 137mM NaCl; 2.7mM KCl; 7.3 mM Na_2HPO_4; 1.4mM KH_2PO_4; pH 7.0

Blocking solutions: 5% BSA in TBS and 5% powdered milk in TBS; 0.02% Tween; 0.02% NaN_3

Washing solution (TBST): 1x TBS; 0.02% Tween

ECL Western Blotting Substrate (Pierce, USA)

4.1.2.3. Buffers and solutions for viral lysis

TE-buffer: 100mM Tris-HCl pH 8.0; 10mM EDTA

VL buffer: 1M TE buffer; 10% SDS

4.1.2.4. Buffers and solutions for production of transformation competent bacteria

$MgCl_2$: 0.1M, ice-cold, sterile

$CaCl_2$: 0.1M, ice-cold, sterile

Glycerol: 99.9%, ice-cold, sterile

4.1.2.5. Buffers and solutions for DNA precipitation

Lithium chloride: 4M

EtOH: 100%

4.1.2.6. Buffers and solutions for caesium chloride equilibrium density ultracentrifugation

CsCl 1.41: 304.6g caesium chloride in deionized water (500 ml) pH 7.8, sterile

CsCl 1.27: 227.2g caesium chloride in deionized water (500 ml) pH 7.8, sterile

Hepes: 5mM, sterile

Glycerol: 99.9 %, sterile

4.1.3. Media

4.1.3.1. Media for bacterial culture

LB-medium: Carl Roth LB (Luria Bertani) Broth, Miller

LBampAgar: LB-Agar (Lennox) with 100mg/l ampicillin

LBkanAgar: LB-Agar (Lennox) with 50mg/l kanamycin

SOC-medium: 2% bactotryptone pH 7.0 (NaOH); 0.5% yeast extract; 10mM NaCl; 2.5mM KCl; 10mM $MgSO_4$; 10mM $MgCl_2$; 20mM glucose

4.1.3.2. Media and solutions for cell culture

DMEM: Dulbecco's Modified Eagle Medium, 4.5g/l glucose without L-glutamine, with sodium pyruvate and pyridoxine (Invitrogen, Karlsruhe)

RPMI: RPMI 1640 without L-glutamine (Invitrogen, Karlsruhe)

OptiMEM (Invitrogen, Karlsruhe)

FBS: foetal bovine serum (PAA, Pasching): was inactivated on 56ºC for 30 min before usage

DPBS: Dulbecco's phosphate buffered saline 0.0095M PO_4 without calcium and magnesium (Lonza, Belgium; Invitrogen, Karlsruhe)

Trypsin-EDTA: 500mg/l trypsin; 200mg/l Versene (EDTA) (Invitrogen, Karlsruhe)

P/S/G: L-glutamine (200mM) with penicillin and streptomycin (100x); (PAA; Pasching)

P/S: 10.000U penicillin/ml; 10.000µg streptomycin/ml (Lonza, Belgium)

Gentamycin: 10mg/ml gentamycin (Sigma, Deisenhofen)

L-Glutamine: 200mM in 0.85% NaCl (BioWhittaker, Verviers, Belgium)

β-Me: 50mM (Invitrogen, Karlsruhe)

Hepes: 1M in 0.85% sodium chloride (Lonza, Belgium)

DMSO: Sigma, Deisenhofen

Crystal violet: 1% crystal violet in 70% ethanol

4.1.4. Cells and Bacteria Strains

4.1.4.1. Bacteria strains

***Escherichia coli* Maximum Efficiency DH5α**: genotype: F^-Φ80ΔlacZΔM15, rec A1, end A1, hsdR17 PhoA, supE44, gyrA96, relA1 (Invitrogen, Karlsruhe)

***Escherichia coli* Electro Maximum DH5α**: genotype: F^-Φ80ΔlacZΔM15, rec A1, end A1, hsdR17 mcrA, mcrB, mcrC, mrr (Invitrogen, Karlsruhe)

***Escherichia coli* BJ5183**: genotype: endA1, sbcBC, recBC, galK, met, thi-1, bioT, hsdR (Stratagene, Amsterdam).

4.1.4.2. Human cells lines

name	cell type	medium	source
A549	Human lung adenocarcinoma epithelial cell line	DMEM/10%FCS/1%P/S/G	ATCC (Manassas, USA)
C8161	Melanoma cell line	DMEM/10%FCS/1%P/S/G	(Welch et al., 1991)
Colo829	Melanoma cell line	RPMI/10%FCS/1%P/S/G 0.04% β-Me	J. Banchereau (Texas, USA)
HaCat	immortalized keratinocyte cell line	DMEM/10%FCS/1%P/S/G	P. Boukamp (Heidelberg, Germany) (Boukamp, Petrussevska et al. 1988)
HFF	Primary normal foreskin fibroblast	MEM/7.5% FCS/1%G/ 1% gentamycin	M. Marschall (Erlangen, Germany)
293	Human embryonic kidney cells (stably transformed with sheared Ad5 DNA)	DMEM/10%FCS/1%P/S/G	G. Fey (Erlangen, Germany)
Mel624	Melanoma cell line	RPMI/10%FCS/1%P/S/G	J. Schlom (Bethesda, USA)
Mel888	Melanoma cell line	RPMI/10%FCS/1%P/S/G	J. Schlom (Bethesda, USA)
pMelL	Low passage melanoma cells	As mentioned above for primary melanoma	D.Dieckmann, M Lüftl (Erlangen, Germany)
SK-Mel-28	Melanoma cell line	DMEM/10%FCS/1%P/S/G	ATCC (Manassas, USA)

Table 1: Human cell lines used in this dissertation

4.1.5. Adenoviruses

Ad5 WT: Ad serotype 5 wild type; provided by D. Curiel, Birmingham, USA (Davydova, Le et al. 2004).

Ad5Δ24Tyr: Ad serotype 5 that expresses E1AΔ24 from an optimized human tyrosinase enhancer/promoter (hTyr2E/P) (Nettelbeck et al. 2002).

4.1.6. Nucleic acids

4.1.6.1. Oligonucleotides

All oligonucleotides were purchased from and were synthesized by MWG-Biotech (Martinsried).

4.1.6.1.1. Oligonucleotides for PCR cloning

Name	Sequence	Description	Fragment size
Mlu for	5'-ACT T**AC GCG T**GC TAG CCC G-3'	mutation of cryptic splice acceptor sequence	1140bp
mut SA rev	5'-CCG AAT AAT CCG GTC GCT ATA GAA AAT GAA ATC AGG TCT AGT TG-3'		
mut SA for	5'-ATA GCG ACC GGA TTA TTC GGT TGT TGG TTG AAG AAG GTT TG-3'		293bp
Age rev	5'-CC**A CCG GT**G GCC AGC ATT G-3'		

Table 2: Oligonucleotides for PCR cloning. Recognition strings for restriction enzymes are marked in bold.

4.1.6.1.2. Oligonucleotides for annealing

Oligonucleotides for insertion of a 50bp spacer between splice acceptor site and FCU gene	
Spacer_1	5'- P-**CTAGC**CCGGGCTCGAGATCTCTGCTAATCTTCCTTTCTCTCTTCAGGCCC-3'
Spacer_2	5'-P-GTCTTCCACGGGGCCTGAAGAGAGAAAGGAAGATTAGCAGAGATCTCGAGCCCGGG-3'
Spacer_3	5'-P-CTTCAGGCCCCGTGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGC-3'
Spacer_4	5'-P-**CATGG**CGGATAGAATGGCGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTC-3'
BPSA ts	5'-P-**GATCT**CTGCTAATCTTCCTTTCTCTCTTCAGGCCAC-3'
BPSA bs	5'-P-**CATGG**TGGCCTGAAGAGAGAAAGGAAGATTAGCAGA-3'

Table 3: Oligonucleotides for PCR annealing. Recognition strings for restriction enzymes are marked in bold.

4.1.6.1.3. Oligonucleotides for splicing analysis

Name	Sequence
TPL for	5'-CTG AGC GAG TCC GCA TCG-3'
FCU 1090-1109 rev	5'-TAT CTG TCA CCA AAG TCA-3`
FCY 430-449 rev	5'-TGA GGT CTT TCA TCG ATA AA-3'

Table 4: Oligonucleotides for PCR splicing analysis of FCU.

4.1.6.1.4. Oligonucleotides for controlling recombinant modified Ad genomes

Name	Sequence
SeqITR for	5'-CGG GAA AAC TGA ATA AGA GGA AGT GA-3`
Ad1124-1100 rev	5'-ATT TTC ACT TAC TGT AGA CAA ACA T-3`
E1AΔ24 rev	5'-AAA GCC AGC CTC GTG GCA GGT AAG-3`
pGL3-Seq3' MCS rev	5'-TTA TGC AGT TGC TCT CCA GCG GTT C-3`
E3 for	5`-CTG CTA GTT GAG CGG GAC AGG GGA C-3`
E3 rev	5`-GGC AAG GAG GTG CTG CTG AAT AAA C-3`
E4-for	5'-ATT GAA GCC AAT ATG ATA ATG AGG G-3`
E4-rev	5'-CAC AGC GGC AGC CTA ACA GTC-3`
5knob for	5'-AGG CAG TTT GGC TCC AAT ATC TGG-3`
RGD Ad5 HI for	5`-TGC CGC GGA GAC TGT TTC TGC-3'
Seq Mfe fiber rev	5'-TGT ATA AGC TAT GTG GTG GTG GGG-3`
FCU1_RT for	5'-GAA ACT GAC ACC AAC GAA AAC-3`
FCU 1090-1109 rev	5'-TAT CTGTCACCAAAGTCA-3`

Table 3: Oligonucleotides for controlling recombinant modified Ad genomes

Combination of oligonucleotides	Ad genome region	DNA fragment size
SeqITR for + Ad1124-1100 rev	E1A-region	Ad5 WT: 844bp Ad5Δ24 viruses: 820bp Ad5TyrΔ24 viruses: 1430bp
E1AΔ24 rev + Seq ITR	E1A-region	Ad5 WT: 666bp Ad5Δ24 viruses: -
pGL3-Seq3' MCS rev + 5knob for	Fiber with luciferase insertion	Ad5 Δ24 fiber IL / TL: 1172bp
E3 for + E3 rev	E3-region	Ad5 WT: 2836bp Ad5 Δ24 E3- viruses: 206bp
E4 for + E4 rev	E4-region	Ad5 WT: 398bp Ad5 Δ24 FCU viruses: 1520bp Ad5 Δ24 SL: 2051bp
RGD Ad5 HI for + Seq Mfe fiber rev	RGD insertion	Insertion in: HI loop: 457bp IJ loop: 401bp EG loop: 538bp
5knob for + Seq Mfe fiber rev	Ad5 fiber knob domain	Ad5 WT: 524bp
FCU1_RT for + FCU1090-1109 rev	FCU insertion	Ad5 Δ24 FCU viruses: 455bp

Table 4: Combination of oligonucleotides for controlling recombinant modified Ad genomes

4.1.6.1.5. Oligonucleotides for sequencing

All sequences obtained from PCR and from annealing with oligonucleotides, as well as all cloning steps were proved by sequencing (MWG-Biotech, Martinsried) to exclude mutations.

Name	Sequence
pGL3-for	5'-CTA GCA AAA TAG GCT GTC CCC TA-3`
pGL-rev	5'-CTT TAT GTT TTT GGC GTC TTC C-3`
SeqITR	5'-CGG GAA AAC TGA ATA AGA GGA AGT GA-3`
Seq Mfe fiber rev	5'-TGT ATA AGC TAT GTG GTG GTG GGG-3`
Seq Luc rev	5'-TTC ATA GCC TTA TGC AGT TGC-3'
RAE antisense	5'-AAC ACG TGG GTC AGA GAG GT-3'

Table 5: Oligonucleotides used for sequencing

4.1.6.1.6. Oligonucleotides for quantitative real time PCR (qPCR)

Human gene	Orientation	Sequence
GAPDH	sense	5'-GGT TTA CAT GTT CCA ATA TGA TTC CA-3`
	antisense	5'-ATG GGA TTT CCA TTG ATG ACA AG-3`
β-Actin	sense	5´-TAA GTA GGC GCA CAG TAG GTC TGA-3`
	antisense	5´-AAA GTG CAA AGA ACA CGG CTA AG-3`

Adenovirus gene/Transgene	Orientation	Sequence
E4	sense	5`-GGT TGA TTC ATC GGT CAG TGC-3`
	antisense	5`-TAC GCC TGC GGG TAT GTA TTC-3`
E1A	sense	5'-AAC CAG TTG CCG TGA GAG TTG-3'
	antisense	5'-CTC GTT AAG CAA GTC CTC GAT ACA-3'
Fiber	sense	5'-TGA TGT TTG ACG CTA CAG CCA TA-3`
	antisense	5'-GAT TTG TGT TTG GTG CAT TAG GTG-3`
Hexon	sense	5`-ACC TGG GCC AAA ACC TTC TC-3`
	antisense	5-CGT CCA TGG GAT CCA CCT C-3`
Luciferase	sense	5'-TGA CCG CCT GAA GTC TCT GA-3'
	antisense	5'-TGG AGC AAG ATG GAT TCC AAT-3'
FCU1	sense	5'-GAA ACT GAC ACC AAC GAA AAC-3'
	antisense	5'-TTT TAC CGA TAC GCA CAG AC-3'

Table 6: Oligonucleotides for quantitative real time PCR

4.1.6.2. Plasmids

pGL3-Basic: luciferase reporter plasmid that lacks eukaryotic promoter and enhancer sequences (Promega, Madison, USA).

pGL3-SV40: luciferase reporter plasmid that contains a SV40 promoter located upstream of the luciferase gene (Promega, Madison, USA).

pGL3-CMV.IE: luciferase reporter plasmid that contains the CMV immediate early promoter upstream of the luciferase gene (Nettelbeck, unpublished).

pShuttle: Ad transfer vector containing a multiple cloning site in which an expression cassette can be inserted. Upon homologous recombination in bacteria with pAdEasy-1 or pVK500-5ts3k the expression cassette is inserted into the original E1 region of the Ad genome (He et al.,1998).

pS-cs-Δ24: shuttle plasmid contains a mutant E1A gene with a 24bp deletion (E1AΔ24) to prevent binding and inactivating pRB and upstream multiple cloning site (Nettelbeck et al.,2002).

pSΔ24_SA_FCU-1: FCU-1 shuttle plasmid that contains a splice acceptor sequence (SA) located upstream of the *FCU-1* gene. Additionally, it contains the mutant E1A gene with a 24bp deletion (obtained from Michael Behr).

pS-hTyr-Δ24: contains a mutated E1A gene under the control of the human Tyr promoter (Nettelbeck et al.,2002).

pAdEasy-1: 33.4kb plasmid containing the Ad5 genome with deletions in the left end of genome including E1 region (He et al.,1998).

pVK500fiber: plasmid containing the Ad5 genome with partial deletion of the fiber gene.

Following plasmids were generated in this thesis by the described cloning procedure:

pSΔ24_SA_spacer_FCU-1: contains a 51bp long, non-coding sequences of the luciferase gene as spacer between the splice acceptor sequence and the *FCU1* gene.

pSΔ24_SA_mFCU-1: contains a SA_FCU1 cassette with a mutation of a cryptic acceptor sequence at position 594 of the *FCU-1* gene.

pNEB.PK.SnaBI: contains the 5 fiber gene with a partial deletion at the C-terminus and the restriction site SnaBI at the C-terminus.

pNEB.5/41sHI/IJ/EG_RGD: contains the fiber chimeric 5/41s incorporated the RGD ligand into the HI/IJ/EG loop of the 41s knob.

pF5/41sHI_eph ΔE3: contains the fiber chimeric 5/41s incorporated the ephrin ligand into the HI loop of the 41s knob. In addition, it contains a deletion of the E3 region.

pAd5Ssp_FCU-1: plasmid contains the Ad5 genome with the therapeutic gene *FCU-1* and 51pb long spacer.

pAd5Ssp_mFCU-1: plasmid contains the Ad5 genome with the therapeutic gene *FCU-1* with a mutation at position 594 and 51pb long spacer.

pAd5/41sHI/IJ/EG_RGD: plasmid contains the Ad5 genome with the fiber chimeric 5/41s incorporated the RGD ligand into the HI/IJ/EG loop of the 41s knob.

pAd5/41sHI_eph ΔE3: plasmid contains the Ad5 genome with the fiber chimeric 5/41s incorporated an EphA2 peptide ligand into the HI loop of the 41s knob and a deletion of the *E3* gene.

4.1.6.3. Antibodies

4.1.6.3.1. Antibodies for western blot analysis

Antigen	Clonality	Species	Dilution	Buffer condition	Source
Ad5 fiber tail (monomeric and trimeric)	monoclonal (4D2)	mouse	1:2000	TBST	Abcam (Cambridge)
Ad5 hexon	polyclonal	rabbit	1:2000	TBST	Abcam (Cambridge)
yeast cytosine deaminase	polyclonal	sheep	1:250	TBST	AbD Serotec (Oxford)
human β-Actin	monoclonal (AC-74)	mouse	1:2000	5%MMP/TBS	Sigma (Deisenhofen)
human α-tubulin	monoclonal	mouse	1:50000	5%MMP/TBS	Sigma (Deisenhofen)
goat IgG H&L-HRP	polyclonal	donkey	1:5000	5%MMP/TBS	Dianova (Hamburg)
mouse IgG-HRP	polyclonal	goat	1:5000	5%MMP/TBS	Cell Signaling Technology (Danvers)
rabbit IgG H&L-HRP	polyclonal	goat	1:10000	5%MMP/TBS	Cell Signaling Technology (Danvers)

Table 7: Antibodies for western blot analysis

4.2. Methods

Standard methods of molecular biology required for this dissertation were performed according to the laboratory manual *Molecular Cloning* by J. Sambrook and D. Russel (Sambrook and Russell 2001).

4.2.1. Nucleic acid methods

4.2.1.1. DNA cloning

Plasmids or amplified PCR products were cleaved with relevant restriction endonucleases in buffers as described by the manufacturer to generate vector and insert fragments for cloning. For the conversion of DNA overhangs, i.e. filling in recessed 5'-overhangs or digesting protruding 3'-overhangs, *DNA Polymerase I, Klenow Fragment* (Invitrogen, Karlsruhe), was utilized following restriction digestion according to the manufacturer's protocol. To prevent self-religation of cutted vector-ends, especially when blunt ends were generated, subsequent dephosphorylation was accomplished with *calf alkaline intestine phosphatase* following the manufacturer's instructions (Invitrogen, Karlsruhe). If required, DNA fragments were purified using *Qiagen® PCR-purification kit* as described by the manufacturer. Subsequently, digested/modified DNA fragments were separated and analyzed by gel electrophoresis on 1% agarose gel at 100V in 1xTAE buffer. Required DNA fragments for ligation were excised from gel and were prepared using *QIAquick® Gel Extraction Kit* (Qiagen, Hilden) following instructions by the manufacturer. Ligation was performed using *Rapid Ligation Kit* (Roche, Mannheim) following instruction manual in a total volume of 20µl with a molar ratio between linearized vector and DNA insert of approx. 1:3 to 1:5. Half of the ligation sample was applied for transformation of bacteria.

4.2.1.1.1. Production of transformation-competent bacteria and transformation

4.2.1.1.1.1. Production of chemical-competent bacteria and transformation by heat shock

For spontaneous uptake of foreign DNA, bacteria were treated with divalent cations (Mg^{2+} and Ca^{2+}). A single colony of DH5α was inoculated in 5ml LB-medium and cultivated over night at 37°C by vigorous shaking in a shaker incubator. On the next day overnight culture was transferred to 300ml of LB-medium and bacteria were grown until they reached OD_{600nm} of 0.4-0.6. After 15 min incubation on ice, bacteria were collected by centrifugation at 4°C and 3000rpm for 5 min. Pellets were

resuspended in 150ml of ice-cold, sterile 0.1M $MgCl_2$-solution and incubated on ice for 1 h. Following centrifugation (see above) bacteria were resuspended in 12ml ice-cold, sterile 0.1M $CaCl_2$-solution and incubated on ice for another hour. Glycerol was added reaching a final concentration of 20%. Chemical-competent bacteria were stored in 200µl aliquots at -80°C.

For heat shock transformation, bacteria were thawed on ice. 1µg of plasmid DNA or 10µl ligation sample was mixed with 100µl chemical-competent bacteria and incubated on ice for 15 min. Afterwards, a heat shock of 60 sec at 42°C was performed. Samples were chilled on ice for 90 sec and 1ml of SOC medium was added. After shaking at 37°C for 1 h, 200µl was used for plating the bacteria on LB-agar-dishes containing amp or kan depending on the resistance gene of the transformed plasmid. Plates were incubated at 37°C over night.

4.2.1.1.1.2. Production of electro-competent bacteria and transformation by electroporation

For the production of electro-competent bacteria a single colony of DH5α or BJ5183 was inoculated in 10ml LB-medium and incubated at 37°C over night by vigorous shaking. Four ml of the overnight culture was added to Erlenmeyer-flasks containing a total volume of 500ml LB-medium. Cells were cultivated until they reached OD_{600nm} of 0.8, were then incubated on ice for 1 h following centrifugation at 3000rpm for 10 min at 4°C. The pellet was resuspended in 200ml ice-cold, sterile 10% glycerol solution and spun down by another centrifugation step. Subsequently, washing of the cells was repeated and cells were resuspended in 20ml 10% glycerol following collection by centrifugation. Five ml of 10% glycerol was used for resuspension and aliquots of 50µl were stored at -80°C.

For transformation 50µl of bacteria were thawed on ice. Electro-competent DH5α were mixed with 2µl Ad-genome plasmids and incubated on ice for 1 minute. Afterwards, this mixture was transferred into a pre-chilled electroporation cuvette. The cuvette was placed in the electroporator and pulse of 2.5kV was applied. Immediately after transformation, 1ml SOC medium was added to the cells. Following 1 h shaking at 30°C the sample was used for plating the cells on LB-agar dishes containing amp or kan. Plates were incubated for at least 30 h at 30°C.

4.2.1.1.1.3. Homologous recombination for the generation of recombinant adenoviral genomes

2µg of pShuttle-plasmids containing modified adenoviral-regions were digested with Pme I for 5 h at 37°C. An aliquot was controlled for complete digestion by DNA

electrophoresis on 1% agarose gel at 100V and the sample was purified using *Qiagen Quickspin Purification Kit* (Qiagen, Hilden) as described by the manufacturer. For homologous recombination electro-competent BJ5183 bacteria were used. An amount of 100-200ng of backbone plasmid (pAdEasy-1 or pVK500) was mixed with 8-10µl linearized pShuttle-plasmid and used for electroporation of 50µl BJ5183. Following 1 h shaking at 30°C samples were plated on LB-agar dishes with amp or kan and incubated for at least 30 h at 30°C.

4.2.1.2. Preparation of DNA and RNA

4.2.1.2.1. Analytical isolation of plasmid DNA (mini lysate)

To analyze modified cloning products after transformation into bacteria, a single colony was inoculated into 5ml of LB-medium containing the relevant antibiotic (notes in parentheses refer to preparation of Ad genomes). A sample of 1.5ml (4ml) of the overnight culture grown at 37°C (30°C) was utilized to prepare DNA. For small plasmids (≤ 15kb) *Qiaprep Spin Miniprep Kit* (Qiagen, Hilden) was applied following manufacturer's instructions. Larger plasmids (pAd plasmids) were isolated using 200µl of P1-buffer (Qiagen) for resuspension. Cells were lysed with 400µl P2-buffer (Qiagen) and incubated at RT for 5 min. By adding 300µl of P3-buffer proteins, cell wall components and genomic DNA were precipitated and pelleted by centrifugation at 13.000rpm for 10 min DNA was precipitated from supernatant with 750µl isopropanole, spun down by centrifugation for 15 min at 13.000rpm and washed with 70% ethanol. DNA was resuspended in 30µl TE buffer pH 8.0. For analysis of plasmid DNA 10µl (17µl) were used for restriction digestion and subsequently analyzed on a 1% (0.5%) agarose gel by electrophoresis.

4.2.1.2.2. Quantitative isolation of plasmid DNA (midi lysate)

For large-scale preparation of plasmid DNA required for cloning and transfection purposes, *Qiagen Plasmid Midi Kit* (Qiagen) was used according to the manufacturer's protocol. DNA was isolated from 100ml bacteria culture. For midi preparation of pAd-plasmids 4ml P1-buffer, 8ml P2-buffer and 6ml P3-buffer was utilized differing from standard protocol. pAd plasmids were resuspended in 100µl TE buffer pH 8.0 and controlled by restriction digestion and PCR followed by agarose gel electrophoresis. DNA plasmids (except pAd plasmids) of all cloning steps were proved by sequencing at MWG Biotech to exclude mutations (see chapter 4.1.6.1.3: Oligonucleotides for sequencing).

4.2.1.2.3. DNA isolation from infected human cell cultures

For preparation of total DNA of infected human cell cultures, *Qiagen Blood Mini Kit* (Qiagen) was used following the instruction protocol. For this purpose, cells were harvested by removing the medium, adding 400µl of PBS and using a cell scraper. Samples were then stored at -20°C.

4.2.1.2.4. RNA isolation

Total cellular RNA was isolated from cell cultures of 50.000 cells using *Qiagen RNeasy Mini RNA Extraction Kit* (Qiagen) according to the manufacturer's manual. To avoid RNA degradation by RNases 10µl β-mercaptoethanol per 1ml Buffer RLT was added.

4.2.1.2.5. RNA isolation and reverse transcription

Total cellular RNA was isolated from cell cultures of 500.000 cells using *Qiagen RNeasy Mini RNA Extraction Kit* (Qiagen) according to the manufacturer's manual. To remove cell debris and shear genomic DNA from lysate, *QIAshredder columns* were utilized. One µg of total RNA was reverse transcribed into single strand cDNA using *SuperScript II Reverse Transcriptase Kit* (Invitrogen) as described by the manufacturer. Specifically, the protocol for first strand synthesis using oligo TT primers was performed.

4.2.1.3. PCR (poymerase chain reaction)

For the amplification of DNA fragments from genomic DNA or plasmid DNA (also pAd plasmids), the PCR method (polymerase chain reaction) was carried out using *Taq Polymerase Kit* (Invitrogen, Karlsruhe) or *Pfu Polymerase* (Promega, Madison) as described by the manufacturer. Standard samples with a total volume of 100µl contained 30ng of DNA template, 50pmol of each oligonucleotide primer, 10µl 10x PCR buffer, 20mM dNTP mixture (end concentration 0.2mM each), 1.5mM $MgCl_2$ and 4U Taq DNA-polymerase. PCR cloning of all fiber modifications were performed with *Pfu Polymerase* (Promega, Madison) and a typical sample had a total volume of 50µl consisting of 30ng DNA template, 10pmol of each oligonucleotide primer, 5µl 10x PCR buffer containing $MgCl_2$, 1µl dNTP mixture (end concentration 0.2mM each) and 1.25U Pfu Polymerase. If not mentioned otherwise, all PCR reactions were performed using *Taq Polymerase Kit* (Invitrogen, Karlsruhe). For the control of constructed recombinant Ad genomes a total PCR volume of 25µl was used. A typical PCR was accomplished under following conditions:

Denaturation	95°C	5 min	
Amplification			
Denaturation	95°C	1 min	←
Annealing	58-62°C	1 min	
Extension	72°C	1 min / 90 sec	⌐
Extension	72°C	10 min	

Annealing temperature, cycle number and extension time was individually defined for the relevant oligonucleotide-primers and fragment. Ten µl of PCR product was analyzed by standard agarose gel electrophoresis. PCR samples for cloning were purified using the *Qiagen®Quickspin Purification Kit* (Qiagen, Hilden).

4.2.1.3.1. Two step PCR

Two step PCR was performed for mutation of a cryptical splice acceptor sequence located at position 594 of the FCU gene. Two µl of PCR product from the first PCR run was applied as DNA template for the second PCR run.

1. PCR step A: primer for: Mlu
   primer rev: SAmut rev
   template: pSΔ24_SA_FCU1
1. PCR step B: primer for: SAmut for
   primer rev: Age
   template: pSΔ24_SA_FCU1
2. PCR step: primer for: Mlu
   primer rev: Age
   template: products from the 1. PCR step A and B

4.2.1.3.2. Quantitative real time PCR (qPCR)

To quantify either intracellular adenoviral mRNA or adenoviral genomes after infection, qPCR with or without reverse transcription was performed with *7300 Real Time PCR System* (Applied Biosystems, Darmstadt) using *MicroAmp® 96 Well Reaction Plates* (Applied Biosystems) at a total volume of 25µl for each PCR reaction. Each probe contained 12.5µl of 2x *Power SYBR Green Master Mix* (Applied Biosystems) consisting of all components necessary for the reaction, 2µl template mRNA or DNA isolated either with *Qiagen RNeasy Mini RNA Extraction Kit* (Qiagen) or *Qiagen Blood Mini Kit* (Qiagen), and 10pmol of each oligonucleotide-primer (see chapter 4.1.6.1.6). For quantification of adenoviral mRNA Reverse Transcriptase and RNase-Inhibitor (both from Applied Biosystems) were additionally added. qPCR was performed with an initial denaturation step of 10 min at 95°C, followed by 50 cycles of 15 sec denaturation at 95°C, 10 sec annealing at 60°C and 15 sec elongation time at

72°C. At the end of each cycle, the fluorescence emitted by the SYBR Green was measured. After completion of the cycling process, samples were subjected to a temperature ramp (from 65°C to 95°C at 0.1°C/s) with continuous fluorescence monitoring for melting curve analysis. Known amount of template DNA pTG3602 (10^8, 10^6, 10^4 and 10^2 copies/µl) was amplified to generate a standard curve for quantification of the copy numbers of unknown samples. For calibration of cellular mRNA amounts, 200, 20, 2 and 0.2ng/µl human RNA isolated from A549 cells, was utilized for creation of standard curves and to determine the GAPDH mRNA concentrations of the samples. Cellular DNA was measured using β-actin oligonucleotides and 200, 20, 2 and 0.2ng/µl human DNA isolated from A549 cells as standard. Data were normalized with cellular genomic DNA or cellular RNA for each sample individually and were analyzed with the *7300 System SDS Software* (Applied Biosystems). Negative controls with no template were carried out for each reaction series.

4.2.1.4. Protein biochemical and immunological methods

4.2.1.4.1. Preparation of total cell lysates

For the preparation of total cell lysate from Ad-infected, cells were harvested using cell scrapers (Sarstedt, Nümbrecht). Afterwards cells were pelleted by 1100rpm. This was followed by two cycles of washing with 5ml ice-cold PBS. Cells were then lysed in 50µl RiPa-buffer containing freshly supplemented protease inhibitors. After shaking at 1000rpm over night in a Thermomixer at 4°C, cell debris were pelleted at 13.000rpm at 4°C and supernatant was stored at -20°C.

4.2.1.4.2. Determination of total protein concentration

To determine the concentration of total protein *Bio-Rad D_c Protein Assay* (Biorad, Munich) was used according to the manufacturer's instructions. For generation of standard curves, known amount (1.4µg/µl, 1.05µg/µl, 0.7µg/µl, 0.35µg/µl and 0.175µg/µl) of BSA (NEB, Frankfurt a. Main) was applied. Samples of unknown protein concentration were diluted (1:4) and all samples mixed with reagent A and B. After 20 min incubation at RT, adsorption at 750nm was determined.

4.2.1.4.3. Discontinous SDS-Polyacrylamidgelelectrophoresis (SDS-Page)

For electrophoretic separation of proteins according to size, SDS-PAGE was performed pursuant to Laemmli's method (Laemmli,1970). Gels were composed of a separating gel (8-12% acrylamide) and a stacking gel (4% acrylamide). Samples

were mixed with 4x sample buffer and after incubation at 95°C for 5 min loaded on the gel. Electrophoresis was carried out using 1x running buffer for 2 h at 100V.

4.2.1.4.4. Western Transfer

For the immunological identification of separated proteins, western transfer was carried out using *Protran-Nitrocellulose-Transfer-Membrane* (Schleicher & Schuell, Dassel) with a pore size of 0.2µm. The transfer was accomplished in a wet blot chamber (Biorad, Munich) for 1 h with 0.8mA/cm^2. Whatman filters (Schleicher & Schuell, Dassel), gel and nitrocellulose membrane were wetted in transfer buffer before assembling in the following order starting from the anode side: 1 sponge, 1 Whatman filter, nitrocellulose membrane, gel, 1 Whatman filter and 1 sponge.

4.2.1.4.5. Immunoblot

Following western transfer, the membrane was incubated on a rocking platform with blocking solution (5% MMP/PBS/Tween/NaN_3) for 2 h at RT. Membranes were incubated with primary antibody diluted in 5% BSA/TBST overnight at 4°C. The next day the membrane was washed 3 times 15 min each with TBST. The HRP-conjugated secondary antibody was incubated in 5% MMP/TBST for 1 h at RT and unbound antibody was washed away 3 times 20 min each with TBST. For chemiluminescent detection, *Pierce ECL Western Blotting Substrate* (Pierce, USA) in a total volume of 5ml was used according to the manufacturer's protocol. Exposition of X-ray films (Fuji X-ray films RX, Kisker-Biotech, Steinfurt) was performed as required for individual antibody for 5 min to 45 min.

4.2.1.5. Cell culture

4.2.1.5.1. Passaging, freezing and thawing cell culture cells

Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Passaging cell culture cells was carried out depending on their growing modality every 2 to 4 days. Cell lines were cultivated for several weeks. For detaching, cells were washed with PBS and subjected to Trypsin-EDTA. After removing Trypsin-EDTA from cells, 10ml of corresponding medium was added in order to inactivate Trypsin-EDTA. Splitting was performed in a ratio of 1:3 to 1:10 according to growing features of individual cell types. For storage, cells were collected by centrifugation at 1200rpm for 3 min and resuspended in 10% DMSO, 90% FCS. One ml aliquots were stored at -80°C for 24 h before freezing in liquid nitrogen at -140°C. To re-cultivate frozen samples, cells were thawed at 37°C quickly before washing with 10ml of relevant

growth medium. Cell pellets were resuspended in growth medium and seeded at appropriate density.

4.2.1.5.2. Luciferase reporter assay

For the determination of luciferase activity in transduced cell lysates the *Promega Luciferase Assay System* (Promega) was utilized as described by the manufacturer. Cells were washed with PBS and lysed in 200µl *Luciferase lysis buffer*. Cell culture plates were incubated at -80°C to freeze samples for complete cell lysis. Following thawing at RT, 10µl cell lysate was mixed with 50µl *Luciferase reagent* and immediately measured in a luminometer (FluoroskanAsentFL, Thermo Scientific, Braunschweig, Germany). For the analysis, background (only lysis buffer) values were subtracted.

4.2.1.6. Recombinant adenovirus

4.2.1.6.1. Generation of recombinant adenovirus

The generation of plasmids containing a recombinant adenoviral genome was carried out using homologous recombination in BJ5183 bacteria with linearized pShuttle plasmids and backbone plasmid (pAdEasy-1 or pVK500). For the generation of replication-competent adenoviruses, either A549 or in case of the melanoma targeted viruses Mel888 / Colo829 cells were used, while replication-deficient adenoviruses were produced in E1-complementing 293 cells (Quantum, Quebec, Canada).

For the generation of recombinant adenovirus, 6µg pAd-plasmid was linearized with Pac I for 5 h at 37°C and precipitated with 4M Lithium chloride and 100% ethanol. The DNA pellet was resuspended in 30µl H_2O, whereof 8µl was controlled by standard gel electrophoresis in a 0.5% agarose gel. Twenty µl of linearized pAd-plasmid diluted in 250µl OptiMEM (Invitrogen) was mixed with 20µl *Lipofectamine* (Invitrogen) solved in 250µl OptiMEM and incubated at RT for 30 min. Meanwhile cells were washed with PBS and 2.5ml OptiMEM was added to the cells. The transfection mixture was dispersed onto 50-60% confluent A549 or 293 cells seeded into T25-cell culture flasks one day prior to transfection. Following 5 h incubation at 37°C, the transfection mixture was replaced with relevant growth medium. Complete cell lysis after formation of viral plaques resulting from recombinant viral particle generation and spread was observed on day 10 to day 12. Cells were collected and recombinant virions were released from the cells by repeating three freeze/thaw cycles at-80°C and 37°C. Following centrifugation at 4°C for 15 min at 4000rpm, virions, situated in the supernatant, were used for large scale production of

recombinant viral particles. To amplify viruses, three further rounds of infection and virus preparation were performed using increasing numbers of cells (15-20 T175 cell culture flasks or 25 cell culture dishes (145cm^2) in the last round of amplification). When complete lysis was observed in the last round of amplification, cells were collected, resuspended in 7ml medium and after three cycles of freezing/thawing, centrifuged at 4000rpm for 15 min (4°C). The supernatant (7ml), containing recombinant viral particles, was applied for purification of adenovirus.

4.2.1.6.2. Caesium chloride gradient equilibrium density ultracentrifugation for the purification of viral particles

For the purification of viral particles from culture supernatants (7ml) two rounds of caesium chloride equilibrium density ultracentrifugation was performed. Three ml CsCl 1.41 (1.41g/ml) was added to sterile ultracentrifugation tubes (Herolab, Wiesloch) and 5ml CsCl 1.27 (1.27g/ml) was carefully overlaid without destroying the border between phases. A volume of 3.5ml of viral cell lysate was pipetted onto the top of two tubes for each virus preparation. Following centrifugation at 4°C for 2 h at 32.000rpm under vacuum infectious viral particles appeared as a white ring at the border of the two phases (Ad5 density: 1.34 g/ml), while empty particles lacking DNA genomes emerged as a ring located above. Virus was harvested by aspirating with a 5ml syringe and a needle (0.4mm x 19mm, BD Microlance) punctured 1cm below the band, while an upper ring containing particles lacking DNA was not aspirated. After adjusting virus suspension to 7ml with 5mM Hepes a second caesium chloride gradient equilibrium density ultracentrifugation was carried out at 32.000rpm for 24 h at 4 °C under vacuum. The isolated virus suspension was adjusted to 2.5ml with PBS and PD 10 columns (Amersham, Munich) were used to remove CsCl following instruction manual. Elutions were performed with 0.5ml of PBS (discarded) and 2ml of PBS (virus stock). Subsequently, 10% glycerol was added to the purified virus and aliquots of 50µl were stored at -80°C.

4.2.1.6.3. Determination of viral particle concentration

4.2.1.6.3.1. Determination of infectious particle concentration using the Tissue Culture Infectious Dose 50 ($TCID_{50}$)-assay

To determine the concentration of infectious particles of purified recombinant adenovirus, $TCID_{50}$ on 293 cells was performed. The principle of this method is the detection of plaques produced by the examined virus preparation applied in serial dilutions for infection. Ten thousand cells per well were seeded in 100µl infection medium (growth medium containing 2% FCS) into 96 well plates. The next day, virus

was serially diluted 10-fold (10^{-5}-10^{12}) and 100µl virus-dilution was used for infection each of 10 wells per dilution. As control, cells were mock infected using only infection medium. Following 10 days incubation at 37°C, cytopathic effect in each well was determined by microscopy analysis. Wells were evaluated as positive, one or more plaques were detected or complete cell lysis was observed. For each virus preparation two dilution series were carried out for precise definition of infectious particle concentration. The virus titer was calculated according to Kaerber's statistical method and stated in $TCID_{50}$/ml:

Titer = $10^{1 + 1(S-0.5)}$ x 10 TCID50/ ml

S = sum of positive wells (starting from the first 10^{-1} dilution)

vp/$TCID_{50}$ tissue culture infective dose ratio was between 15-20.

4.2.1.6.3.2. Determination of physical viral particles by reading optical density

To determine absolute concentration of adenoviral particles (vp/ml), optical adsorption of adenoviral DNA at λ= 260nm was quantified. Therefore purified virus was lysed by incubation in VLB buffer for 10 min at 56°C and different dilutions were measured. Viral particle concentration was calculated multiplying the absorbance by the appropriate dilution factor and the extinction coefficient (1.1 x 10^{11} vp/ml).

4.2.1.6.3.3. Verification of recombinant adenoviral genomes

For the verification of recombinant adenoviral genomes and the exclusion of Ad WT contamination, a PCR was performed. Purified virus was diluted 1:50 in H_2O and incubated at 95°C for 10 min. Five µl was used as DNA template for PCR with a total volume of 25µl to control recombinant modified adenoviral genomes (see chapter 4.1.6.1.4).

4.2.1.6.4. Transduction and infections of cells with recombinant adenovirus

4.2.1.6.4.1. Transduction with replication-deficient adenovirus for the analysis of luciferase activities

Fifty-thousand cells were seeded per well into 24-well plates in 500µl medium (DMEM with 10% FCS). The following day, cells were infected in triplicates with titers dependent on their transduction efficiency (10-1000 $TCID_{50}$/cell or vp/cell) in 250µl infection medium. Following 2 h incubation at 37°C, 500µl relevant growth medium was added to the cells. Forty-eight hours post infection cells were lysed for Luciferase assay.

4.2.1.6.4.2. Infection with replication-competent adenovirus with subsequent inhibition of virus genome replication by AraC

For inhibition of virus genome replication, 50.000 cells were seeded in 24-well plates in 500µl growth media or 500.000 cells were seeded in 6-well plates in 3ml growth media containing 10 % FBS. The following day growth medium was removed and cells were infected with adenoviruses at 1-10 TCID50/cell in 250µl or 1ml growth medium containing 2 % FBS. To inhibit virus genome replication cells were incubated with AraC (Sigma-Aldrich) at 2µM, which was added at 1 h, 12 h, 24 h and 36 h post-infection.

4.2.1.6.4.3. Infection for cytotoxicity assay

For the determination of virus-mediated cytotoxicity by crystal violet staining, 30.000 cells per well were seeded in 48-plates. The following day, cells were infected in 200µl of growth medium containing 2% FBS with adenoviruses at 10-fold increasing titers of 0.001-100$TCID_{50}$/cell or 0.01–1000 vp/cell or were mock infected. Two hours post infection 500µl growth medium was added. Medium was replaced with growth medium every two or three days. When cell lysis was observed for virus at 0.01-0.1 $TCID_{50}$/cell or 0.1-1 vp/cell, cells were stained with crystal violet (see next chapter).

4.2.1.6.4.4. Crystal violet staining of infected cells

To document cell lysis, medium was removed from infected cells seeded into 48-well plates. For staining of living cells, approximately 50µl of 1% cystal violet/70% EtOH was added to the cells and incubated at RT for 20 min. Plates were rinsed with tap water to remove any excess colour. Afterwards, plates were dried and documented by digital photography.

4.2.1.6.4.5. Infection for splicing analysis of the transgene FCU-1 by reverse transcription

For the analysis of splicing by reverse transcription, 500.000 cells (SK-MEL-28) per well were seeded in 6-well-plates in 3ml growth medium. The following day, cells were infected with adenoviruses at 100 TCID50/cell in 1ml infection medium. Two hours post infection 2ml growth medium was added. One day post infection cells were washed once with PBS before and total cellular RNA was isolated using *Qiagen RNeasy Mini RNA Extraction Kit* (Qiagen) according to the manufacturer's manual. To remove cell debris and shear genomic DNA from lysate, *QIAshredder columns* were utilized. Three µg of total RNA was reverse transcribed into single strand cDNA using *SuperScript II Reverse Transcriptase Kit* (Invitrogen) and oligo (dT) primers (Invitrogen) as described by the manufacturer. The *FCU-1* gene was then amplified

from 2µl of cDNA in a final volume of 25µl, containing 1.5mM $MgCl_2$, 0.2 mM dNTP mixture each, 1.25U taq polymerase (Invitrogen, Karlsruhe) and 10pmol forward (5'-CTG AGC GAG TCC GCA TCG) and reverse (5'-TAT CTG TCA CCA AAG TCA) primers. The amplicons were analyzed by agarose gel electrophoresis.

4.2.1.6.4.6. Infection for quantification of adenoviral mRNA or adenoviral genomes by qPCR

To quantify intracellular adenoviral mRNA or adenoviral genomes in proliferating cells, 50.000 cells were seeded in 24-well plates in 500µl growth medium (containing 10 % FCS). Cells were infected the next day at 10vp/cell in 250µl infection medium. After 1 h of incubation at 37°C, 500µl of growth medium was added. Samples were harvested at indicated time points by removing the medium, adding 400µl of PBS (for genome copy numbers) or 350µl Buffer RLT (for adenoviral mRNA) and using cell scrapers (Sarstedt, Nümbrecht). Samples were stored at -20°C (genome copy numbers) or at -80°C (adenoviral mRNA).

4.2.1.6.4.7. Infection for the analysis of protein-expression

For the detection of adenoviral protein expression 500.000 cells were seeded in 6-well-plates in 3ml of growth medium. The following day cells were infected at 10-300$TCID_{50}$/cell of adenovirus in 1ml infection medium. Two hours post infection 2ml growth medium was added. After indicated time points cells were harvested with cell scrapers and used for preparation of total cell lysates (see chapter 4.2.1.4.1 Preparation of total cell lysates).

4.2.1.6.4.8. Infection for the quantification of infectious particles of oncolytic adenoviruses

To analyze the infectious particle production of oncolytic adenoviruses burst assays were performed. Therefore 50.000 cells were seeded per well in 24-well plates in 500µl of growth medium. The day after, cells were infected at 10vp/cell of adenovirus in 250µl infection medium. Two hours post infection the medium was removed, cells were washed twice with PBS to remove all unbound adenoviruses and then 1ml of growth medium was added. After 2, 3 or 4 days supernatants and cells were harvested separately. Supernatants were collected in a 15ml tubes, spun down for 5min at 1300rpm to remove residual cells and transferred into a fresh tube. After supernatants were removed 1ml of ice-cold PBS was added to the cells, cells were scraped off, transferred to a tube and lysates prepared by three cycles of freezing and thawing. Serial dilutions on the supernatants and lysates were titred on 293 cells using the $TCID_{50}$ assay.

4.2.1.6.4.9. Infection for enzyme prodrug therapy

For the determination of enzyme-produrg mediated cytotoxicity, cells were seeded in 96-well-plates at a concentration of 10.000 cells / 100µl of growth medium (containing 2 % FCS) per well and grown overnight at 37°C. After 24 h, cells were infected with serial dilutions of the indicated viruses in 50µl of medium (containing 2 % FCS). Two days post-infection either the prodrug FC at a final concentration at 10mM (HaCat, A549 and SK-MEL-28) or 5mM (Mel624) or medium was added in a volume of 50µl to the cells. Five (A549) or six (HaCat, SK-MEL-28, Mel624) days post-infection, cells were fixed and stained with 1% crystal violet in 70% ethanol for 10min, followed by washing with tap water to remove excess color. The plates were dried and images were captured with a LabsystemsMultiscanMS (Thermo, Braunschweig) at 595nm. The absorbance of control wells, which were exposed only to the medium, defined 100% viability and effect of the prodrug on cell survival was expressed as a percentage of cell viability relative to control cells.

4.2.1.6.4.10. Infection for the analysis of bystander effect of the enzyme prodrug therapy

For analysis of bystander effect, 50.000 cells (SK-MEL-28) were seeded in 24-well plates in 500µl growth media containing 2 % FBS. The following day, cells were infected at 10$TCID_{50}$/cell in 500µl growth media containing 2 % FBS. Two days post-infection, infection media was removed and cells were incubated with either 5-fluorocytosine at a concentration of 10mM or with medium alone. The day after, collected supernatants and 5-fluorouracil (5-FU cat. no.: A13456, Alfa Aesar, Karlsruhe, Germany) at a concentration of 10mM were heated up for 10 min at 50°C. Serial dilutions of the supernatants and 5-FU were added to cells, which were plated for cytotoxicity assay.

4.2.1.6.4.11. Infection for the analysis of effect of FC metabolites to viral life cycle

For analysis of the influence of FC metabolites to viral life cycle, 50.000 cells (SK-MEL-28) were seeded in 24-well plates in 500µl growth media containing 2 % FBS. The following day, cells were infected with Ad5TyrSsp_mFCU, Ad5TyrSL or Ad5CMVLUC at 1$TCID_{50}$/cell in 250µl growth media containing 2 % FBS. Two hours post infection the medium was removed, cells were washed twice with PBS to remove all unbound adenoviruses and then 1ml of growth medium was added. One day post infection cells were incubated with 10mM FC. After two days supernatants and cells were harvested separately. Supernatants were collected in a 15ml tubes, spun down for 5min at 1300rpm to remove residual cells and transferred into a fresh

tube. After supernatants were removed 1ml of ice-cold PBS was added to the cells, cells were scraped off, transferred to a tube and lysates prepared by three cycles of freezing and thawing. Serial dilutions on the supernatants and lysates were titred on 293 cells using the $TCID_{50}$ assay.

5 RESULTS

5.1 Late transgene expression by transcriptionally targeted oncolytic adenoviruses is dependent on the transgene insertion strategy

Towards the goal to develop oncolytic adenoviruses (OAds) that feature expression of therapeutic genes in a tumour-specific manner, transgene expression by exploitation of the late viral gene expression machinery might be a promising tool. Promoter control of E1A could be used to indirectly control late transgene expression which should be replication-dependent. As the restriction of replication and cytotoxicity of the TyrE/P targeted OAds to melanoma cells was shown (Nettelbeck et al. 2002, Banerjee et al. 2004), the exceptionally high selectivity of TyrE/P offers a promising opportunity to combine transcriptional targeting of the E1A gene with the transgene expression to achieve indirectly melanoma selective transgene expression.

5.1.1 Activity of optimized tyrosinase enhancer/promoter (TyrE/P) in replication-deficient adenoviral vectors

To analyze the specificity of the TyrE/P promoter a tandem enhancer (2x203bp) and core promoter (260pb) of the human tyrosinase gene and an upstream polyadenylation signal (Nettelbeck et al. 2002) was cloned into a replication deficient, E1-and E3 deleted adenoviral vector.

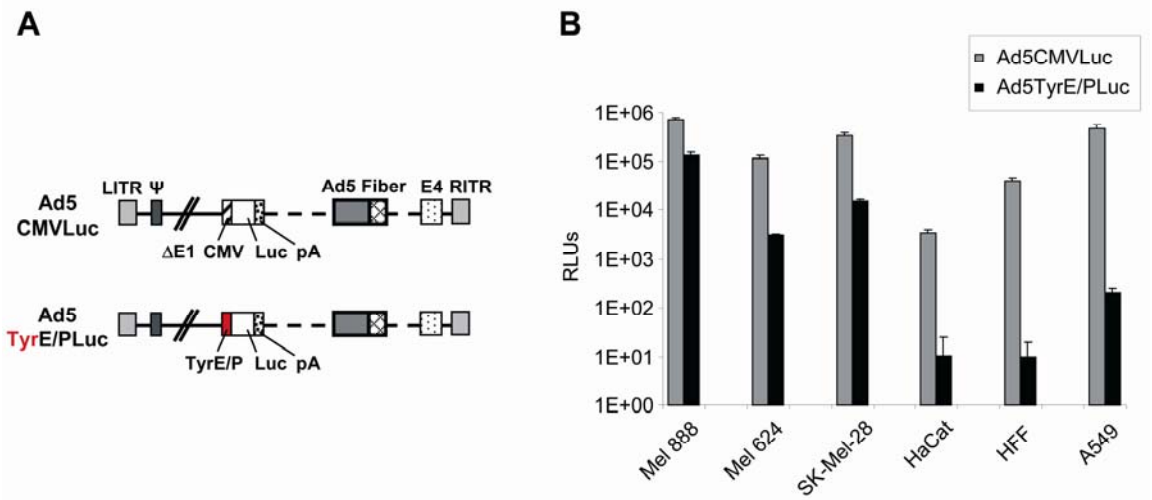


Fig.8: **Activity of optimized tyrosinase enhancer/promoter (TyrE/P) in replication-deficient adenoviral vectors**. (A) Schematic outline of replication deficient adenovirus vectors (Ad5CMVLuc, Ad5TyrE/PLuc) with either the Tyrosinase enhancer/promoter or the CMV promoter driving the luciferase gene. Luc, Luciferase reporter gene; pA, polyadenylation signal; ΔE1, E1 region deleted. (B) Cells were transduced with Ad5CMVLuc or Ad5TyrE/PLuc. Two days post transduction, luciferase activity was determined with a luminescence assay. Columns show mean RLU values of triplicate transductions; error bars show standard deviations; RLU, relative luminescence units. Background measurement readings from the lysis puffer were subtracted. One representative experiment out of at least three is shown.

In this regard, the TyrE/P controls the expression of the reporter gene luciferase, which was also placed into the E1 region of the viral genome. In addition, a viral vector that expresses the luciferase gene from the strong and constitutive CMV promoter (Reynolds et al. 2001) was also constructed (Fig. 8A). These viral vectors were used to analyze the selectivity of the TyrE/P measuring luciferase activity 48h post transduction of melanoma cell lines (Mel888, Mel624, SK-MEL-28) and control cells such as primary normal fibroblasts (HFF), immortalized keratinocytes (HaCat) and the lung adenocarcinoma epithelial cell line (A549). As shown in figure 8B, the luciferase gene expression level resulting form Ad5CMVLuc transduction yielded similar results of approximately 1.00E+05 relative light units (RLU) in melanoma and non-melanoma cells except in the HaCat cells. The luciferase expression in HaCat cells was approximately one order of magnitude reduced compared to the other cell types. The luciferase expression level resulting from AdTyrE/PLuc was 5.5-fold (Mel888), 22.6-fold (SK-MEL-28) and 26.4-fold (Mel624) less in comparison to Ad5CMVLuc. As expected, the luciferase activity resulting from the AdTyrE/PLuc was dramatically reduced in control cells. RLUs were 331-fold (HaCat), 2222-fold (A549) and 3968-fold (HFF) lower in these cells as compared with the Ad5CMVLuc. Still, the TyrE/P showed minimal basal activity after viral transduction, especially in A549 cells. In summary, the TyrE/P allowed for strongly melanoma specific transgene expression by replication deficient adenoviral vectors.

5.1.2 Adenovirus constructs used for investigation of melanoma selectivity of transgene expression by oncolytic adenoviruses

Previously, *Rivera et al.* (2004) showed that the mode of transgene expression and the locale of transgene insertion into the virus genome of replication competent oncolytic adenoviruses critically determine the efficacy of their expression. Hence, the potency of the transgene, such as a therapeutic gene, is depended on the insertion strategy. Furthermore, it is important to note that timing of therapeutic gene expression are critical determinate of the outcome of the approach (see chapter 2.4.3.3.). In light of these results, three different strategies for transgene insertion in the late transcription which might be resulting in replication-dependent expression pattern were analyzed. I chose an internal ribosomal entry site (IRES), a picornaviral 2A sequences or an additional splice acceptor site to insert the reporter gene luciferase into the late transcription unit of Ad5 genome. For IL viruses, the 600 bp EMCV IRES sequence was used for bicistronic expression of the viral fiber and luciferase genes as described before (Rivera et al. 2004). TL viruses were generated

by fusion of the fiber and luciferase genes via the 54 bp 2A sequence of the insect virus *Thosea asigna* (Szymczak et al. 2004) for expression of both proteins from a single ORF by ribosomal skip (Fig. 9).

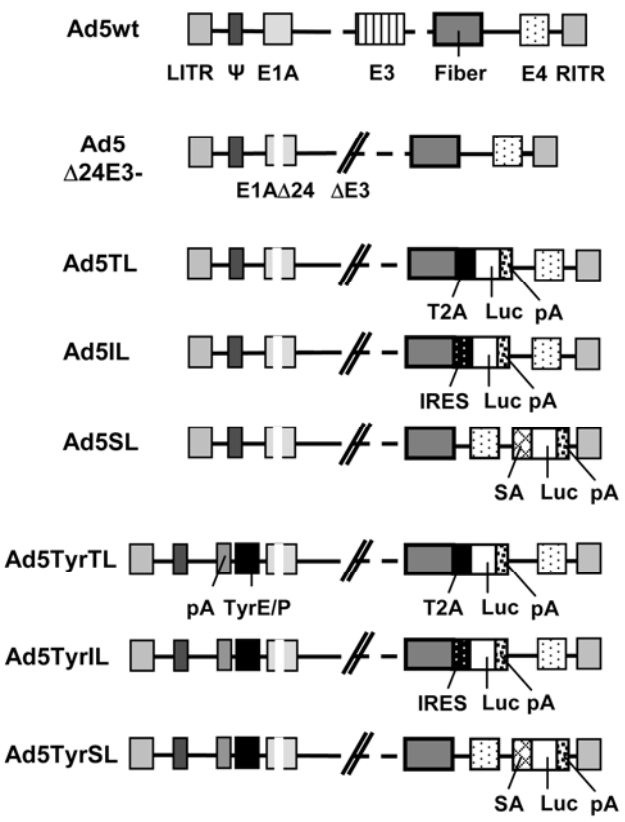


Fig.9: **Adenovirus constructs used for investigation of melanoma selectivity of transgene expression by OAds.** Schematic outline of the genome of the 6 luciferase reporter viruses, of the parental E3-deleted recombinant adenoviruses without transgene (Ad5Δ24E3-) and of the wild-type adenovirus serotype 5 (Ad5wt). ΔE3, E3 region deleted; E1AΔ24; E1A mutant featuring a deletion of the pocket protein-binding conserved region two; IRES, internal ribosome entry site; SA, splice acceptor site (branch point, polypyrimidine tract, CAG); T2A, self-cleaving motif of *Thosea asigna* virus (Asp-Ile-Glu-X-Asn-Pro-Gly-Pro); Luc, luciferase gene; pA, polyadenylation signal for transcription termination; LITR/RITR, left/right inverted terminal repeat; ψ, packaging signal; black boxes in the left termini of the genomes highlight the optimized tyrosinase enhancer/promoter (TyrE/P)

The SL viruses were generated by inserting the luciferase gene between E4 genes and RITR using a 26 bp artificial splice acceptor (SA) element consisting of branchpoint, polypyrimidine tract and splice acceptor element (Jin et al., 2005). The endogenous E1 is controlled by the highly pigment cell selective promoter TyrE/P aiming selective viral replication and indirect targeting of transgene expression to malignant melanoma. As shown in figure 9, the control viruses Ad5TL, Ad5IL, Ad5SL and Ad5Δ24E3- encode the endogenous E1 promoter. Ad5TL, Ad5IL, Ad5SL, Ad5TyrTL, Ad5TyrIL and Ad5TyrSL were derived from parental virus Ad5Δ24E3- carrying Δ24 and E3 deletion. Because of the size of the inserted DNA sequence all recombinant viruses are carrying deletion of E3 region and of the pRb-binding domain in the E1A gene.

5.1.3 Spread-dependent cell killing of recombinant oncolytic adenoviruses in different melanoma cell cultures and control cells

First, cytolytic activity and specificity of cell lysis by Ad5TyrTL, Ad5TyrIL and Ad5TyrSL were analyzed after infection of melanoma (SK-MEL-28, Mel888) and non-melanoma cells (HaCat, HFF). The different cell lines were infected with the total panel of recombinant adenoviruses (Ad5TL, Ad5IL, Ad5SL, Ad5TyrTL, Ad5TyrIL, Ad5TyrSL) and the parental virus Ad5Δ24E3-, which offers the replicating control, at virus titer of 0.001 – 100 TCID50/cell in 10-fold dilutions. The replication-deficient Ad5CMVLuc served as a non-replicating control. Cell killing was detected by staining of viable, adherent cells with crystal violet (Fig. 10).

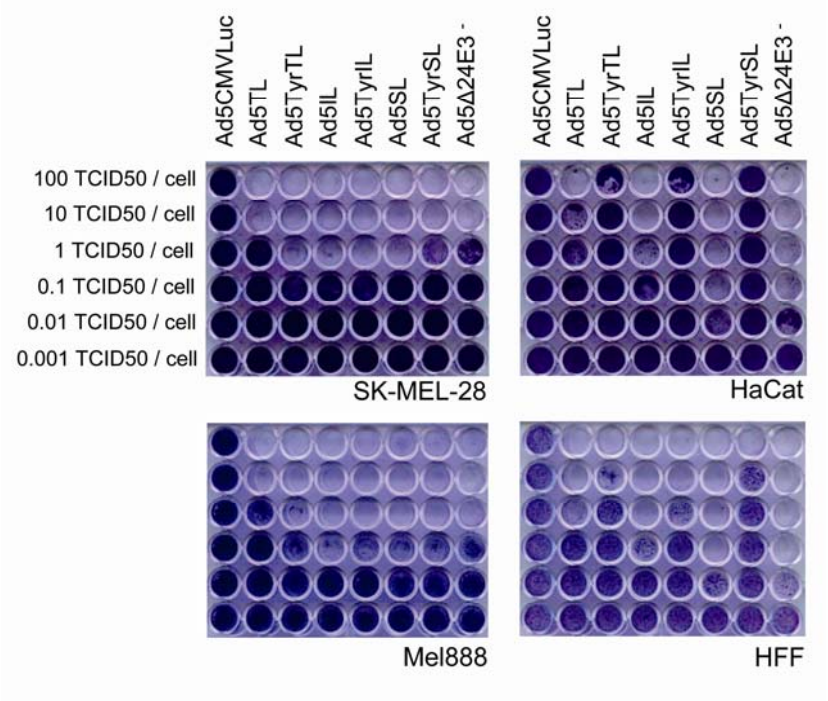


Fig.10: **Spread-dependent cell killing of recombinant oncolytic adenoviruses in different melanoma cell cultures and control cells.** SK-MEL-28, Mel888, melanoma cell lines; HaCat, immortalized keratinocyte cell line; HFF, primary normal foreskin fibroblast; cell cultures were infected at 100$TCID_{50}$/cell and 10-fold dilutions until 0.001$TCID_{50}$/cell with indicated viruses carrying the adenoviral E1A gene under the transcriptional control of native E1A- or TyrE/P-promoter. Virus spread-dependent cytotoxicity was visualized by crystal violet staining of surviving cells. Therefore, staining was performed when cytotoxicity was observed for the most potent virus at 0.01 or 0.001$TCID_{50}$/cell for each cell type individually. $TCID_{50}$, tissue culture infectious dose 50. One representative experiment out of at least three is shown.

No cytotoxic effect was shown for replication-deficient Ad5CMVLuc. However, a replication dependent cytotoxicity in melanoma cells was observed for all recombinant oncolytic adenoviruses including the TyrE/P recombinant viruses (Ad5TyrTL, Ad5TyrIL, Ad5TyrSL) indicating that replacing the promoter of the E1A by the TyrE/P construct is feasible. After infection of the melanoma cell lines SK-MEL-28 and Mel888, the total panel of recombinant viruses and Ad5Δ24E3- showed similar

level of cytotoxicity. Almost identical cell killing by Ad5IL, Ad5SL and the corresponding Ad5TyrIL, Ad5TyrSL was observed in SK-MEL-28 and Mel888. One order of magnitude attenuated cell killing relative to the matching Ad5TyrTL was shown for Ad5TL in the tested melanoma cell lines. High cytolysis of all untargeted oncolytic viruses was detected in control cells HaCat and HFF, which were completely lysed at 10TCID50/cell (Ad5TL), 1TCID50/cell (Ad5IL) or 0.1TCID50/cell (Ad5SL). Importantly, in HaCat cells, the recombinant melanoma-targeted viruses, showed no (Ad5TyrSL) or only minimal (Ad5TyrTL, Ad5TyrIL) cell killing, even when infected with high virus titers. The cytolytic effect was 2 - 4 orders of magnitude attenuated compared to matching untargeted control viruses. Also in HFF the oncolytic potency of Ad5TyrTL, Ad5TyrIL and Ad5TyrSL was attenuated 10 – 1000 fold when compared to Ad5TL, Ad5IL and Ad5SL. Thus, infection of Ad5TyrTL, Ad5TyrIL and Ad5TyrSL in melanoma cell lines resulted in potent cytotoxicity and is clearly attenuated in control cells. By comparison, highest selectivity of cytolysis was shown for Ad5TyrSL. Of note, insertion of the luciferase gene into the late transcription unit of the viral genome per se did reduce the oncolytic activity of Ad5TL in Mel888, SK-MEL-28, HaCat and HFF, and of Ad5IL in the control cells. In conclusion, melanoma-selectivity of cell lysis and spread by tyrosinase promoter-regulated oncolytic adenoviruses was retained for viruses with transgene insertion into the late transcription unit via IRES and SA sequences, but less so when the 2A sequence was used.

5.1.4 Investigation of dependence of transgene expression by oncolytic adenoviruses on the viral DNA replication

My hypothesis was that transcriptional targeting of adenovirus replication indirectly confers specificity of the expression of transgenes inserted into the viral genome by targeted amplification of the transgene template. Besides regulation of virus genome numbers, indirect control should be achieved when transgenes are expressed from the major late promoter, which is activated only after virus genome replication. To further investigate whether the transgene expression by IRES, 2A or SA insertion strategies is restricted to the period after viral DNA replication, the melanoma cell line SK-MEL-28 was infected at 1TCID50/cell with the total panel of recombinant adenoviruses and the replication-deficient Ad5CMVLuc that served as a non-replicating control. Cells were treated with cytosine arabinoside (AraC) to inhibit viral

replication. At 48 hours post infection, cells were harvested and luciferase activity was measured (Fig. 11).

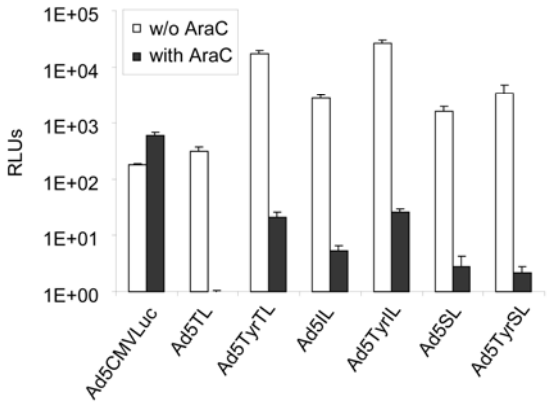


Fig.11: **Investigation of dependence of transgene expression by OAds on the viral DNA replication.** The melanoma cell line SK-MEL-28 was infected with indicated viruses. Cells were treated with 2 µM of AraC which was added 1 h, 12 h, 24 h and 36 h post infection or were mock treated. At 48 h after infection, luciferase activity was determined. Columns show mean RLU values of triplicate infections; error bars show standard deviations; RLUs, relative luminescence units. This Experiment was performed three times and one representative result is shown.

The measured luciferase activities showed that all recombinant oncolytic adenoviruses expressed luciferase independent from the insertion strategies, but the levels of luciferase expression differ. This demonstrated that the mode of transgene expression and the replacement of the viral E1 promoter with the TyrE/P obviously have an influence on the level of transgene expression. The luciferase expression resulting from the Ad5TyrSL was approximately 7-fold attenuated in comparison to Ad5TyrTL and Ad5TyrIL. However, only minimal differences between the Ad5TyrSL and Ad5SL were observed in comparison to Ad5TyrTL and Ad5TL (55.7-fold), Ad5TyrIL and Ad5IL (9.5-fold). Blocking viral replication by adding AraC clearly inhibited the transgene activity of all recombinant oncolytic viruses, showing that the luciferase behaves as late genes when inserted via IRES, 2A or SA site. The results of the replication-deficient Ad5CMVLuc demonstrate that AraC has no effect on expression of luciferase itself, which is independent of viral replication for Ad5CMVLuc. For Ad5TyrSL, repression of viral replication resulted in the strongest reduction of transgene activity by AraC.

5.1.5 Efficacy and specificity of transgene activity of melanoma targeted OAds compared to untargeted viruses

Having demonstrated that expression of the transgene by the recombinant viruses is efficient and replication-dependent in the melanoma cell line SK-MEL-28, the magnitude and the specificity of transgene expression was analyzed in two additional melanoma cell lines (Mel888, Mel624) and control cells such as HFF, HaCat and A549. For this purpose these cell lines were infected with Ad5TL, Ad5IL, Ad5SL,

Ad5TyrTL, Ad5TyrIL and Ad5TyrSL with a virus titer of 0.1TCID$_{50}$/cell and luciferase activity was determined 48 hours post infection (Fig. 12A).

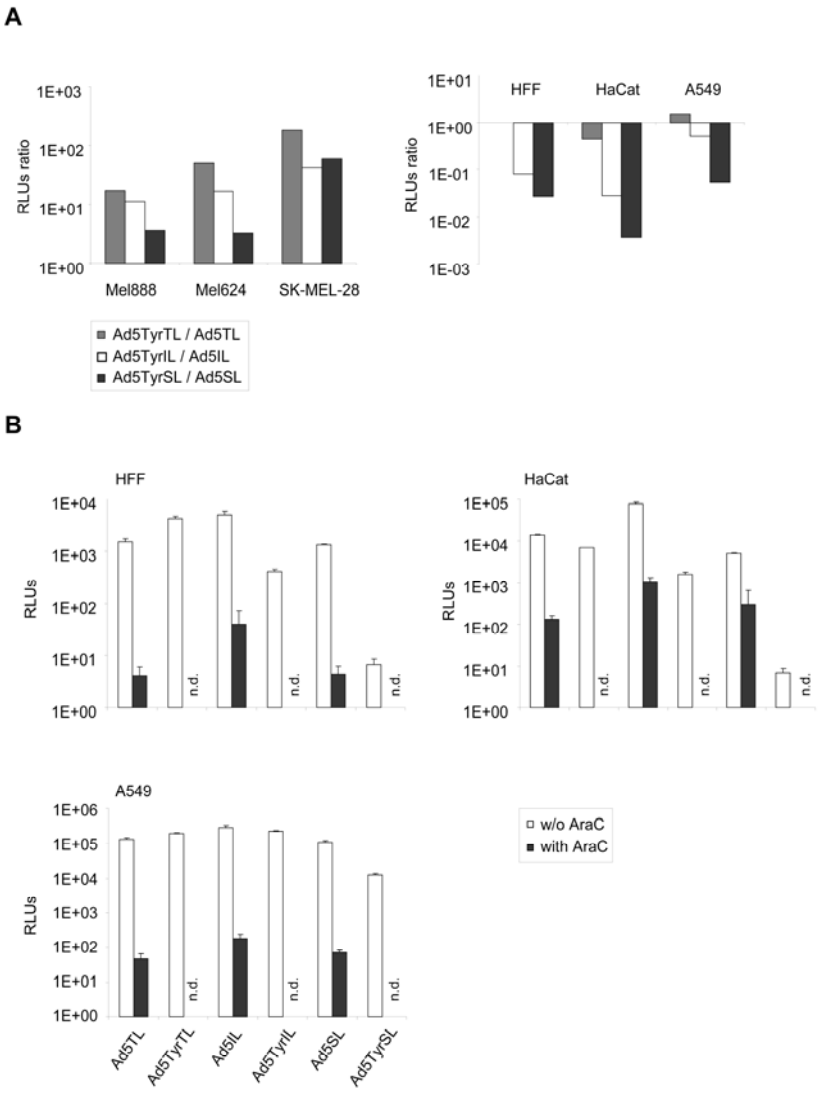


Fig.12: **Efficacy and kinetic of transgene expression by melanoma targeted OAds compared with untargeted viruses.** (A/B) Cells were infected with the recombinant OAds expressing luciferase after transgene insertion into the late transcription unit of the viral genome. Two days post infection, luciferase activities were determined with a luminescence assay. (A) Luciferase activities in melanoma cell lines (Mel888, Mel624, SK-MEL-28) and control cells (HFF, HaCat, A549). Cells were infected at 0.1TCID$_{50}$/cell. Ratios, RLUs Ad5TyrT/I/SL / RLUs Ad5T/I/SL. (B) Luciferase activities in control cells (HFF, HaCat, A549). Cells were infected at 1TCID$_{50}$/cell and either treated with 2 µM of AraC which was added 1 h, 12 h, 24 h and 36 h post infection or were mock treated. Columns show mean RLU values of triplicate infections; error bars show standard deviations; RLUs, relative luminescence units. One experiment out of at least three is shown.

The values of luciferase activity for cells infected with TyrE/P recombinant viruses (Ad5TyrTL, Ad5TyrIL and Ad5TyrSL) were normalized with readings from the corresponding untargeted virus (Ad5TL, Ad5IL, and Ad5SL) to compare the efficacy and specificity of transgene expression. As shown in figure 12A for all melanoma cell lines the ratios of luciferase activity were positive indicating a higher level of luciferase activity was detectable for TyrE/P recombinant viruses in comparison to untargeted adenoviruses. In contrast, in control cells the ratios Ad5TyrIL / Ad5IL and Ad5TyrSL / Ad5SL revealed a significant reduction of luciferase activity resulting from a lower transgene expression by the TyrE/P recombinant viruses. This reduction of the luciferase ratios indicates a melanoma selective transgene expression depended on the TyrE/P. Of note, the ratio of luciferase activity for Ad5TyrSL / Ad5SL was

decreased 2.85-fold in HFF, 8-fold in HaCat and 9.8-fold in A549 when compared to Ad5TyrIL / Ad5IL, as well as in the melanoma cell lines Mel888 and Mel624 the ratio of luciferase expression Ad5TyrSL / Ad5SL was also reduced compared to Ad5TyrIL / Ad5IL. Interestingly, the ratio of transgene activity by the Ad5TyrTL was in the control cells around one indicating a similar level of transgene expression for Ad5TyrTL and Ad5TL. In conclusion, the mode of transgene expression critically determines the specificity resulting in descending order of melanoma selective luciferase activity from Ad5TyrSL to Ad5TyrIL to Ad5TyrTL. Accordingly the minimal residual expression of luciferase by Ad5TyrSL in HFF and HaCat argued for a specifically “off” status of transgene expression indirectly regulated by TyrE/P in the context of the Ad5TyrSL genome.

Furthermore, for detailed analysis of the specificity of transgene expression, HaCat, HFF and A549 cells were infected at 1$TCID_{50}$/cell with the total panel of recombinant adenoviruses and genome replication was either blocked by adding AraC at a concentration of 2μM or cells were mock treated. Of note, this showed whether the targeting of adenoviral replication and indirectly the controlling of luciferase expression by the TyrE/P “mimic” the blocking of viral replication by adding the inhibitor AraC resulting in reduced luciferase activity in control cells. Cells were harvested 48 hours post infection and luciferase activity was determined (Fig. 12B). Efficient luciferase activity was detected for Ad5TL, Ad5IL and Ad5SL and surprisingly, for the melanoma targeted Ad5TyrTL and Ad5TyrIL. As depicted in figure 12B, no significant difference of transgene activity was observed for Ad5TyrTL and Ad5TL in the different cell types. Luciferase activity of Ad5TyrIL reaches nearly similar level when compared to Ad5IL in A549 cells, but decreased in the control cells yielding RLUs 47.1 (HaCat) - and 11.89 (HFF) -fold lower than for the Ad5IL. Depending on the cell line the level of transgene activity resulted from the Ad5TyrSL was 712.50-fold (HaCat), 199.17-fold (HFF) or 8.45-fold (A549) reduced in comparison to Ad5Δ24SL. In addition, the inhibitor AraC blocked transgene activity of Ad5TL, Ad5IL and Ad5SL in HaCat, HFF and A549 (Fig 12B). Note, however, I observed that the AraC did not completely block adenovirus genome replication in HaCat cells (Fig. 13B). With the exception of Ad5TyrSL in HaCat cells, adding AraC led to lower levels of luciferase activity as the corresponding melanoma targeted adenoviruses. This tight control of transgene activity of Ad5TyrSL in comparison to Ad5SL inhibited by AraC showed the high selectivity of transgene activity.

5.1.6 Efficacy and kinetics of luciferase mRNA expression for the recombinant oncolytic viruses

Next, magnitude and kinetics of luciferase mRNA expression by the recombinant oncolytic viruses were evaluated over the first 30h of the viral replication cycle. In addition, the specificity of transgene expression by the TyrE/P recombinant viruses was compared to the corresponding untargeted viruses in the control cell line HaCat. In this regard, HaCat cells were infected at 10TCID$_{50}$/cell with the total panel of recombinant adenoviruses. Luciferase mRNA was quantified 6, 12, 18, 24, and 30 h post infection by real time PCR (Fig. 13A).

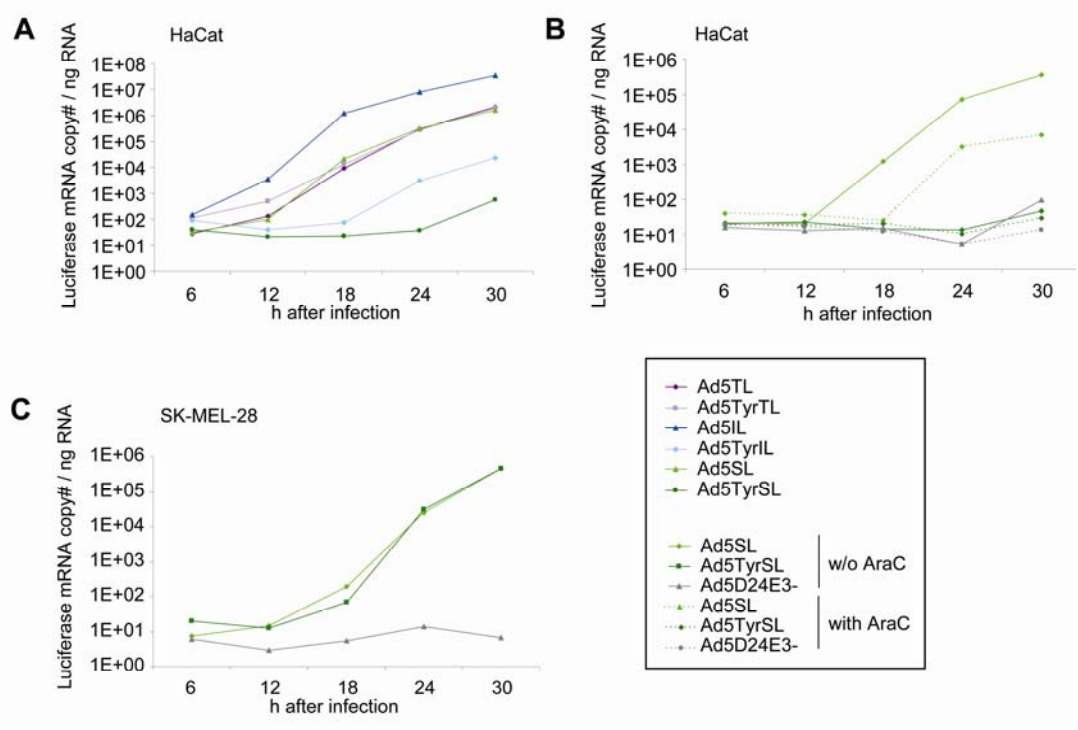


Fig.13: **Efficacy and kinetics of mRNA expression from the transgene.** (A/B/C) SK-MEL-28 and HaCat cells were infected with the indicated OAds at 10TCID$_{50}$/cell. When indicated, viral replication in HaCat cells was inhibited by adding 2 µM of AraC 1 h, 12 h and 24 h post infection. Luciferase messenger RNA copies were quantified 6, 12, 18, 24, and 30 h after infection by Real Time PCR and are shown after normalization with mass of cellular RNAs determined by GAPDH messenger RNA copy numbers. One representative experiment out of at least three is shown.

An increase of luciferase activity was determined from 6 to 30 h post infection for all recombinant viruses. However, for Ad5IL and Ad5SL, copy numbers for luciferase message were approximately 1500-fold or 2800-fold of those of the corresponding melanoma targeted adenoviruses Ad5TyrIL and Ad5TyrSL, respectively, over the time interval 18 – 30 h post infection in HaCat cells. This observation shows a TyrE/P dependent reduction of transgene expression in the control cells HaCat. On the contrary, after infection of HaCat cells with Ad5TyrTL and Ad5TL the same luciferase

expression pattern was observed for both viruses, representing an ineffective indirect transcriptional targeting.

Due to the high specificity of luciferase activity by Ad5TyrSL evaluated in HaCat cells, in the following experiments I focused on the more detailed analysis of luciferase expression by Ad5TyrSL and the corresponding untargeted Ad5SL. Since, luciferase expression by Ad5TyrSL should be dependent on the transcriptional targeting of adenoviral replication by the TyrE/P, the levels of luciferase mRNA in Ad5TyrSL-, Ad5SL- and the parental virus Ad5Δ24E3- infected cells were analyzed either after blocking viral replication by adding inhibitor AraC at a concentration of 2μM or in untreated HaCaT cells (Fig. 13B). Luciferase mRNA was quantified 6, 12, 18, 24, and 30 h post infection by real time PCR. For Ad5SL, expression of luciferase mRNA started 6 h later and was severely attenuated by the replication inhibitor AraC. Compared with the Ad5TyrSL, expression of luciferase messages by Ad5SL was still 156.41-fold (with AraC) and 8041.80-fold (without AraC) increased 30 h post infection. Independent of AraC, luciferase expression by Ad5TyrSL and the control virus Ad5Δ24E3-, which has no luciferase gene, was nearly similar in HaCat cells indicating background readings which cannot be attributed to transgene expression.

To exclude that the high specificity of luciferase expression in HaCat cells by Ad5TyrSL results from a general reduction of transgene expression of Ad5TyrSL in comparison to Ad5SL, I evaluated luciferase mRNA expression after infection of the melanoma cell line SK-MEL-28 at 10TCID50/cell with Ad5TyrSL, Ad5SL and Ad5Δ24E3-. In contrast to the parental virus Ad5Δ24E3-, increasing mRNA copy numbers of luciferase were detected over the first 30 h post infection of Ad5TyrSL and Ad5SL in SK-MEL-28 (Fig. 13C). Of note, the same mRNA expression pattern for Ad5TyrSL and Ad5SL in SK-MEL-28 cells reflects that insertion of TyrE/P into the adenoviral genome of Ad5SL does not interfere with the luciferase expression.

5.1.7 Efficacy and specificity of expression of the viral early E1A gene and the late fiber gene and quantification of virus genome copy numbers

I could show that the specific cellular TyrE/P construct facilitated indirect transcriptional targeting of luciferase expression by the recombinant oncolytic adenoviruses Ad5TyrIL and Ad5TyrSL. Further, I investigated how the different insertion strategies of the luciferase gene influence the specificity of E1A expression controlled by the TyrE/P, the selective amplification of the viral genome as well as efficacy and specificity of the viral fiber gene expression.

In this regard, E1A mRNA expression was measured over the first 30h of the viral replication cycle (Fig. 14A).

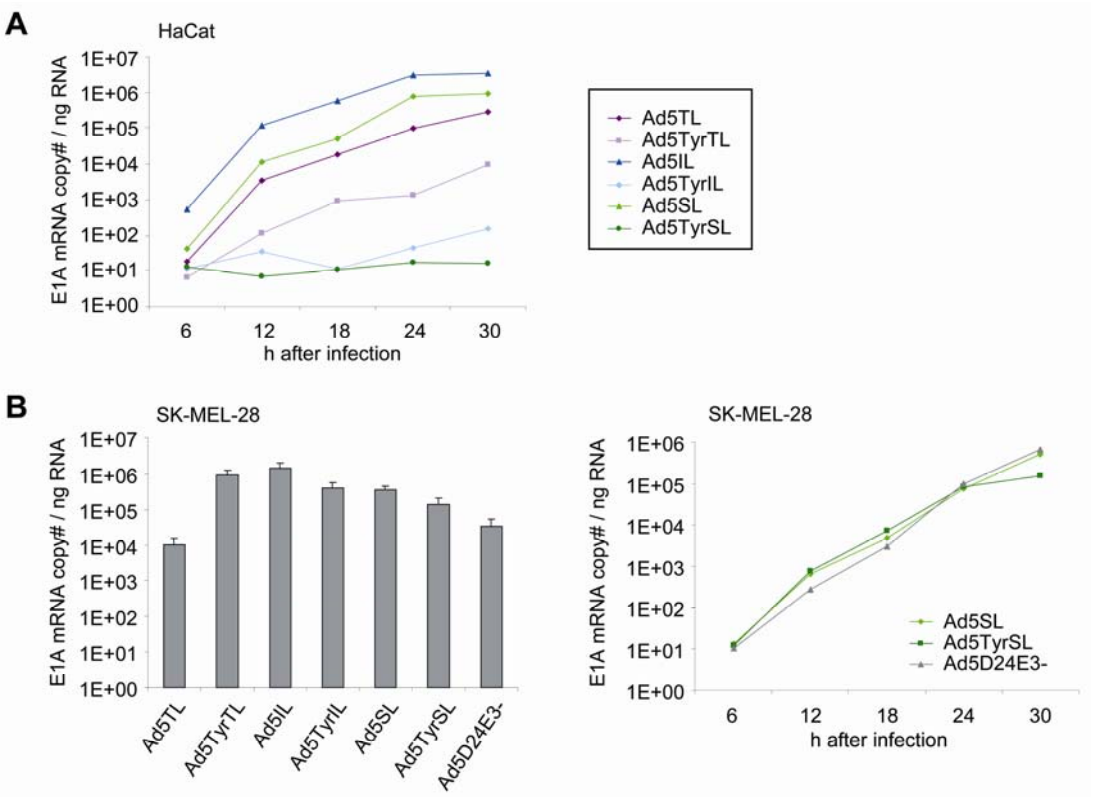


Fig.14: **Kinetics and efficiency of messenger RNA expression form the viral early gene E1A of recombinant OAds** (A) For quantification of E1A messenger RNA copy numbers in a period of 30 h, HaCat cells were infected with the indicated viruses at 10TCID$_{50}$/cell. At 6, 12, 18, 24 and 30 h after infection the RNA copy numbers for viral E1A were determined by Real Time PCR and are shown after normalization with mass of cellular RNAs determined by GAPDH messenger RNA copy numbers. (B) The left panel shows the quantification of copy numbers of early E1A mRNA 24 h after infection of SK-MEL-28. Columns show mean values of triplicate infection and error bars show standard deviations. In the right panel is the quantification of E1A messenger RNA copy numbers in a period of 30 h after infection of SK-MEL-28 cells with the indicated viruses at 10TCID$_{50}$/cell shown. Data are presented as described in (A) One representative experiment out of at least three is shown.

Therefore, HaCat cells were infected with the total panel of recombinant adenoviruses. Values were quantified 6, 12, 18, 24, and 30 h post infection by real time PCR. In HaCat cells a high E1A mRNA copy number for Ad5IL and Ad5SL were quantified 30 h after virus infection. However, E1A mRNA copy numbers were dramatically lower for Ad5TyrIL (21946.9-fold) and Ad5TyrSL (57582.3-fold) at late time points compared to the untargeted, recombinant adenoviruses (Ad5IL, Ad5SL). For Ad5TyrTL, the E1A mRNA level was only minimal attenuated in comparison to Ad5TL and still 29-fold higher as for Ad5TyrIL and Ad5TyrSL. Relative to the values of transgene expression (Fig. 13A), specificity of E1A expression by the melanoma targeted recombinant viruses correlated with the selectivity of luciferase expression

which is ordered from Ad5TyrSL > Ad5TyrIL > Ad5TyrTL. Of note, the mode of transgene expression has an influence on the specificity of the TyrE/P and therefore on E1A mRNA expression.

As the expression of E1A mRNA reflected the highest selectivity of indirect transcriptional control by the TyrE/P within the context of Ad5TyrSL in HaCat cells, E1A expression was analyzed in SK-MEL-28 cells to investigate the efficacy of E1A mRNA expression resulting from Ad5TyrSL compared the corresponding unspecific Ad5SL and the parental virus, Ad5Δ24E3-. E1A mRNA copy numbers were quantified 6, 12, 18, 24, and 30 h post infection of the melanoma cell line SK-MEL-28 by real time PCR. The 30 h kinetics of E1A messages by Ad5TyrSL showed an almost similar increase compared to Ad5SL and Ad5Δ24E3- early after adenoviral infection but was 3.2-fold reduced at the later time point (Fig. 14B).

To analyze the E1A expression values by the Ad5TyrSL, Ad5SL and Ad5Δ24E3- in comparison to Ad5TL, Ad5IL, Ad5TyrTL and Ad5TyrIL, SK-MEL-28 were additionally infected with the total panel of recombinant adenoviruses and E1A mRNA was measured 24 h post infection. E1A mRNA copy numbers were 26.7-fold (Ad5TyrTL), 12-fold (Ad5TyrIL), 40.3-fold (Ad5IL), 4.1-fold (Ad5TyrSL), 10.5-fold (Ad5SL) increased and 3.3-fold (Ad5TL) attenuated relative to the parental virus Ad5Δ24E3- in SK-MEL-28 (Fig. 14B). These results correlate with the oncolysis data of the recombinant viruses in SK-MEL-28 (Fig. 10).

Next, the effect of the different strategies of transgene integration into the viral genome on viral replication was analyzed. Hence, amplification of the viral genome was measured over the first 30h of the viral replication cycle. Therefore, HaCat cells were infected with the total panel of recombinant viruses. Genome copy numbers were quantified 6, 12, 18, 24, and 30 h post infection by real time PCR. As expected, genome copy numbers of melanoma targeted adenoviruses Ad5TyrIL and Ad5TyrSL were approximately 2.5 orders of magnitude reduced 30h post infection in HaCat cells (Fig. 15A) compared with Ad5IL and Ad5SL, respectively. In contrast, at late time points genome copy numbers were almost identical for Ad5TyrTL and Ad5TL. Of note, the analysis of the genome copy number correlates with the E1A mRNA expression pattern indicating that viral genome replication is dependent on the expression of E1A and that the different transgene insertion strategies resulted in a downward order of selectivity of viral replication from Ad5TyrSL > Ad5TyrIL > Ad5TyrTL.

Moreover, to further investigate the specificity of viral replication by the Ad5TyrSL, genome copy numbers of Ad5TyrSL, Ad5SL and Ad5Δ24E3- were evaluated in 30 h kinetics in absence or presence of the viral replication inhibitor AraC. As depicted in figure 15B, viral replication of Ad5SL and Ad5Δ24E3- could not be completely blocked by the inhibitor AraC. However, addition of AraC led to a reduction of genome copy numbers for Ad5SL and Ad5Δ24E3- starting form 18 h after infection of HaCat cells. For Ad5TyrSL replication efficiency was severely attenuated compared with Ad5SL and Ad5Δ24E3-. This observation was independent of the inhibitor AraC and indicates melanoma specific replication of Ad5TyrSL conditioned on the expression of E1A.

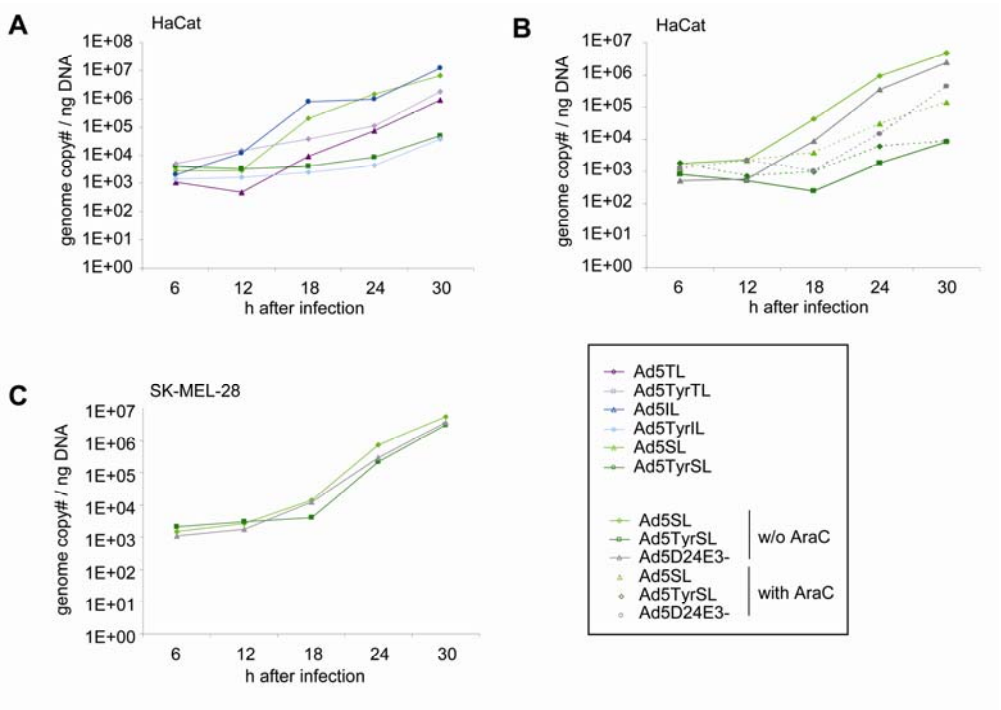


Fig.15: **Quantification of virus genome copy numbers of recombinant OAds in SK-MEL-28 and HaCat cells**. (A/B/C) To analyze adenovirus genome copy numbers HaCat and SK-MEL-28 cells were infected with the indicated viruses at 10TCID$_{50}$/cell. If indicated, viral replication in HaCat cells was inhibited by AraC added 1 h post infection. At 6, 12, 18, 24 and 30 h after infection genome copy numbers were determined by Real Time PCR and are shown after normalization with nanograms cellular genomic DNA determined by β-actin messenger DNA copy numbers.

To underline that the reduction of genome copy numbers of Ad5TyrSL in HaCat cells comes from a high specificity of viral replication and is not a result of a generally lower replication rate of Ad5TyrSL, genome copy numbers of this recombinant virus was compared with Ad5SL and Ad5Δ24E3- in melanoma cells. In this regard, SK-MEL-28 was infected at 10TCID50/cell with Ad5TyrSL, Ad5SL and Ad5Δ24E3- and genome copy numbers were quantified at different time points by real time PCR. The

genome copy numbers for Ad5TyrSL, Ad5SL and Ad5Δ24E3- increased continuously over time with the sharpest increase from 18 – 30 h and ended up at the same level. This shows, that the transgene insertion by an additional splice acceptor site and the TyrE/P do not have an effect on the viral replication in SK-MEL-28. Consequently, all analyzed viruses showed the same efficacy of genome replication in melanoma cells. Next, it was analyzed whether the insertion of the transgene into the late transcription unit of the adenoviral genome interferes with the expression of the neighbouring late viral genes such as fiber. Therefore, expression of fiber mRNA was measured by real time PCR 6, 12, 18, 24 and 30 h after infection of HaCat cells with the total panel of recombinant viruses. 30 h after infection values of fiber messages were near four orders of magnitude increased compared to the early time point for Ad5IL and Ad5SL. According to the specific E1A expression, transgene expression and viral replication by Ad5TyrIL and Ad5TyrSL, fiber mRNA expression was reduced three orders of magnitudes 30 h post infection of HaCaT cells compared to cells infected with the corresponding, untargeted viruses. In contrast, the Ad5TyrTL and Ad5TL showed a similar level of fiber mRNA expression starting from 18 h after infection indicating that the late gene expression is independent of TyrE/P and not specific for melanoma cells. Furthermore, the expression of the structural protein fiber was analyzed by western blot (Fig. 16A).

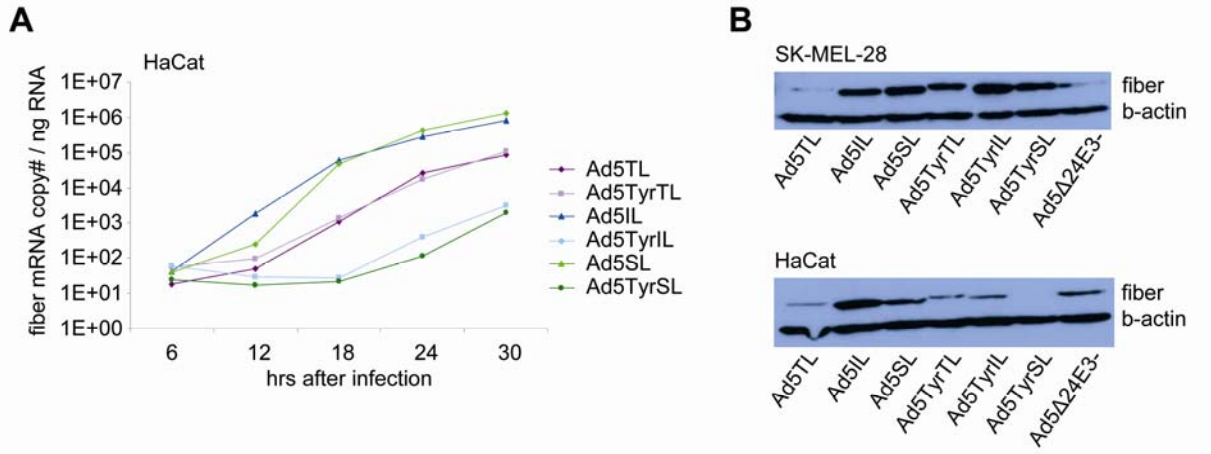


Fig.16: **Kinetics and efficiency of expression of the viral late gene fiber by determination of fiber messenger RNA and protein.** (A) For quantification of late fiber messenger RNA copy numbers, HaCat cells were infected with the indicated viruses at 10TCID$_{50}$/cell. At 6, 12, 18, 24 and 30 h after infection mRNA copy numbers for the viral fiber gene were determined by Real Time PCR and are shown after normalization with mass of cellular RNA determined by GAPDH messenger RNA copy numbers. (B) SK-MEL-28 and HaCat cells were infected at 100TCID$_{50}$/cell with the indicated viruses and were harvested one day post infection. Immunoblots were performed to detect viral Ad5 fiber or β-actin.

HaCat cells were infected at 100 TCID50/cell with the total panel of recombinant viruses including Ad5Δ24E3-. 24 h post infection total cell lysates were applied for SDS gel electrophoresis, followed by immunoblotting and detection of viral fiber protein. ß-actin was used as loading control. The results (Fig. 16B) demonstrated expression of fiber protein in HaCat cells infected with the recombinant viruses except the Ad5TyrSL. However, strongest protein expression was detectable after infection of Ad5TyrTL. Infection of HaCat cells yielded a high amount of fiber protein level by Ad5IL reducing in compare to Ad5SL. For Ad5TyrIL and Ad5TyrSL, fiber expression is clearly decreased or completely blocked compared to the Ad5IL and Ad5SL, whereas Ad5TyrTL and Ad5TL showed similar fiber expression. To determine the specificity of the protein expression in the context of recombinant viruses, fiber expression was analyzed 24 h post infection of the total panel of recombinant viruses as well as Ad5Δ24E3- in the melanoma cell line SK-MEL-28. Western blot analysis demonstrated strong expression of fiber after infection with the recombinant viruses, except for Ad5TL and the parental virus Ad5Δ24E3-. Of note, this observation implies that insertion of the luciferase-cassette per se does not reduce fiber expression by Ad5IL, Ad5SL, Ad5TyrTL, Ad5TyrIL and Ad5TyrSL in melanoma cells. As expected, infection with Ad5TyrSL resulted in highly specific expression of fiber in melanoma cells in comparison to control cells HaCat, reflecting the specificity of E1A and luciferase expression by Ad5TyrSL. In comparison to Ad5TyrSL the specificity of fiber expression by AdTyrIL and AdTyrTL was reduced or unregulated, respectively.

5.1.8 Analysis of interference between splice acceptor site and E4 expression in the context of Ad5SL

Next, I analyzed whether insertion of the luciferase gene into the genome of Ad5SL interferes with expression of the neighbouring E4 genes, which are located between the fiber gene and the transgene (Fig. 9). This locale of transgene insertion differs from the other insertion strategies and could critically determine the efficacy of E4 expression, depending on mRNA stability and splicing. For this purpose E4 mRNA expression by Ad5SL was compared to mRNA expression of the same genes in the parental virus Ad5Δ24E3- in the SK-MEL-28 cell line by real time PCR (Fig. 17). E4 mRNA copy numbers were similar for Ad5SL and Ad5Δ24E3- over the whole time interval evaluated in this experiment. The increase in E4 messages from 6 to 30 h

was 26790-fold for both viruses indicating E4 mRNA expression was not affected by transgene insertion.

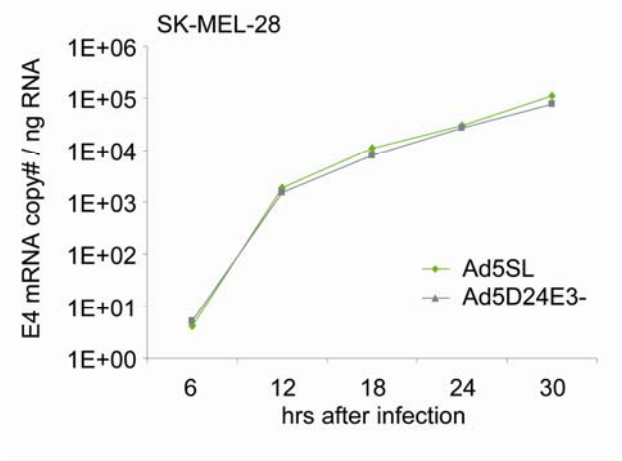


Fig.17: **Analysis of possible interference of splice acceptor site with E4 expression in the context of Ad5SL.** SK-MEL-28 cells were infected with Ad5SL or Ad5D24E3- at 10TCID$_{50}$/cell and messenger RNA copy numbers were determined for the E4 genes at indicated time points. Values are shown after normalization with mass of cellular RNA determined by GAPDH messenger RNA copy numbers. One representative experiment out of at least two is shown.

5.1.9 Expression of therapeutic genes by oncolytic adenoviruses via alternative splicing depends on the transgene

My studies of the recombinant oncolytic adenoviruses have shown that the splice acceptor sequences can be used to achieve melanoma selective transgene expression in the context of Ad5TyrSL and Ad5TyrIL. AdTyrIL showed higher transgene expression level, however, transgene expression by AdTyrSL was more specific. Based on the required specificity of therapeutic gene expression for therapeutic application I decided to concentrate on Ad5TyrSL. This virus represents a genomically tool for arming oncolytic adenoviruses with therapeutic genes.

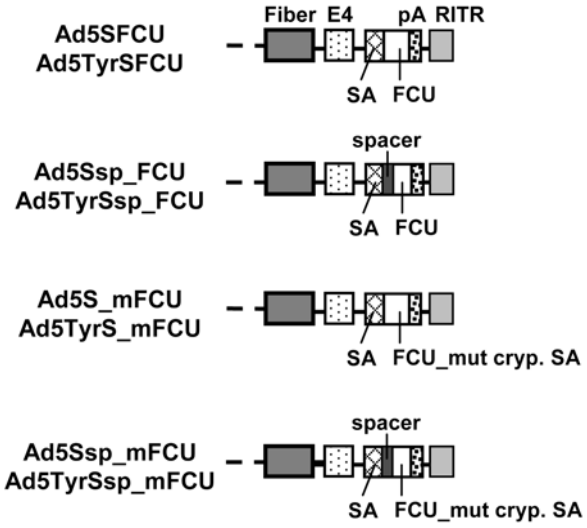


Fig.18: **Adenovirus constructs with *CD-FUR-1* fusion gene inserted into the late transcription unit via SA.** Schematic outline of the genomes of the oncolytic viruses with insertion of *FCU-1* gene encoding the yeast CD-FUR-1 fusion protein. Shown are the right termini of the genomes (see Fig. 9 for left termini of Ad5 and Ad5Tyr genomes). E4, early adenoviral gene; FCU_mut crypt. SA, *FCU-1* gene with mutation of cryptic splice acceptor site; pA, polyadenylation signal; RITR, right inverted terminal repeat; SA, splice acceptor site.

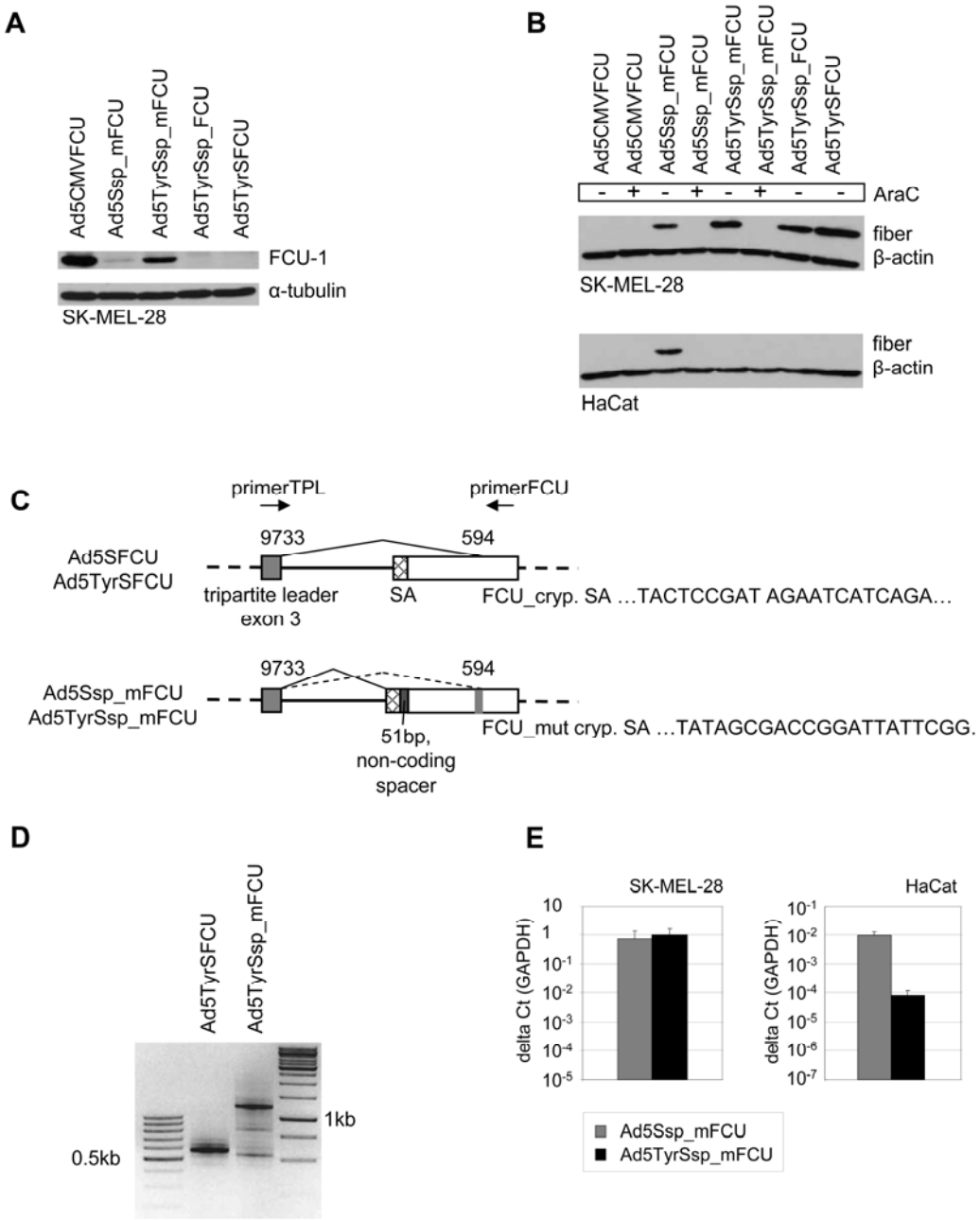


Fig.19: **Expression of the *CD-FUR-1* fusion gene inserted into the late transcription unit via SA requires sequence optimization.** (A) SK-MEL-28 cells were infected at 100TCID$_{50}$/cell and expression of the *CD-FUR-1* fusion protein was detected by Immunoblot two days post-infection. As loading control α-tubulin was detected. (B) SK-MEL-28 cells were infected at 10TCID$_{50}$/cell in the absence or presence of AraC and fiber expression was detected by Immunoblot one day post-infection. As loading control β-actin was detected. (C) Schematic outline of the *FCU-1* gene insertion via SA into the late virus transcription unit and sequence optimization in Ad5Ssp_mFCU/Ad5TyrSsp_mFCU. Numbers indicate nucleotide positions in the Ad5 genome or *FCU-1* gene. The non-coding spacer corresponds to nucleotides 4 to 54 of the luciferase gene.Nucleotide sequences indicate the cryptic splice acceptor site (FCU_cryp. SA) and the mutations we introduced (FCU_ mut cryp. SA). (D) RT-PCR analysis of *FCU-1* mRNA expression by SK-MEL-28 harvested one day post-infection with adenoviruses at 100TCID$_{50}$/cell and using the primer pair indicated in C. The correct splice product is 1185bp. (E) qPCR analysis of *FCU-1* mRNA expression in melanoma cells and keratinocytes harvested one day post-infection with adenoviruses at 10TCID$_{50}$/cell.

In this regard, the prodrug activating enzyme *cytosine deaminase / uracil phosphoribosyltransferase* fusion gene (*FCU-1*) from yeast (Erbs et al., 2000) was inserted into the late transcription unit of the Ad5TyrSL genome by replacing the luciferase gene (Ad5SFCU and Ad5TyrSFCU, Fig. 18).

To my surprise, analysis of *FCU-1* expression demonstrated that enzyme expression via alternative splicing was insufficient (Fig. 19A), even though the fiber gene was expressed (Fig. 19B) and lytic activity was not affected (Fig. 20). As their genomes outside the *FCU-1* gene matched those of Ad5SL and Ad5TyrSL, I speculated that the different nucleotide sequence downstream of the SA in Ad5SFCU and Ad5TyrSFCU interfered with proper splicing. To optimize the splicing machinery a 51bp long, non coding sequences of the luciferase gene were inserted as spacer between the splice acceptor sequence and the *FCU-1* gene. These Ad5Ssp_FCU and AdTyrSsp_FCU viruses contain a SA nucleotide environment which is more similar to the surrounding sequences of the splice acceptor site of Ad5SL and AdTyrSL in comparison to Ad5SFCU and Ad5TyrSFCU (Fig. 18 and 19C). However, these viruses only showed marginal *FCU-1* expression (Fig. 19A). I then analyzed *FCU-1* mRNA expression and splicing in Ad5TyrSFCU-infected SK-MEL-28 cells. By RT-PCR using a forward primer binding in the tripartite leader and a reverse primer binding in the 3' end of the *FCU-1* gene a 1185 bp product was expected if splicing was correct, however we obtained a band of approximately 550bp (Fig. 19D). Sequencing of the PCR products revealed a cryptic splice acceptor site at position 594 of the *FCU-1* gene, that prevented synthesis of the proper fusion protein-encoding mRNA. This position was also found in a search for splice sites using *EMBnet / Scientific Computing Service* software. In the light of these results I generated Ad5Ssp_mFCU and Ad5TyrSsp_mFCU by completely changing the sequence surrounding the cryptic splice acceptor site within the *FCU-1* gene with silent mutations (Fig. 18, 19C).

After infection of SK-MEL-28 cells, Ad5Ssp_mFCU and Ad5TyrSsp_mFCU expressed the CD-FUR-1 fusion protein (Fig. 19A). CD-FUR-1 expression was stronger for Ad5TyrSsp_mFCU than for Ad5Ssp_mFCU, which corresponded to the strength of luciferase expression observed for Ad5TyrSL and Ad5SL (Fig. 12). Viruses with mutated *FCU-1* gene but without spacer (Ad5TyrS_mFCU and Ad5S_mFCU) showed lower CD-FUR-1 expression than Ad5TyrSsp_mFCU and Ad5Ssp_mFCU (not shown); indicating that the combination of cryptic splice site mutation and spacer ensured strongest transgene expression. As expected,

expression of fiber protein by Ad5TyrSsp_mFCU and Ad5Ssp_mFCU was dependent on virus replication and specific for melanoma cells (Fig. 19A and B). Immunoblot analysis was not sensitive enough to detect CD-FUR-1 protein in HaCat cells; however, I could demonstrate by qRT-PCR that *FCU-1* mRNA expression by Ad5TyrSsp_mFCU was highly melanoma-specific (Fig. 19E). I conclude that insertion of the prodrug convertase fusion gene *FCU-1* via SA into the late transcription unit of oncolytic adenovirus Ad5Tyr requires sequence optimization, but results in transcriptionally targeted *FCU-1* expression.

5.1.10 Lytic activity and specificity of armed oncolytic adenoviruses in melanoma and non-melanoma cells

First, the lytic activity and specificity of the *FCU1*-armed oncolytic adenoviruses was determined by infection of melanoma and non melanoma cells. In this regard, cells were infected with the FCU1-armed oncolytic adenoviruses (Ad5TyrSFCU, Ad5TyrSsp_FCU, Ad5TyrSsp_mFCU and Ad5Ssp_mFCU) and the Ad5CMVFCU that served as a non-replicating control at virus titers of 0.001 to 100TCID50/cell in 10-fold dilutions. Cell killing was detected by staining of surviving, adherent cells with crystal violet (Fig. 20). As expected, no cell killing was observed for all cells infected with Ad5CMVFCU regardless of which titer was used. Cytopathic effect of Ad5TyrSFCU, Ad5TyrSsp_FCU and Ad5TyrSsp_mFCU was similar to Ad5Ssp_mFCU in the melanoma cell lines Mel888 and SK-MEL-28. Of note, Ad5Ssp_mFCU and Ad5Ssp_mFCU were 10-fold superior to the melanoma targeted armed viruses Ad5TyrSFCU and Ad5TyrSsp_FCU in the cell killing of Mel624. Besides, Ad5Ssp_mFCU showed the most potent cytotoxicity in the tested control cell lines HaCat, A549 and HFF, which was approximately 2 to 3 orders of magnitude stronger compared to the melanoma targeted armed viruses. Importantly, the targeted armed viruses showed no cell killing of HaCat cells, even when infected at high titers. Minimal cell lysis by Ad5TyrSFCU, Ad5TyrSsp_FCU and Ad5TyrSsp_mFCU at 100TCID50/cell was observed in A549 and HFF. These results show that insertion of the mutated *FCU-1* gene, SA and spacer did not interfere with selective replication of the virus.

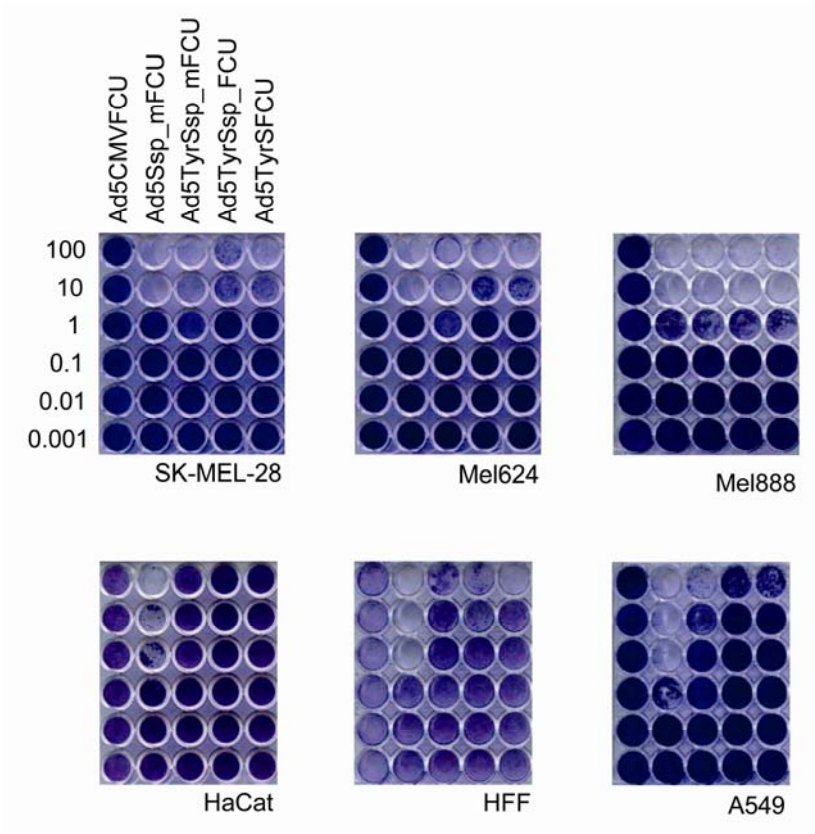


Fig.20: **Oncolytic activity of adenovirus constructs with *FCU-1* gene inserted via SA into the late transcription.** Melanoma cells (SK-MEL-28, Mel624, Mel888), HaCat, HFF and A549 were infected with adenoviruses and cytotoxicity was determined by crystal violet staining of surviving cells when cytotoxicity was observed for the most potent virus at 1 or 0.1TCID$_{50}$/cell for each cell type individually. Numbers are viral titers in TCID$_{50}$/cell. Ad5CMVFCU is an E1/E3-deleted replication-deficient adenovirus with CMV-*FCU-1* expression cassette.

5.1.11 Combining of adenoviral oncolysis with suicide gene therapy by indirect transcriptional targeting of genetic prodrug activation

In a virus burst assay, which over a single round of virus replication measure the infectious virus particles in a cell population, I first assessed the effect of 5-FC on virus production and release. For that purpose, SK-MEL-28 cells were infected at 1TCID$_{50}$/cell with Ad5TyrSsp_mFCU or Ad5Ssp_mFCU in the presence or absence of the prodrug 5-FC. Three days post infection supernatant and cells were harvested and amounts of total infectious virus particles were measured (Fig. 21).

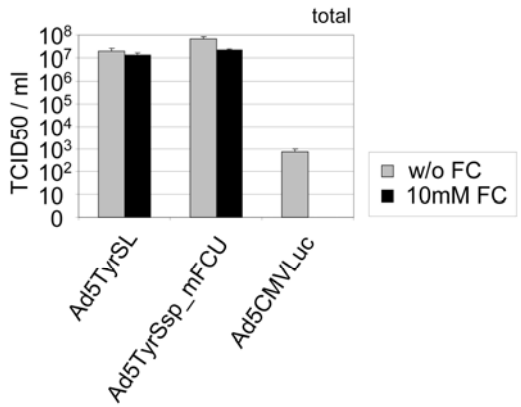
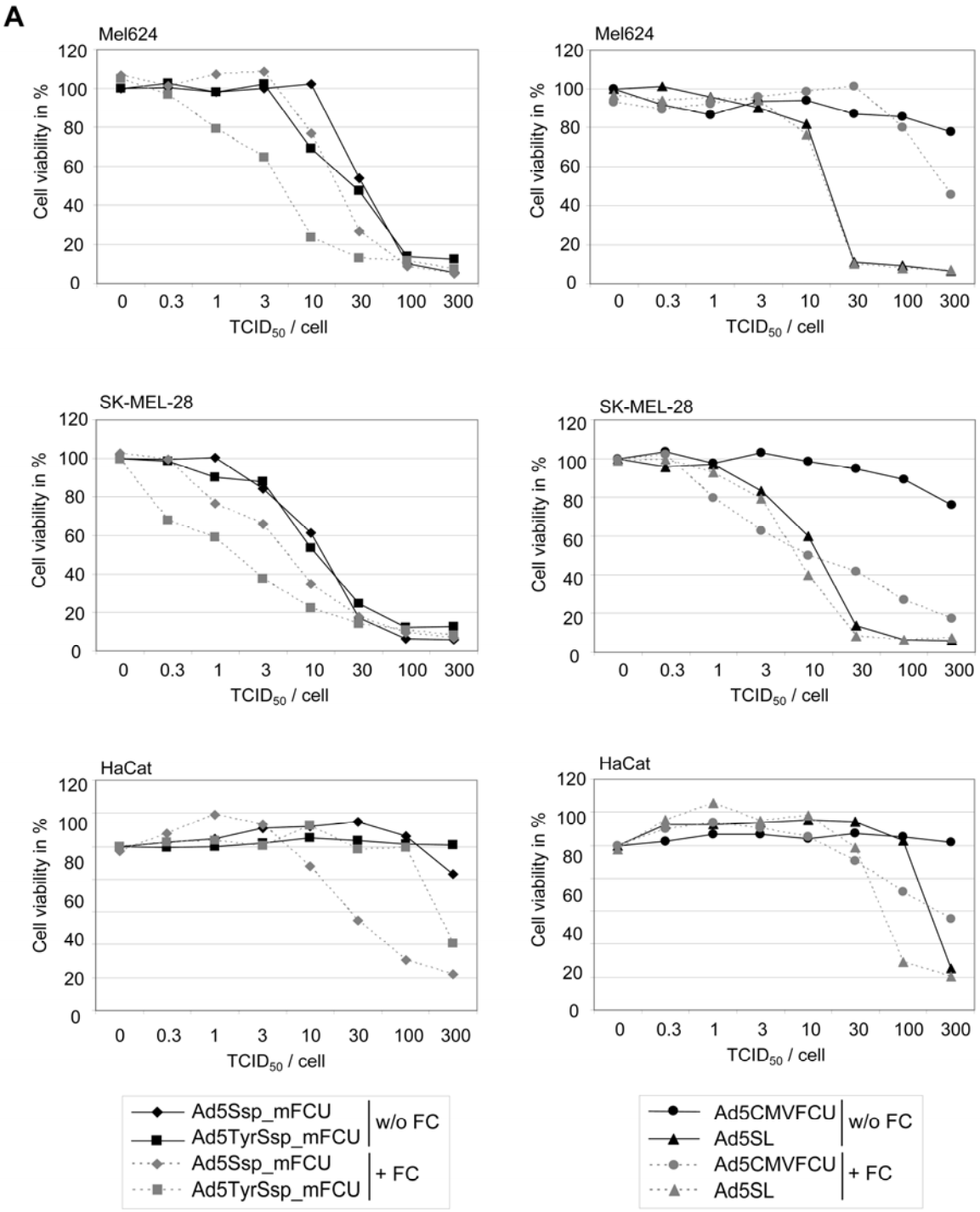


Fig.21: **Progeny production of adenovirus constructs with *FCU-1* gene inserted via SA into the late transcription.** SK-MEL-28 cells were infected with the indicated viruses at 1TCID$_{50}$/cell and one day post infection cells were incubated with 10mM FC. Three days post infection, cells and supernatant were harvested separately and titers of infectious viral particles were determined by TCID$_{50}$ assay. Infections were performed in triplicates. Columns show mean titers, arrow bars show standard deviations.

The results showed that application of 5-FC was compatible with the production of infectious particles of Ad5TyrSsp_mFCU or Ad5Ssp_mFCU at high titers in melanoma cells, although virus yields were reduced 8.2- and 1.5-fold, respectively. I next investigated a combination therapy of viral oncolysis and tumour-targeted prodrug activation mediated by Ad5TyrSsp_mFCU. Therefore, I infected melanoma cells SK-MEL-28 and Mel624, and HaCat cells with dilutions of Ad5TyrSsp_mFCU, with Ad5Ssp_mFCU, Ad5SL or replication-deficient Ad5CMVFCU expressing *FCU-1* from the strong CMV enhancer/promoter.



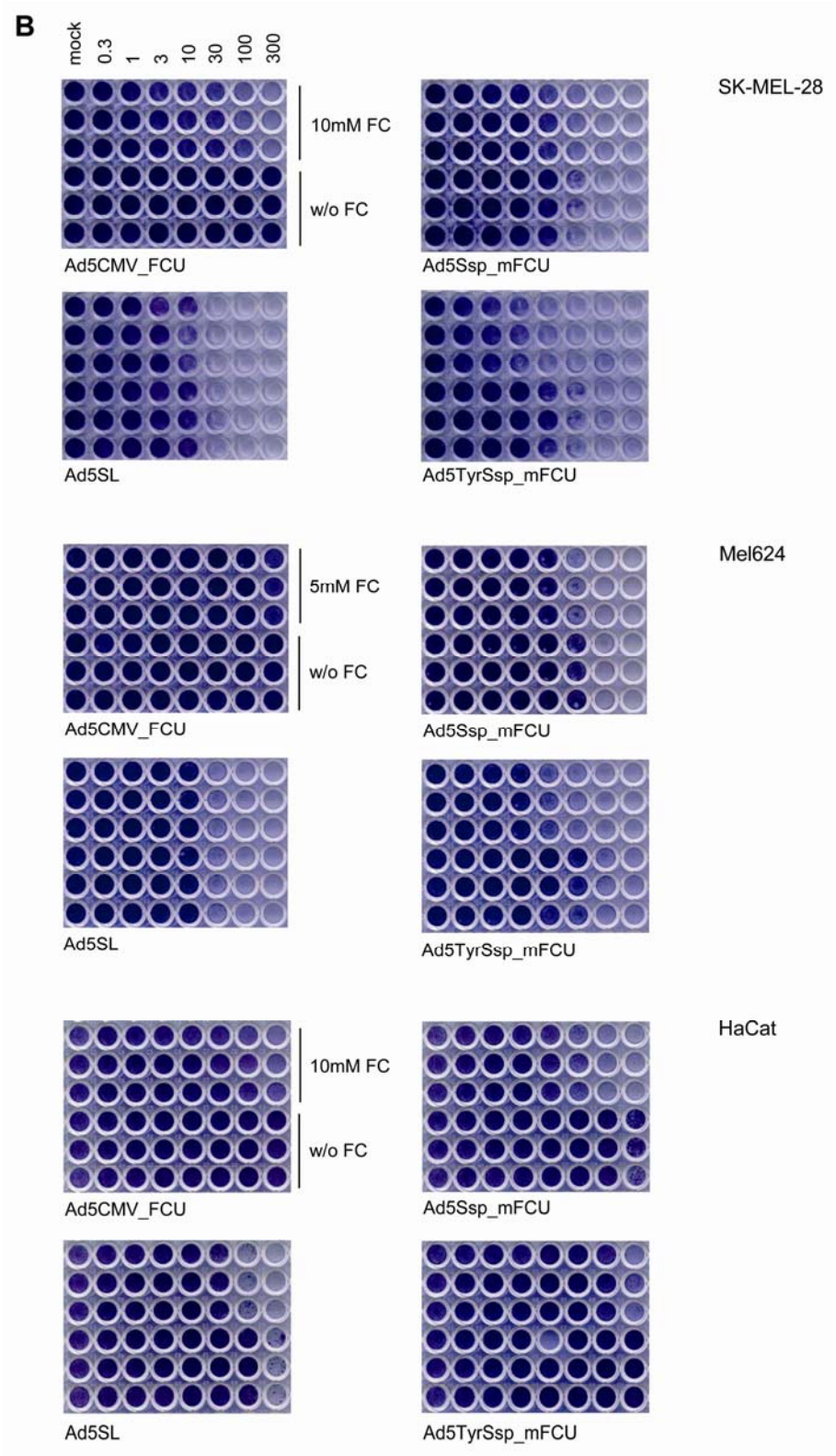


Fig.22: **Combined oncolysis and prodrug activation therapy by adenovirus constructs with *FCU-1* gene inserted via SA into the late transcription unit.** (A/B) Melanoma cells (SK-MEL-28, Mel624), and keratinocytes (HaCat) were infected with with adenoviruses at indicated titers or were mock-infected in triplicates. 5-FC or medium was added two day post-infection at 10mM (SK-MEL-28, HaCat) and at 5mM (Mel624) concentration. Cytotoxicity was determined six days post-infection by staining of surviving cells with crystal violet. (A) Cell content was determined by measuring OD_{595} and cell viability was plotted in % of mock-infected cells that did not obtain 5-FC. Mean values of triplicates are shown. For clarity of presentation, standard deviations are not shown and were below 16.6%. (B) Crystal violet-stained plates for the experiments with SK-MEL-28, Mel624 and HaCat cells.

The prodrug 5-FC or mock were added two days post-infection, thus before virus spread, and cytotoxicity was determined four days later (Fig. 20/21). As expected, the replication-competent adenoviruses Ad5Ssp_mFCU, Ad5TyrSsp_mFCU and Ad5SL showed similar dose-dependent cytotoxicity in melanoma cells, which was not observed for replication-deficient AdCMVFCU-1. Addition of 5-FC resulted in increased cytotoxicity for all *FCU-1*-encoding adenoviruses, but not for Ad5SL in melanoma cells. In both melanoma cells, the strongest cytotoxicity was observed for Ad5TyrSsp_mFCU in the presence of 5-FC, which reduced the viral IC_{50} dose approximately 10-fold. The stronger prodrug effect in comparison to Ad5Ssp_mFCU + 5-FC corresponds with the higher expression of CD-FUR-1 by Ad5TyrSsp_mFCU (Fig. 22). In contrast to melanoma cells, the untargeted Ad5Ssp_mFCU + 5-FC showed the strongest cytotoxicity in HaCat cells. For Ad5TyrSsp_mFCU no cytotoxicity was observed in the absence of 5-FC and only at the highest virus titer we observed a prodrug effect. Note that in these cells 5-FC mediated also an increase in cytotoxicity of Ad5SL, which does not encode the prodrug convertase. I conclude that Ad5TyrSsp_mFCU induces melanoma-selective oncolysis and selectively enhanced cytotoxicity in combination with 5-FC.

When combined with viral oncolysis, a bystander effect of genetic prodrug activation is essential in order to kill those tumour cells that the lytic virus cannot reach, for example due to connective tissue barriers in the tumour. We therefore investigated whether Ad5TyrSsp_mFCU + 5-FC has the potential to induce bystander killing in melanoma cells (Fig. 23).

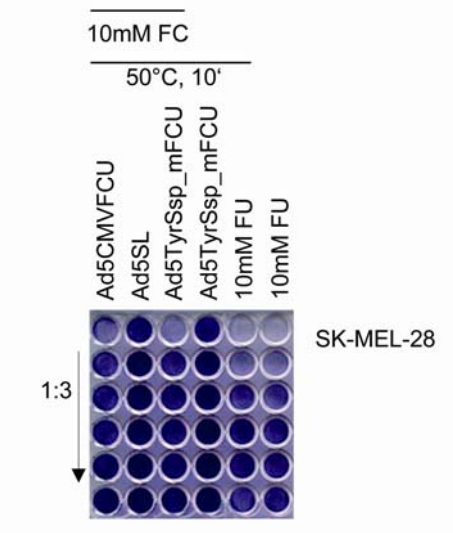


Fig. 23: **Bystander effect of prodrug activation by adenovirus constructs with *CD-FUR-1* fusion gene inserted via SA into the late transcription unit.** SK-MEL-28 cells were infected with Ad5TyrSsp_mFCU, Ad5CMVFCU or Ad5SL at 10 $TCID_{50}$/cell. 5-FC at a concentration of 10mM was added two days post-infection; as control, Ad5TyrSsp_mFCU-infected cells were treated with media alone. Supernatants were harvested three days post-infection and were incubation for 10 min at 50°C for virus inactivation. Then, each heat-treated supernatant was added in a 3-fold dilution series to fresh SK-MEL-28 cell monolayer. In parallel, SK-MEL-28 cells were treated with a 3-fold dilution series of 10 mM 5-FU or of 10 mM 5-FU after 10 min incubation at 50°C. The arrow indicates increasing dilutions of supernatants/5-FU. Cytotoxicity was determined four days post-infection by staining of surviving cells with crystal violet.

Supernatants of Ad5TyrSsp_mFCU-infected and 5-FC-treated SK-MEL-28 cells showed a clear cytotoxicity for uninfected SK-MEL-28 cells when viruses in the supernatants were heat-inactivated. Cytotoxicity of supernatants was nearly as efficient as 10 mM of the drug 5-FU. As the prodrug 5-FC was added at 10 mM to the Ad5TyrSsp_mFCU-infected cells, these results demonstrate efficient activation of the prodrug and release of the drug into the media by Ad5TyrSsp_mFCU-infected SK-MEL-28 cells. No bystander killing was observed for Ad5SL/5-FC or for Ad5TyrSsp_mFCU without 5-FC, indicating that virus particles in the supernatants were effectively inactivated by heat-treatment and that the detected cytotoxicity was dependent on prodrug-activation.

5.2 Targeting Oncolytic Adenoviruses based on Tumour Selective Cell Entry

Recombinant adenoviruses have emerged as promising agents in therapeutic gene transfer, genetic vaccination and viral oncolysis. For therapeutic applications of adenoviruses cell type-selective viral cell entry, for example into cancer or immune cells, should be engineered. Such tropism-modification of adenoviruses requires the ablation of their natural cell binding properties and the incorporation of cell-binding ligands. The short fiber of subgroup F (F41s) adenoviruses has recently been suggested as a tool for genetic adenovirus detargeting based on the reduced liver infectivity of corresponding fiber chimeric adenovectors *in vitro* and *in vivo*. In regard to this, the aim of my project was to generate an oncolytic adenovirus that features the Ad41 short fiber to ablate the native tropism. To retarget the virus cell-binding ligands were inserted into the ablated Ad41 short fiber regaining cell-specific infectivity.

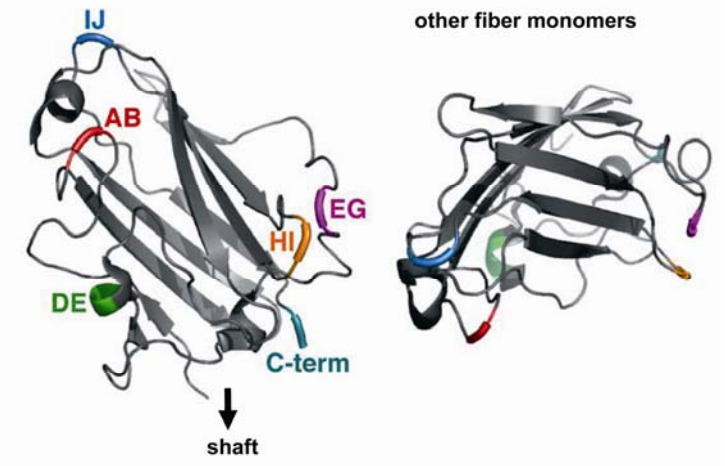


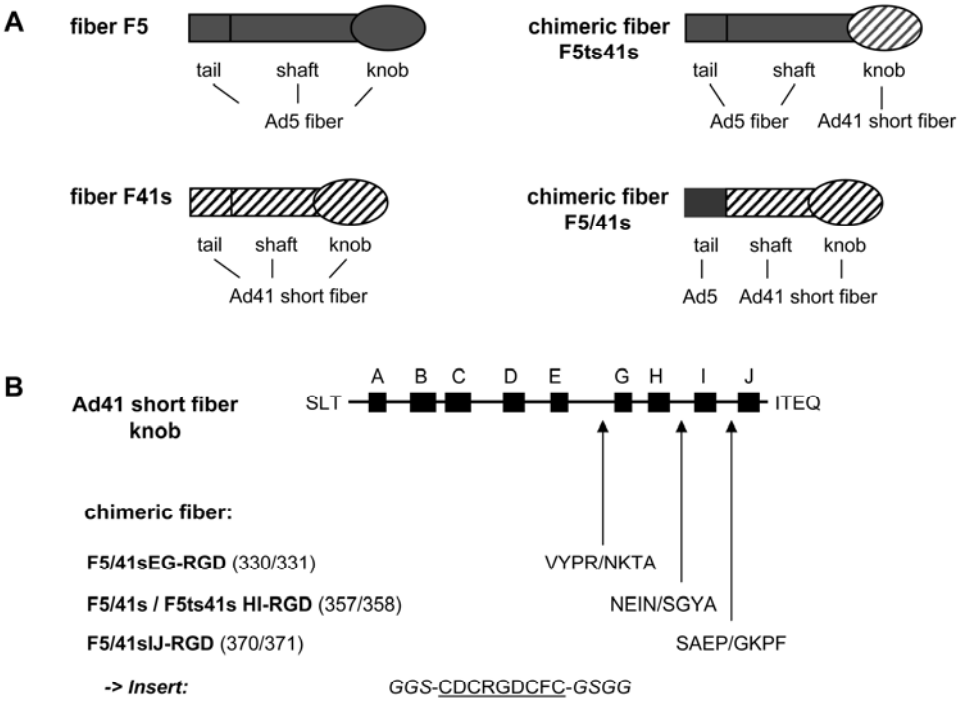
Fig.24: **Structure of the Ad41s fiber knob indicating the positions chosen for RGD insertion (Hesse et al., 2007).** (left panel) knob monomer seen from the side (its position relative to the fiber shaft is indicated); (right panel) knob monomer seen from the top.

Based on previous studies on functional insertion sites for a RGD model peptide into the Ad41 short fiber knob of replication-deficient adenovectors (Hesse et al. 2007; Fig. 24) I investigated (I) the F41s in context of oncolytic adenoviruses using a RGD model peptide and (ii) targeting cell entry by insertion of a described tumour cell-binding peptide ligand into this fiber format.

5.2.1 F41s-pseudotyped recombinant adenoviruses as platform for insertion of RGD model peptide

5.2.1.1 Generation of F41s-pseudotyped recombinant oncolytic adenoviruses

Towards the goal to engineer the capsid of an oncolytic virus for optimal ablation of the native tropism and insertion of the RGD model peptide, I generated F41s-pseudotyped oncolytic adenoviruses (Ad5/41sHI-RGD, Ad5/41sIJ-RGD, Ad5/41sEG-RGD) with genetic insertion of the RGD peptide into the EG, HI and IJ loops of the 41s knob domain. All recombinant adenoviruses code for chimeric F5/41s fiber constructs containing the tail domain of the Ad5 fiber fused to the shaft and knob domain of Ad41s fiber (Fig. 25). To investigate the role of the Ad41s shaft domain in context of oncolytic adenoviruses, a recombinant virus with a fiber construct matching Ad5/41sHI-RGD with the shaft domain of Ad5 fiber (Ad5ts41sHI-RGD) was generated. I hypothesize that both, length and flexibility of the shaft domain determines the efficiency and affinity of the interaction of ligands inserted into the fiber knob domain with their cellular receptors. The recombinant virus Ad5HI-RGD was produced as the corresponding control coding for the Ad5 fiber with the RGD model peptide insertion into the HI loop of the Ad5 knob domain. Concerning former reports that double mutants lacking both CAR- and integrin binding showed reduced liver tropism (Koizumi et al. 2003; Einfeld et al. 2001) I decided to mutate the integrin-binding motif within the virus penton base of Ad5/41sHI-RGD. My focus was to analyze this Ad5RAE41sHI-RGD virus regarding the efficiency of viral cytotoxicity and as further steps, after replacing the RGD model peptide by a tumour cell-binding peptide ligand, to investigate the cell-specificity. All viruses could be produced in 293 cells at high titers (Fig. 25C). However, the ratio of vp/ml versus $TCID_{50}$/ml of Ad5/41sHI-RGD and Ad5/41sEG-RGD was approximately 10-fold higher in comparison to the other viruses indicating a higher concentration of non-functional virus particles in these virus preparations. After production of Ad5RAE41sHI-RGD no further detailed analysis was done.



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virus	fiber format	penton base	vp / ml	TCID50 / ml
Ad5HI-RGD	F5	RGD	$6{,}27 \times 10^{12}$	$3{,}98 \times 10^{11}$
Ad5/41sEG-RGD	F5/41s	RGD	$1{,}78 \times 10^{12}$	$1{,}78 \times 10^{10}$
Ad5/41sHI-RGD	F5/41s	RGD	$3{,}13 \times 10^{12}$	$3{,}16 \times 10^{10}$
Ad5/41sIJ-RGD	F5/41s	RGD	$2{,}3 \times 10^{12}$	$1{,}58 \times 10^{11}$
Ad5ts41sHI-RGD	F5ts41s	RGD	$1{,}26 \times 10^{11}$	$1{,}99 \times 10^{10}$
Ad5RAE41sHI-RGD	F5/41s	RAE	$2{,}12 \times 10^{12}$	$3{,}55 \times 10^{10}$

Fig.25: **Generation of F41s-pseudotyped recombinant oncolytic adenoviruses carrying the RGD peptide**. (A) Schematic representation of adenovirus serotype 5 (F5), 41s (F41s) fiber and the chimeric fiber F5/41s and F5ts41s. To generate the chimeric fiber F5/41s the Ad5 fiber tail domain was fused to the Ad41s fiber shaft and knob domain. To generate the chimeric fiber F5ts41s the Ad5 fiber tail and shaft domain was fused to the Ad41s fiber knob domain. (B) Outline of RGD peptide insertions into various position of the Ad41s fiber knob. A CDCRGDCFC peptide with the indicated linkers (italic) was inserted into the EG, HI and IJ loop of Ad41s fiber knob (numbers in parenthesis refer to the amino acid positions of the Ad41s fiber that flank the insertion site; amino acid that flank the insertion site are shown in single letter code). (C) Tabular overview of the produced recombinant oncolytic viruses.

5.2.1.2 Incorporation of the chimeric fiber F5/41s with RGD peptide insertion into the capsid of recombinant viruses

For examination of chimeric fiber F5/41s incorporation into the viral capsid, 10^{10} virus particles of Ad5HI-RGD, Ad5/41sHI-RGD, Ad5/41sIJ-RGD, Ad5/41sEG-RGD, Ad5ts41sHI-RGD and Ad5RAE41sHI-RGD were boiled and analyzed by immunoblot analysis with mouse monoclonal antibody clone 4D2, which binds to the Ad5 fiber tail, and an anti-hexon antibody. As shown in Fig. 26, all recombinant fiber constructs

with the RGD peptide inserted into the HI, IJ or EG loop were incorporated into the viral capsid with similar efficiency.

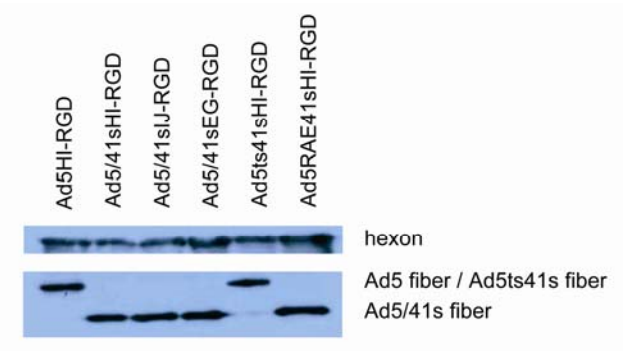

Fig.26: **Incorporation of the chimeric F5/41s fibers with RGD peptide insertion into the viral capsid.** 10^{10} purified particles of the indicated viruses were boiled and analyzed by SDS-polyacrylamide gel elcetrophoresis and western blotting with antibody 4D2 (lower panel) or with an anti-hexon antibody (upper panel) as a loading control. The calculated molecular mass of the F5/41s monomer is 42kDa and the F5 and F5ts41s monomer are 50kDa.

Notably, these results demonstrated that it is possible to insert peptides into Ad41s fiber knob in such a way that they face the top (IJ loop) or the side (EG and HI loop) without destroying the virus assembly including fiber incorporation. Almost similar amounts of the viral capsid protein hexon were detectable for the different viruses indicating that equal amount of virus particles were loaded.

5.2.1.3 Spread-dependent cell killing of chimeric fiber F5/41s viruses in melanoma cells

A cytotoxicity and spread assay was performed to analyze whether the chimeric fiber F5/41s has an influence on viral spread of the recombinant viruses in melanoma cells. Therefore, SK-MEL-28 and the CAR-negative cells C8161 and pMelL were infected with the total panel of recombinant viruses (Ad5HI-RGD, Ad5/41sHI-RGD, Ad5/41sIJ-RGD, Ad5/41sEG-RGD and Ad5ts41sHI-RGD) at virus titers of 0.01 to 1000vp/cell in 10-fold dilutions. The parental Ad5wt was used as replication control virus and the Ad5RGDLuc represents a replication deficient control virus. When lysis of melanoma cells was observed for the most potent virus at 1-0.1vp/cell, surviving cells were fixed and stained with crystal violet (Fig. 27). As expected, no cell killing was detectable for all cells infected with the replication deficient AdRGDLuc. Cytotoxicity was observed for all chimeric fiber F5/41s viruses and for Ad5HI-RGD. Of note, in principle genetic insertion of the RGD model peptide into the Ad41s knob domain of recombinant viruses is feasible without loss of viral spread. However, efficiency of cytotoxicity of chimeric fiber F5/41s viruses was reduced. In comparison to Ad5HI-RGD, cytopathic effect of Ad5/41sHI-RGD, Ad5/41sIJ-RGD and

Ad5/41sEG-RGD was 10-100 fold attenuated depending on the cell line. Independent from the insertion loop of RGD into Ad41s knob, almost similar level of cytotoxicity was shown for Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5/41sEG-RGD in the different melanoma cells. Comparable or 10-fold increased cell killing to the chimeric fiber F5/41s viruses (Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5/41sEG-RGD) was shown for Ad5ts41sHI-RGD.

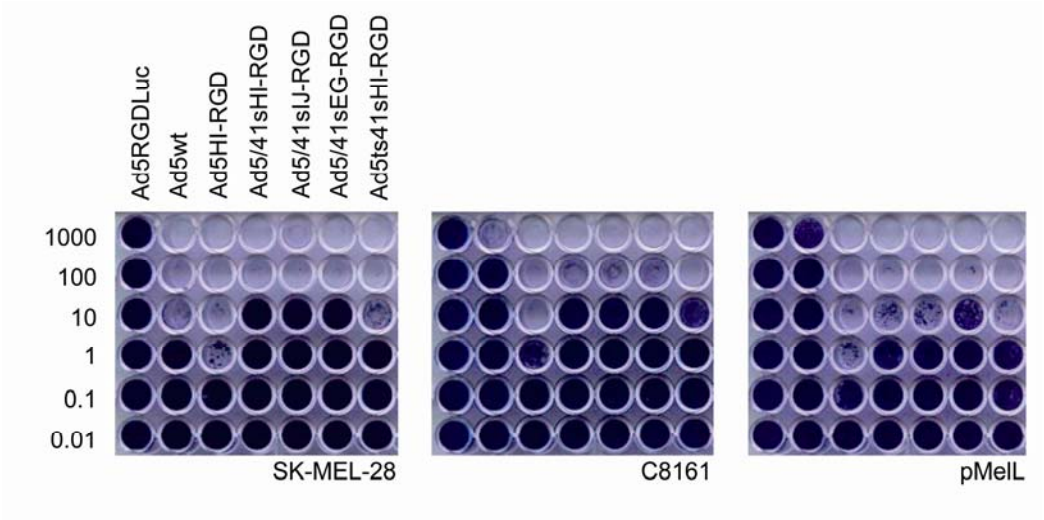

Fig.27: **Cytopathic effect of chimeric fiber F5/41s viruses in melanoma cells.** C8161, SK-MEL-28, melanoma cell lines; pMelL, low passage melanoma culture; cell cultures were infected with the indicated viruses at 1000vp/cell and 10-fold dilutions until 0.01vp/cell. Attached cells were stained with crystal violet when cell lysis was observed at 0.1vp/cell for each cell types individually. One representative experiment out of at least three is shown.

Low or no cytotoxicity of Ad5wt was detected in C8161 and pMelL due to the lack of the Adenovirus serotype 5 primary receptor CAR. Notably, cell entry of the total panel of recombinant viruses is mediated by the RGD motive inserted into the knob domain. In this case, viral cell entry is independent of the CAR receptor. As expected, potent cell killing of SK-MEL-28 was observed by Ad5wt resulting in a 10-fold stronger cytopathic effect than obtained with the chimeric fiber F5/41s viruses. Taken together, oncolysis by chimeric fiber F5/41s viruses is feasible in all tested cell lines. However, the lytic activity is reduced, independent of the insertion site, in comparison to Ad5HI-RGD.

5.2.1.4 Efficacy of replication of chimeric fiber F5/41s viruses in melanoma cells

To analyze the reason for the attenuation of viral spread by the recombinant oncolytic viruses cytotoxicity assay was performed. Therefore, viral replication of the recombinant viruses was studied over a time period of 7 days in melanoma cells. In this regard, the melanoma cell line C8161 was infected with the total panel of recombinant viruses (Ad5HI-RGD, Ad5/41sHI-RGD, Ad5/41sIJ-RGD, Ad5/41sEG-RGD and Ad5ts41sHI-RGD), the parental Ad5wt and a replication deficient virus Ad5RGDLuc at virus titers of 10vp/cell. Cells were harvested 3 h and 7 days post infection, DNA was purified and virus genome numbers were quantified by real time PCR.

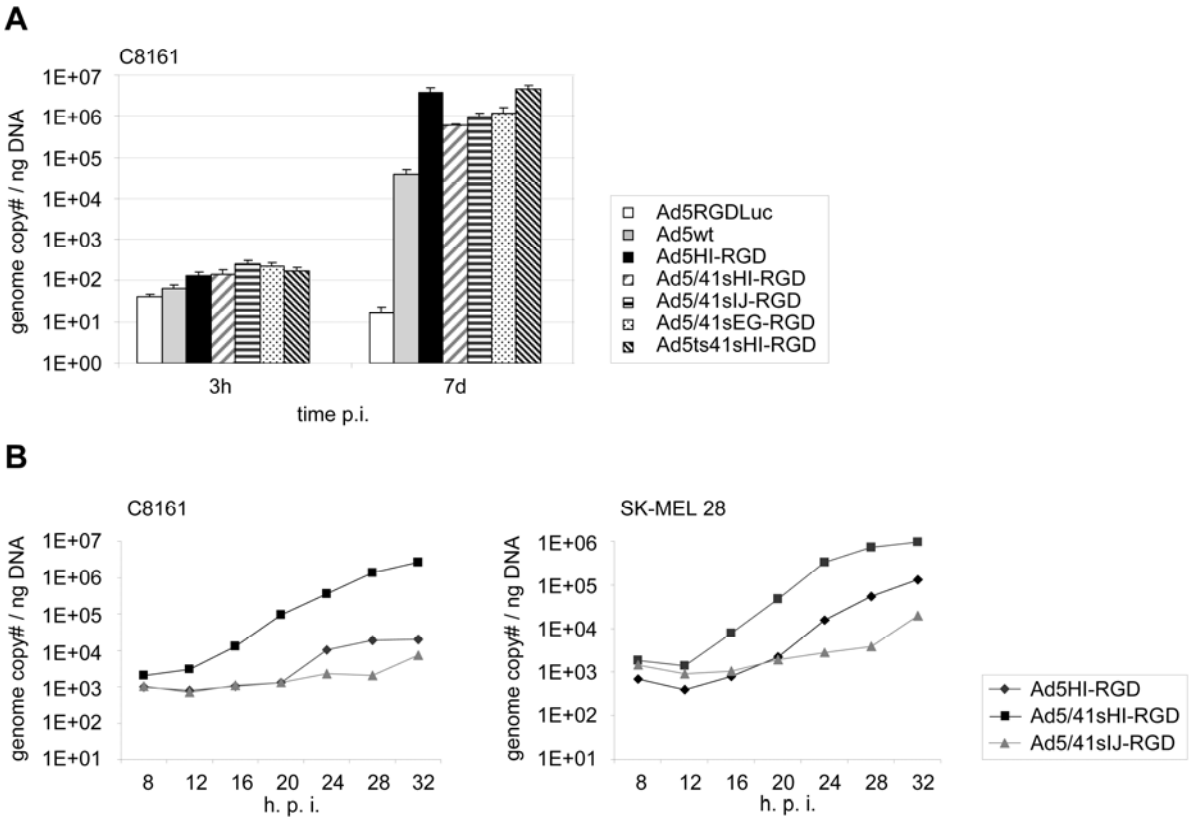

Fig.28: **Efficacy of replication of chimeric fiber F5/41s viruses in melanoma cells.** (A) To analyze adenovirus genome replication, C8161 cells were infected with the indicated viruses at 10vp/cell in triplicates. Three hours and seven days post infection DNA was purified and ratio of adenovirus genome copy numbers to nanograms cellular genomic DNA was determined by real time PCR. Columns show mean values. Error bars show standard deviation. One representative experiment out of three is shown. (B) For quantification of adenovirus genome copy numbers during the first replication cycle C8161 and SK-MEL-28 cells were infected with Ad5HI-RGD, Ad5/41sHI-RGD and Ad5/41sIJ-RGD at 10vp/cell. At 8, 12, 16, 20, 24, 28 and 32 h post infection genome copy numbers were determined by real time PCR and are shown after normalization with mass of cellular DNA. Experiments were repeated at least three times; one representative experiment is shown. p.i., post infection; h, hours.

As shown in Fig. 28A, genome copy numbers of the replication-deficient Ad5RGDLuc were nearly constant within one week revealing replication-independent background of virus genomes. As expected, genome copy numbers of all recombinant viruses dramatically increased from 3 h to 7 days post infection. Due to the lack of the primary receptor CAR on the surface of C8161 genome copy numbers for Ad5wt was 16-fold lower than for Ad5HI-RGD at day 7 indicating a lower genome amplification rate. Genome copy numbers of chimeric fiber F5/41s viruses (Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5/41sEG-RGD) were similar at day 7, which was lower than the genome copy numbers of Ad5HI-RGD but higher than Ad5wt. Of note, highest genome copy numbers were observed for Ad5HI-RGD and Ad5ts41sHI-RGD 7 days post infection.

To further investigate the cause for the fiber-dependent differences in virus spread, the amplification of the genome copy numbers during the first replication cycle was analyzed in more detail (Fig. 28B). Therefore, C8161 and SK-MEL-28 were infected with Ad5HI-RGD, Ad5/41sHI-RGD and Ad5/41sIJ-RGD at 10vp/cell. Values were quantified 8, 12, 16, 20, 24, 28 and 32 h post infection by real time PCR. Amplification of adenovirus genomes for Ad5HI-RGD, Ad5/41sHI-RGD and Ad5/41sIJ-RGD was detected in both melanoma cell lines. Genome copy numbers by Ad5/41sHI-RGD increased continuously over time with the sharpest increase between 12 and 28 h post infection resulting surprisingly in the highest genome copy numbers at all time points in both cell lines. The increase in genome copy numbers from 8 to 32 h was 1306-fold (Ad5/41sHI-RGD), 21-fold (Ad5HI-RGD) and 7-fold (Ad5/41sIJ-RGD) in C8161 and 531-fold (Ad5/41sHI-RGD), 187-fold (Ad5HI-RGD) and 13-fold (Ad5/41sIJ-RGD) in SK-MEL-28. Genome copy numbers of viruses Ad5HI-RGD and Ad5/41sIJ-RGD were nearly similar among each other from 8 to 20 h post infection in C8161 and showed the same level of genome copy numbers 20 h post infection in SK-MEL-28 indicating same input of genome copy numbers after viral cell entry. Genome copy numbers for Ad5HI-RGD increased significantly from 20 to 32 h post infection in comparison to Ad5/41sIJ-RGD. Notably, the differences of the efficacy of viral replication are dependent on the fiber format, but also the insertion loop of RGD into the Ad41s knob domain seems to play a critical role. Referring to this, the recombinant virus Ad5/41sHI-RGD showed the highest efficacy of genome replication whereas genome copy numbers increased only slightly for

Ad5/41sIJ-RGD coding both for the chimeric fiber F5/41s, but with different insertion sites for the RGD peptide.

5.2.1.5 Efficacy of late viral genes expression by chimeric fiber F5/41s viruses in melanoma cells

For Ad5/41sHI-RGD and Ad5/41sIJ-RGD, representing exemplary two varied chimeric fiber F5/41s viruses, efficacy of late genes expression was analyzed in comparison with Ad5HI-RGD over an extended time interval of 7 days after infection (Fig. 29A). For this purpose, C8161 were infected with Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5HI-RDG at 10vp/cell. 8 h and 7 days post infection cells were harvested; RNA was isolated and quantified by real time PCR. Expression of hexon mRNA of Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5HI-RDG increased from 8 h to 7 days post infection 80976, 263711, and 1472566-fold, respectively. Highest hexon messages were detected for Ad5HI-RGD at day 7 compared to Ad5/41sHI-RGD and Ad5/41sIJ-RGD. In regard to the investigation of cytotoxicity and viral replication, this result confirmed that chimeric fiber F5/41s influenced viral spread and consequently, led to the attenuation of cell killing, viral genome copies and late gene expression independently of the RGD insertion loop. Furthermore, I analyzed at what step of the viral replication cycle chimeric fiber F5/41s viruses were attenuated in comparison to Ad5HI-RGD. I quantified the level of late gene expression during the initial replication cycle. Therefore, SK-MEL-28 and C8161 were infected with Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5HI-RDG at 10vp/cell. Values were quantified 8, 12, 16, 20, 24, 28 and 32 h post infection by real time PCR. Hexon mRNA expression of the chimeric fiber F5/41s viruses was weak early after infection, as expected for a late gene, and from 8 to 20 h post infection almost similar to Ad5HI-RGD. However, expression of hexon messages by Ad5/41sHI-RGD increased dramatically from 20 to 32 h post infection resulting in the highest hexon expression over the total time period. The increase between 8 and 32 h was 241-fold or 15618-fold for Ad5/41sHI-RGD after infection of C8161 or SK-MEL-28, respectively. Increase in copy numbers of hexon mRNA by Ad5HI-RGD started 24 (C8161) or 28 h (SK-MEL-28) post infection. Nevertheless, they were significant attenuated 32 h post infection compared to Ad5/41sHI-RGD. This observation demonstrated a delayed onset of 4 or 8 h, depending on the cell line, of hexon expression from Ad5HI-RGD relative to Ad5/41sHI-RGD. Surprisingly, expression of hexon mRNA by Ad5/41sIJ-RGD was

only slightly higher as background measurement over the total time period. Overall, these patterns resembled the kinetic and efficacy of viral DNA replication by Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5HI-RDG.

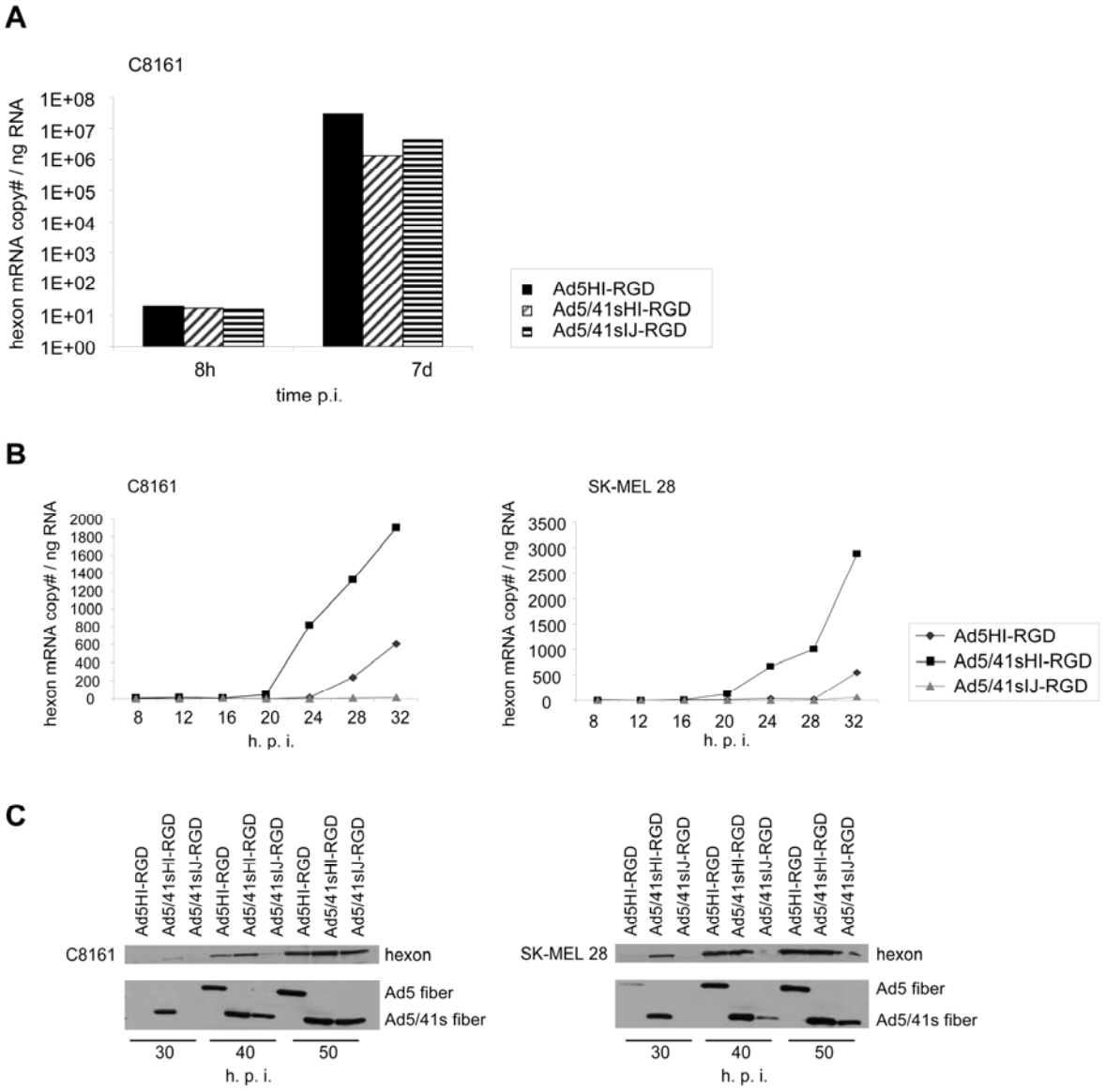

Fig.29: **Efficacy of late viral genes expression by chimeric fiber F5/41s viruses in melanoma cells.** (A) For quantification of messenger RNA copy numbers of hexon gene C8161 cells were infected with Ad5HI-RGD, Ad5/41sHI-RGD and Ad5/41sIJ-RGD at 10vp/cell. Eight hours and seven days post infection RNA was purified and ratio of RNA copy numbers to nanograms cellular RNA was determined by real time PCR. Experiments were repeated at least three times; one representative experiment is shown. (B) Melanoma cell lines were infected with indicated viruses at 10vp/cell. At 8, 12, 16, 20, 24, 28 and 32 h post infection cells were harvested for RNA purification to determine hexon mRNA numbers by real time PCR. Data are presented as hexon mRNA copy numbers / ng cellular RNA. Experiments were repeated at least three times; one representative experiment is shown. (C) C8161 and SK-MEL-28 were infected with Ad5HI-RGD, Ad5/41sHI-RGD and Ad5/41sIJ-RGD at 100vp/cell. 30, 40 and 50 hours after infection, cells were harvested and total cell lysate was used to perform immunoblots for hexon and fiber detection. The calculated molecular mass of the F5/41s monomer is 42kDa, the F5 monomer is 50kDa and the hexon monomer is 105kDa.

Next, I assessed the efficiency of late viral gene expression by detection of fiber and hexon protein by immunoblotting (Fig. 29C). In this regard, SK-MEL-28 and C8161 were infected with Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5HI-RDG at 100vp/cell. 30, 40 and 50 h post infection cells were harvested, total cell lysates were applied for SDS gel electrophoresis, followed by immunoblotting and detection of viral fiber and hexon proteins. The results demonstrated strong expression of fiber for Ad5/41sHI-RGD at all time points. Delayed fiber protein expression levels compared to Ad5/41sHI-RGD were observed for Ad5HI-RGD and Ad5/41sIJ-RGD. 30 h post infection only weak or no expression of fiber was detectable for Ad5HI-RGD and Ad5/41sIJ-RGD. At later time points expression of fiber protein was similar when infected with Ad5HI-RGD and Ad5/41sHI-RGD. Low fiber expression was observed at 40 and 50 h post infected for Ad5/41sIJ-RGD in SK-MEL-28. Expression of hexon protein of Ad5/41sHI-RGD, Ad5/41sIJ-RGD and Ad5HI-RDG at all 3 time points matched those of the expression of fiber protein.

In summary, I concluded that late viral gene expression was affected by the fiber format and the RGD model peptide insertion site. However, the expression of hexon and fiber by Ad5/41sHI-RGD was still expressed with earlier kinetics compared to Ad5HI-RGD and Ad5/41sIJ-RGD. Therefore, reduced viral spread and toxicity could not be explained by reduced / delayed genome replication or gene expression for Ad5/41sHI-RGD.

5.2.1.6 Effect of chimeric fiber F5/41s on progeny production and viral release

Next, I investigated whether the observed effects of chimeric fiber F5/41s on viral spread and cell killing were the result of a reduction of viral progeny production or release after infection of chimeric fiber F5/41s viruses (Fig. 30). For this purpose, SK-MEL-28 cells were infected with Ad5/41sHI-RGD and Ad5HI-RDG. 2, 3 and 4 days post infection supernatants and cells were harvested separately and cell lysates were prepared by three cycles of freezing and thawing. Serial dilutions of the lysates were then titered on 293 cells using the $TCID_{50}$ assay. Surprisingly, production of total infectious particles for Ad5HI-RGD was significantly higher compared to Ad5/41sHI-RGD at day 2, 3 and 4 post infections. The Ad5HI-RGD infection resulted in an approximately 10-fold increase in total virus particle production and an earlier onset of viral release concluding from the lower ratio $TCID_{50}$/ml_cells / $TCID_{50}$/ml_supernatant of Ad5HI-RGD in comparison to Ad5/41sHI-RGD at day 2

and 3 post infection. These results argue that the fiber format has an influence on production of infectious virus particles and viral release. Finally, this observation could be an explanation for the attenuation of viral spread and cell killing by chimeric fiber F5/41s viruses.

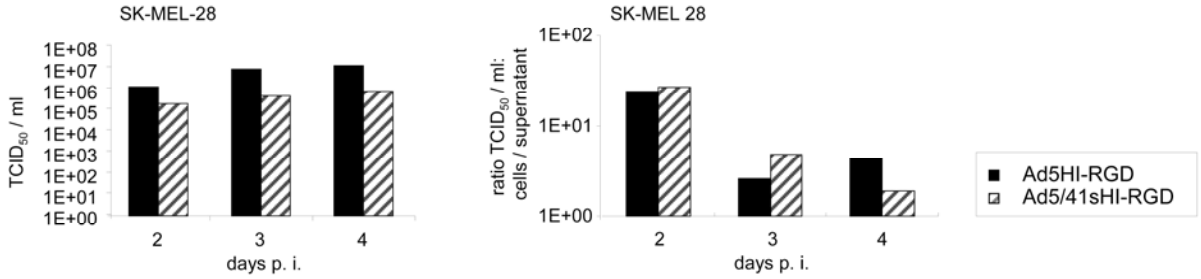

Fig.30: **Effect of chimeric fiber F5/41s on viral progeny production and release.** SK-MEL-28 cells were infected with Ad5HI-RGD and Ad5/41sHI-RGD at 10vp/cell. Two, three and four days post infection, supernatants and cells were harvested separately and titers of infectious viral particles were determined by $TCID_{50}$ assay. Left panel shows total infectious viral particles (cells + supernatants). Right panel shows the ratio of infectious viral particles in cells to infectious viral particles in supernatants to indicate virus release. Experiments were repeated at least two times; one representative experiment is shown. p.i., post infection.

5.2.1.7 Thermal stability of chimeric fiber F5/41s viral capsids

To assess the thermal stability of the chimeric fiber viruses as a potential cause of reduced virus titers of Ad5/41sHI-RGD in comparison to Ad5HI-RGD and to investigated the relation of stability of viral capsid and viral spread, Ad5/41sHI-RGD and Ad5HI-RGD were incubated for 0, 12 or 24 minutes at 45°C.

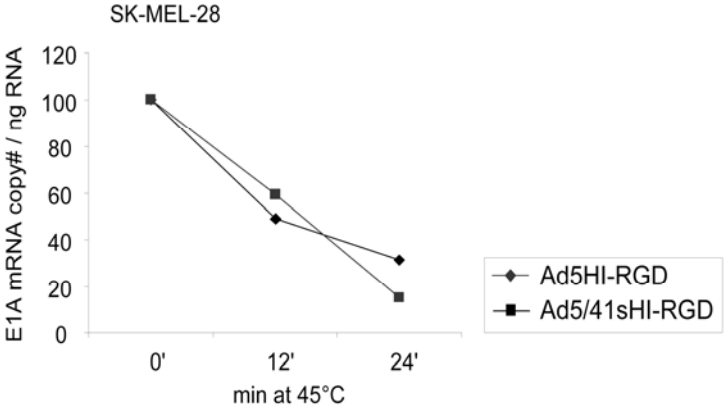

Fig.31: **Thermal stability of chimeric fiber F5/41s viral capsids.** To determine the thermal stability of chimeric fiber F5/41s, 1000vp/cell of Ad5HI-RGD and Ad5/41sHI-RGD were preincubated for 0, 12, or 24 minutes at 45°C. Subsequently, SK-MEL-28 cells were infected with the heat-treated viruses. 12 h post infection cells were harvested for RNA purification to determine E1A mRNA numbers by real time PCR. Data are presented as E1A mRNA copy numbers / ng cellular RNA and obtained values are normalized with readings for untreated. Experiment was repeated at least two times.

Afterwards, SK-MEL-28 cells were infected with these viruses and 12 h post infection cells were harvested, RNA was purified and expression of the E1A gene was analysed by quantitative real time PCR. The ability of the virus to enter the cell and express the early gene E1A required a functional viral capsid structure. Consequently, the efficacy of E1A expression correlates with the stability and viability of the heat-treated virus particles. The level of E1A copy numbers correlated inversely with time of treatment. However, only minor differences of E1A expression by Ad5/41sHI-RGD and Ad5HI-RGD were shown. In conclusion, the fiber format has no major influence on the thermal stability of the virus particles.

5.2.2 F41s-pseudotyped recombinant adenoviruses as platform for insertion of a tumour cell-binding peptide ligand that binds to the EphA2 receptor

5.2.2.1 Generation of F41s-pseudotyped recombinant adenoviruses incorporated an EphA2 ligand peptide into the HI loop of the Ad41s knob domain

This work was based on results of our lab that a highly affinitive peptide that binds to EphA2 receptor (Zelinski et al. 2001) could be used for specific transduction of replication-deficient adenovirus vectors. In the light of these results, I investigated the lytic efficiency and specificity of oncolytic adenoviruses engineered with an EphA2 ligand peptide inserted into the HI loop of the Ad41 fiber knob (Fig. 32).

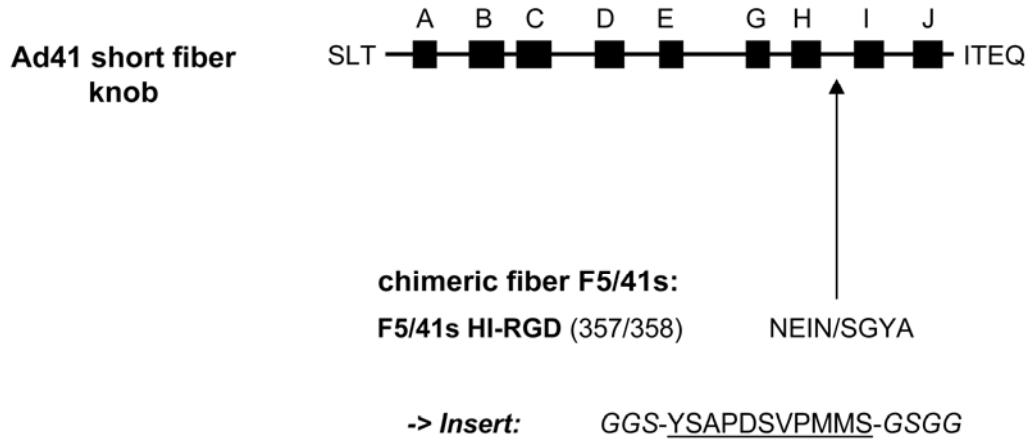

Fig.32: **Generation of F41s-pseudotyped recombinant oncolytic adenoviruses carrying an EphA2 peptide ligand**. Outline of EphA2 peptide ligand insertions into the Ad41s fiber knob. An YSAPDSVPMMS peptide was inserted into the HI loop of Ad41s fiber knob (numbers in parenthesis refer to the amino acid positions of the Ad41s fiber that flank the insertion site; amino acid that flank the insertion site are shown in single letter code).

In this regard, the model peptide RGD was replaced by a tumour cell-binding EphA2 ligand peptide generating the oncolytic adenovirus Ad5/41sHI-ephA2. Additional, I investigated whether the native cellular tropism could be further ablated, beside the F5/41s fiber format, by mutation of the integrin-binding motifs in the penton base (double-ablated virus Ad5RAE/41sHI-ephA2). To generate the F41s-pseudotyped oncolytic adenoviruses as tool for viro- and gene therapy the E3 region was already deleted to obtain space for future transgene insertion.

5.2.2.2 Spread-dependent cell killing by the EphA2-targeted chimeric fiber F5/41s viruses

Due to former surface expression analysis of the EphA2 receptor on human melanoma cell lines, the CAR-negative cell line C8161 were suitable as a model to study viral targeting. Since the EphA2 receptor is absent on SK-MEL-28, I used these as negative control cells. For the analysis of the cytolytic activity and the specificity of cell lysis of the targeted chimeric fiber F5/41s viruses, C8161 and SK-MEL-28 were infected with Ad5/41sHI-ephA2 and Ad5RAE/41sHI-ephA2 at virus titer of 1000-0.01vp/cell in 10-fold dilutions. Ad5wt, Ad5Δ24E3-, Ad5/41sHI-RGD and the replication-deficient virus Ad5CMVLuc served as control viruses. Cell killing was detected by staining of viable cells with crystal violet (Fig. 33).

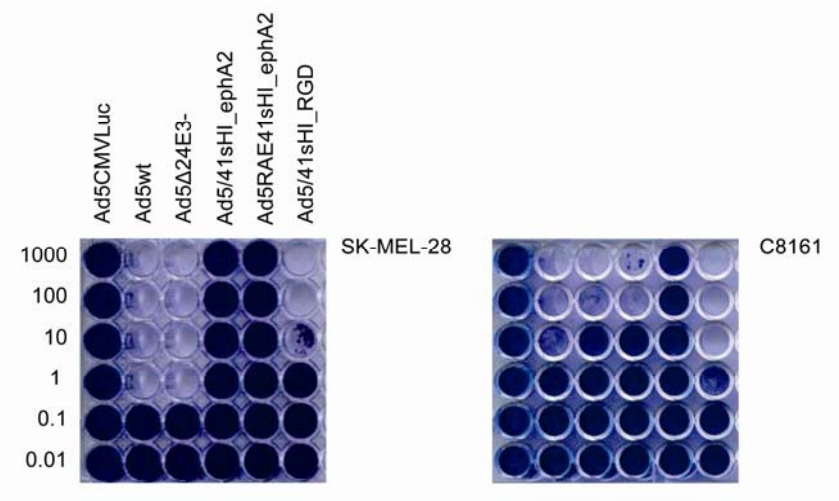

Fig.33: **Spread-dependent cell killing by the targeted chimeric fiber F5/41s viruses.** SK-MEL-28 and C8161 cells were infected with the indicated viruses at 1000-0.01vp/cells. Virus spread-dependent cytotoxicity was visualized by crystal violet staining of surviving cells. Therefore, staining was performed when cytotoxicity was observed for the most potent virus at 0.1vp/cell (SK-MEL-28) or 1vp/cell (C8161). Experiments were repeated at least two times; one representative experiment is shown.

Cytotoxicity by Ad5/41sHI-ephA2 was observed in C8161. However, Ad5RAE41sHI-ephA2 showed no cell killing of C8161 indicating that penton-integrin interaction is needed. Most potent cytotoxicity in C8161 cells was observed for Ad5/41sHI-RGD, which was approximately 10 to 100 fold enhanced when compared to Ad5/41sHI-ephA2. Of note, Ad5wt and Ad5Δ24E3- were 10-fold superior or identical to Ad5/41sHI-ephA2 in cell killing of C8161. This was not expected due to the low expression of the CAR-receptor on C8161. As expected, cell killing was observed for SK-MEL-28 infected with Ad5wt, Ad5Δ24E3- and Ad5/41sHI-RGD confirming my analysis of spread-dependent cell killing of chimeric fiber F5/41s viruses (see chapter 5.2.1.3). Of note, EphA2 receptor-deficient SK-MEL-28 cells showed no lysis by Ad5/41sHI-ephA2 and Ad5RAE/41sHI-ephA2 indicating specific cell lysis of EphA2 positive cells. Taken together, targeted chimeric fiber F5/41s virus Ad5/41sHI-ephA2 clearly demonstrated specific cell killing, though attenuated compared to Ad5/41sHI-RGD. For Ad5RAE/41sHI-ephA2 even no cell killing of C8161 was observed.

5.2.2.3 Efficacy and specificity of the late fiber gene expression by the EphA2-targeted chimeric fiber F5/41s viruses

Towards the goal to engineer highly potent and tumour specific viruses, the efficacy and specificity of the late fiber gene expression by the targeted chimeric fiber F5/41s viruses were analyzed. For this purpose, SK-MEL-28 and C8161 were infected with Ad5/41sHI-ephA2, Ad5RAE/41sHI-ephA2, Ad5/41sHI-RGD and Ad5Δ24E3-. 40 h post infection cells were harvested, total cell lysates were applied for SDS gel electrophoresis, followed by immunoblotting and detection of viral fiber (Fig. 34).

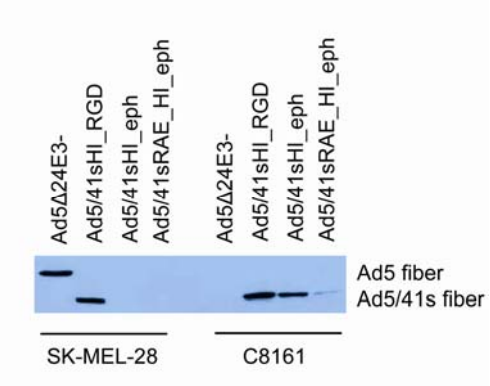

Fig.34: **Efficacy and specificity of late fiber gene expression by the targeted chimeric fiber F5/41s viruses.** Melanoma cells were infected with the indicated viruses at 100vp/cell. Cells were lysed at 40 h post infection and were analyzed for fiber expression by SDS-polyacrylamide gel electrophoresis and western blotting using monoclonal antibody clone 4D2, which binds to the Ad5 fiber tail. Lysates with 30µg of protein were boiled before being loaded. The calculated molecular mass of the F5/41s monomer is 42kDa and the F5 monomer is 50kDa.

For all chimeric fiber F5/41s viruses (Ad5/41sHI-ephA2, Ad5RAE/41sHI-ephA2 and Ad5/41sHI-RGD), infection of C8161 cells resulted in fiber expression. However, the efficacy of fiber scaled down from Ad5/41sHI-RGD to Ad5/41sHI-ephA2 to Ad5RAE/41sHI-ephA2. Consequently, merely low fiber expression was observed for Ad5RAE/41sHI-ephA2 in C8161. As expected, no fiber expression was found for CAR-negative C8161 cells infected with Ad5Δ24E3-, which binds to cells via CAR receptor. Furthermore, the results demonstrated strong expression of fiber in SK-MEL-28 infected with Ad5Δ24E3- and Ad5/41sHI-RGD. In contrast, no fiber expression was detected after infection of the EphA2-negative cell line SK-MEL-28 by Ad5/41sHI-ephA2 and Ad5RAE/41sHI-ephA2 confirming the analysis of cytotoxicity in SK-MEL-28 (see chapter 5.2.2.2). To conclude, these data demonstrated an efficient and specific fiber expression mediated by Ad5/41sHI-ephA2 EphA2-positive C8161 cells. Late fiber gene expression by the targeted Ad5RAE/41sHI-ephA2 was selective for C8161 but less efficient than for Ad5/41sHI-ephA2.

6 DISCUSSION

6.1 Combination of oncolysis with targeted prodrug activation therapy of melanoma

The development of tumour-specific drugs and the implementation of potent multimodal treatment regimens are of critical importance for cancer therapy. Oncolytic adenoviruses have the potential to realize both tumour-specificity and multimodality by restricting virus replication to tumour cells and by insertion of therapeutic genes into the virus genome, respectively. Using the luciferase gene as a sensitive reporter for monitoring transgene expression, I showed in this study that expression of E1A from a cell-type-specific promoter not only mediates targeted virus replication, but also facilitates the expression of transgenes inserted in the late transcription unit with remarkable selectivity (selectivity up to 1500-fold). Notably, I revealed that selectivity and efficiency of transgene expression depend on the strategy of transgene insertion into the late transcription unit and might require optimization for individual transgenes. Specifically, my results demonstrated that both the IRES and SA strategies of transgene insertion facilitate indirect transcriptional targeting of transgene expression by tyrosinase promoter-dependent expression of E1A without interfering with virus replication. When comparing melanoma cells with fibroblasts and keratinocytes, the SA strategy showed highest selectivity of up to 1500-fold (Fig. 12); for the IRES strategy selectivity was up to 450-fold. However, the IRES strategy mediated approx. 10-fold stronger transgene expression. In consequence, I suggest the IRES strategy for expression of therapeutic genes for which high expression levels are necessary in order to reach therapeutic efficacy, whereas the SA strategy is especially advantageous whenever side effects of therapeutic genes are limiting. These “add-in” strategies for transgene insertion into the adenoviral genome, in contrast to replacing late viral genes with transgenes (Nettelbeck 2009), in principle allow for retaining all viral genes – at least if transgene sequences are short (the viruses used in this study were E3-deleted due to the large size of the luciferase gene). Both the EMCV IRES and different splice acceptor sequences have been previously used for late therapeutic gene expression (Sauthoff et al. 2002 and 2006; Fuerer and Iggo 2004; Lukashev et al. 2005; Carette et al. 2005; Robinson et al. 2007; Cascante et al. 2007). My results suggest that comparing both strategies in therapeutic studies might indeed be of interest to improve a given therapeutic approach.

Lower transgene expression by the SA strategy (Fig. 11/13) observed in my study might reflect the locale of transgene insertion between the E4 genes and right ITR of the adenovirus genome. Other groups have inserted transgenes with different splice acceptor sites directly downstream of the fiber gene (Fuerer and Iggo 2004; Cascante et al. 2007). Fuerer and Iggo reported that therapeutic gene expression by the EMCV IRES resulted in stronger gene expression than the splice acceptor sequence of the Ad41 long fiber, when both were inserted at the end of the L5 transcript (Fuerer and Iggo 2004). It remains to be investigated in direct comparisons whether this fiber locale results in more efficient transgene expression than the E4 site and whether the selectivity I reported in the present study is dependent on the specific splice acceptor sequence used. However, in a transposon-based scan for insertion sites for SA-transgene cassettes in the adenovirus genome, Hermiston and colleagues reported that an E4 insertion site, similar to the one I used, afforded strongest transgene expression and reflected late transgene expression kinetics best (Jin et al. 2005).

An additional important finding of my study with respect to the necessity to optimize transgene insertion into oncolytic adenovirus genomes was that transgenes can interfere in a sequence-specific manner with splicing (Fig. 19A). Thus, efficient transgene expression was lost by replacing the luciferase cDNA of SL viruses with the *FCU-1* gene or the therapeutic gene *purine nucleoside phosphorylase* (data not shown). Interestingly, this was not only due to insufficient activity of the inserted SA sequence (caused by a different sequence downstream of the SA site), but was also resulting from usage of a cryptic splice acceptor site within the transgene (Fig. 19B). In this regard it is important to note that the SA sequence used in my study, containing branch point and polypyrimidine tract elements, was already optimized for more “tight” splicing than shorter sequences (Jin et al. 2005). Having revealed the reasons for ineffective splicing, I could restore *FCU-1* expression by insertion of a luciferase gene-derived spacer downstream of the SA sequence in combination with switching codons in and around the identified cryptic splice site. The former might restore a favourable sequence environment around the SA site. One could envision that cryptic splicing of transgenes could also interfere with viral gene expression and thus with virus replication, although this was not the case for the viruses generated in this study (Fig. 17). These results suggest that, when “arming” viruses with therapeutic genes, it is advisable to perform splicing analyses.

To optimize transgene insertion into oncolytic adenovirus genomes, I also investigated a viral 2A sequence as a tool for co-expression of two or more proteins from one transcription unit (Szymczak et al. 2004). I showed that the short 2A sequence of the *Thosea asigna* virus facilitates replication-dependent co-expression of adenoviral fiber and luciferase proteins as observed via inhibition of virus replication with AraC, with no fusion protein detectable (data not shown). However, my data also revealed that indirect promoter control was not effective using the 2A strategy, as Ad5TyrTL, in contrast to Ad5TyrIL and Ad5TyrSL, showed strong luciferase expression in fibroblasts and keratinocytes (Fig. 12). Thus, an important result of my study is that replication-dependence of transgene expression, as demonstrated using for example by AraC block, does not always correctly predict selectivity of transgene expression by targeted oncolytic adenoviruses. In consequence, it is necessary to investigate selectivity of armed oncolytic adenoviruses in a biologically relevant system, e.g. in both target and non-target cells, with an appropriate control of a matching but non-targeted, transgene-encoding virus. This was often not done in previous studies. For reasons that remain to be determined, the 2A strategy also resulted in reduced E1A expression, replication and transgene expression for the Ad5TL virus, but not the Ad5TyrTL virus. Iggo and co-workers recently reported feasibility of 2A sequences derived from the foot-and-mouth disease virus (F2A) and from the porcine teschovirus-1 (P2A) for co-expression of GFP with the adenoviral pIX (Funston et al. 2008). They also observed reduced replication and spread for the 2A viruses, which they attributed to interference with pIX activity of the 2A-derived amino acids that remain in the pIX protein. In our study both Ad5TL and Ad5TyrTL feature the same fiber modification but a different replication phenotype. Thus, interference of the 2A-derived amino acids with capsid stability or virus entry into cells is unlikely. I conclude that the 2A strategy, facilitating efficient co-expression in other systems (Szymczak et al. 2004; Fang et al. 2005), can affect both replication and targeted transgene expression in armed oncolytic adenoviruses.

Iggo and co-workers previously investigated selective expression of therapeutic genes inserted via IRES or splice site into the viral late transcription unit of an adenovirus transcriptionally targeted to colon cancer via TCF4 transcription factor binding sites in E1 and E4 promoters (Fuerer and Iggo, 2004; Lukashev et al. 2005). Comparing tumour and normal cells, they could also demonstrate tumour-selective

expression of the therapeutic protein by immunoblot; however, they did not quantify the level of specificity. Other studies reporting late therapeutic gene insertion into transcriptionally targeted oncolytic adenoviruses frequently lacked a proof of indirect promoter regulation of transgene expression.

I reported that the SA strategy, after optimizing splice-dependent *FCU-1* expression, facilitates the transcriptionally targeted combination therapy of efficient oncolysis and molecular chemotherapy. When the prodrug 5-FC was added during the first virus replication cycle after infection with Ad5TyrSsp_mFCU, production of infectious virus particles was only modestly reduced by less than 10-fold (Fig. 21), showing that late expression of *CD-FUR-1* in the presence of 5-FC is compatible with virus replication, which is in agreement with previous reports in other tumour cells (Fuerrer and Iggo, 2004). Importantly, cytotoxicity of the oncolytic virus was strongly increased by 5-FC in a melanoma-specific manner and was superior to Ad5CMVFCU treatment in melanoma cells; for Mel624 cells this difference was quite remarkable, more than 30-fold (Fig. 22). Interestingly, I observed that Ad5TyrSsp_mFCU showed higher *CD-FUR-1* fusion protein expression in melanoma cells than Ad5Ssp_mFCU (Fig. 19A), which translated in stronger sensitization to the prodrug 5-FC. This was in agreement with the results of luciferase reporter assays for IL and SL viruses, that also showed stronger luciferase expression when E1A was expressed from the tyrosinase promoter (Fig. 11). E1A expression kinetics of Ad5SL and Ad5TyrSL were similar during the first 30 h after infection of melanoma cells, the reason for this difference is not clear and might be due to different expression kinetics at later times. Whereas my results clearly establish the efficacy and specificity of the Ad5TyrSsp_mFCU/5-FC therapy for melanoma, there is further opportunity for improvement. I always added the prodrug during the first virus replication cycle after infection in order to show the selectivity of the system. However, an important advantage of the genetic prodrug activation therapy is that kinetics of gene transfer and prodrug application can be optimized. This is especially of interest for armed oncolytic viruses: both efficacy and selectivity of oncolytic virus-delivered genetic prodrug therapy can be improved by adding the prodrug later after infection, allowing for (tumour-specific) virus spread and thus more widespread expression of the prodrug convertase. My observation that supernatants of Ad5TyrSsp_mFCU/5-FC-treated melanoma cells, after virus-inactivation, can efficiently kill uninfected melanoma cells demonstrated the potential of the system for effective prodrug conversion and for killing of uninfected “bystander”

tumour cells. Again, this is of critical importance for combination therapies with "armed" oncolytic adenovirus, as the killing of uninfected cells is the goal of the arming strategy. Previous studies investigated oncolytic adenoviruses expressing *E. coli CD* directly from tyrosinase promoters, either by expressing E1A and *CD* from two identical copies of the promoter (Liu and Deisseroth, 2006) or by expressing a *CD*-IRES-E1A fusion gene (Liu et al. 2006). In agreement with my results, these studies also reported synergy between adenoviral oncolysis and CD/5-FC therapy. However, the virus described in the present study possesses qualities that differ from these viruses in several aspects. It encodes the yeast *CD-FUR-1* fusion protein, which has been reported to be a more effective prodrug convertase (Erbs et al. 2000), which goes along with the efficient bystander effect we observed. This protein is expressed with late kinetics, which is advantageous to prevent interference with viral replication. A single copy of the tyrosinase promoter ensures stability of the virus genome by avoiding homologous recombination. Direct control and thus strong expression of E1A ensures efficient virus replication and oncolysis, in contrast to the CD-IRES-E1A strategy, while indirect control of late gene expression still facilitates melanoma-specific expression of the *CD-FUR-1* fusion protein.

5-FU is not used in clinical routine for chemotherapy of malignant melanoma. However, I believe that local application of the drug at high doses, as I proposed by targeted prodrug activation after infection with "armed" oncolytic adenoviruses, holds promise as a novel regimen for targeted treatment of malignant melanoma. This is in agreement with previous reports on CD/5-FC gene therapy by non-viral, viral, bacterial or cellular vectors (Szala et al. 1996; Cao et al. 1998; Aboody et al. 2006; Stritzker et al. 2008; Kucerova et al. 2008). Moreover, several studies indicate that treatment of melanoma with 5-FU improves cellular natural or therapeutic immune responses to the tumour (Ryan et al. 1988; Neefe and Glass, 1991; Gazit et al. 1992; Cao et al. 1998; Yang and Haluska, 2004). In this regard, activation of prolonged anti-tumour immunity by oncolytic adenoviruses is considered a promising strategy to complement viral tumour cell lysis for increased therapeutic potency.

In summary, my study established Ad5TyrSsp_mFCU/5-FC as an effective strategy for targeted killing of melanoma cells by combined viral oncolysis and molecular chemotherapy. I reported several steps for genetic engineering of the therapeutic virus that were required to optimize late therapeutic gene expression. This, in turn,

facilitated targeting of both viral replication and therapeutic gene expression to melanoma cells from the cellular tyrosinase promoter.

6.2 Peptide-dependent cell entry for targeted oncolysis

6.2.1 Ad41 short fiber as platform for insertion of RGD model peptide in context of oncolytic adenoviruses

Recombinant adenoviruses have emerged as promising agents in therapeutic gene transfer, genetic vaccination, and viral oncolysis (Alemany and Curiel et al. 2000; Basak et al. 2004; McConnell et al. 2004; Zang et al. 1999). However, therapeutic applications of adenoviruses would benefit substantially from targeted virus cell entry. Notably, all oncolytic adenoviruses described to date are based on post-entry restriction of adenoviral life cycle. To target viral oncolysis by a specific cell entry genetic modification is needed. Such modification of the natural adenoviral tropism requires the deletion of the natural cell binding properties and achievement of tumour specific targeting for example by incorporation of cancer cell binding ligands. Recently, the replacement of the Ad5 fiber with the short fiber of subgroup F adenovirus Ad40 and Ad41 has been shown to achieve substantially reduced transduction of the liver and other organs after intravenous injection into mice and rats (Nakamura et al. 2003; Nicol et al. 2004). Therefore, the short fiber of Ad40 and Ad41 represent promising “detargeted” platforms for retargeting of adenovirus cell entry by insertion of specific peptide ligands. It was previously shown by our group that peptide ligand insertions into various positions of the Ad41s knob domain is feasible resulting in molecules that retain both fiber / capsid integrity and the potency of the inserted peptide to bind to its cellular receptor (Hesse et al. 2007). Of note, this was the first study that reported the feasibility of internal ligand insertions into a short-shafted adenovirus fiber. In this regard, I investigated the short fiber of Ad41 (F41s) in context of oncolytic adenoviruses by using the integrin-binding motif RGD as a prototype of the cell binding ligand. I inserted the model peptide RGD into the Ad41s fiber knob in such a way that they face the top (IJ loop) or the side (EG and HI loop) of the fiber molecule (Fig. 24). The production and amplification of the recombinant viruses represents a critical step towards the implementation of viral targeting. Referring to this, I retained that the conservation of the Ad5 fiber tail in my recombinant adenoviruses might lead to a more efficient incorporation of the fiber chimera with Ad5 tail fused to Ad41 shaft and knob domains into the Ad5 capsid (Hesse et al. 2007). All recombinant viruses were able to be produced at high titers

(Fig. 25C). According to the successful viral production, the chimeric fiber retains the fiber's essential properties of trimerization, incorporation into adenovirus capsids and binding to its cellular receptor which is important for viral spread and amplification. Together with the western blot data (Fig. 26) my results indicate that all fibers can be incorporated into the viral capsid with efficiency comparable to Ad5wt.

It was shown previously (de Jong et al. 1983) that the subgroup F adenoviruses grow poorly in cell cultures that are highly permissive to other human adenoviruses. It is possible that the unique property of enteric adenoviruses missing the RGD motif in the penton base (Albinsson et al. 1999) might be related to the special ability of these viruses to mainly infect cells in the human gastric system with high efficiency. In my study, all recombinant viruses code for the chimeric fiber F5/41 containing an RGD motif in the Ad5 tail and in addition in the Ad41s knob domain. Though, the cytotoxicity of all chimeric fiber F5/41s viruses was 10-100 fold attenuated in comparison to the corresponding virus Ad5HI-RGD with the same ligand inserted into the Ad5 fiber in the tested melanoma cells (Fig. 27). This finding suggests that the presence of the Ad41s shaft and knob domain irrespective of the RGD insertion site has a negative effect on the lytic potency and spread of the chimeric fiber F5/41s viruses. At which step of the viral replication cycle this effect could take place needs to be further characterized. My results argue that the reduced lytic potency of the chimeric fiber F5/41s viruses is independent from the insertion site of the model ligand RGD into the Ad41s fiber knob. The analysis of viral gene expression and replication of the viral genome during the first replication cycle demonstrated that infection by Ad5/41sHI-RGD resulted in the earliest onset of viral replication and in the sharpest increase of genome copy numbers over the time period (Fig. 28B). In this regard, an attenuated transactivation capacity of the E1A proteins resulting from reduced replication efficiency was not observed for Ad5/41sHI-RGD in comparison to Ad5HI-RGD. In contrast, genome copy numbers of Ad5/41sIJ-RGD showed only a slightly increase from 8 to 32 h post infection and in comparison to Ad5/41sHI-RGD and Ad5HI-RGD the lowest replication rate (Fig. 28B). This is surprising, especially in consideration of the fiber format F5/41s which is used by both viruses, Ad5/41sHI-RGD and Ad5/41sIJ-RGD, for cell entry. I conclude that the efficacy of viral replication is dependent on the fiber format, but also the insertion site of RGD into the Ad41s knob domain seems to play a critical role. Note that the HI loop is located on the side and the IJ loop face the top of the Ad41s fiber knob. I hypothesize that the

insertion of the model peptide RGD into different orientated loops of the Ad41s fiber knob domain might influence the binding behaviour and / or the cellular signalling which could affect viral replication. In previous study it was shown that loops located on the side (HI and EG loop) might be a favourable location for insertion of targeting ligands resulting in high transduction efficiency (Hesse et al. 2007). It has been reported that enteric adenoviruses Ad40 and Ad41 do not use integrins for cell entry and may use an alternative, unknown mechanism for facilitating endocytosis (Albinsson et al. 1999). It is possible that due to the different orientation of the RGD motif Ad5/41sHI-RGD could infect the cell via integrin-mediated cell entry. However, Ad5/41sIJ-RGD could show a different manner of binding in comparison to Ad5/41sHI-RGD using the alternative mechanism for endocytosis of the enteric adenoviruses.

Further studies of the expression of the late viral genes (Fig. 29) underline the data of the viral replication experiments. In accord with the high efficiency of viral replication by Ad5/41sHI-RGD, this recombinant viruses resulted in higher expression of hexon mRNA and the late viral proteins hexon and fiber compared to Ad5HI-RGD and Ad5/41sIJ-RGD. However, in contrast to the analysis of viral replication and late gene expression during the first replication cycle, genome copy numbers and late gene expression by the chimeric fiber F5/41s viruses was reduced in comparison to Ad5HI-RGD 7 days post infection (Fig. 28A/29A). These observations might indicate that some steps following late gene expression are influenced by the chimeric fiber F5/41s. In addition to cellular binding, the fiber and its knob domain have been suggested to have several functions important for intracellular trafficking, membrane lysis and virus maturation (Legrand et al. 1999; Miyazawa et al. 2001; Gaden et al. 2004). In regard to this, the analysis of viral release and progenies production demonstrated a significant reduction of the amount of infectious viral particles 2, 3, and 4 days post infection of Ad5/41sHI-RGD compared to Ad5HI-RGD (Fig. 30). I could also show a delayed release of Ad5/41sHI-RGD viral particles from the cell into the supernatant. This low titer of infectious particles of Ad5/41sHI-RGD might be explained by the absence of the Ad5 fiber shaft and knob domain. As discussed above, this domain can be involved in some additional steps of viral life cycle beside cell binding and entry which cannot be adopted by Ad41s fiber shaft and knob domain. For example, it has been suggested that the deletion of the knob domain of serotype 5 fiber might have a negative effect on the intracellular concentration of the

fiber protein (Henning et al. 2006) resulting either in a delayed viral assembly or in a delayed encapsidation. Importantly, it was also shown that glycosylation of the fiber shaft might play a role in the stabilization of the fiber and may also protect it from proteolytic degradation (Mullis et al. 1990). This aspect could have an influence on the kinetic of the intracellular accumulation of the fiber protein. Note that only the central shaft domains of serotype 2 and 5 fibers are glycosylated by O-linked N-acetylglucosamine (Mullis et al. 1990). In the light of our results for thermal stability of chimeric fiber F5/41s viral capsids, I could not detect an influence of the fiber format on the thermal stability of the virus capsid (Fig. 31). However, the lytic potency of the chimeric fiber F5/41s was increased when the fiber shaft domain of the Ad41s was replaced by the Ad5 shaft (Fig. 27). This could be explained either by stabilization of the fiber due to the Ad5 shaft glycosylation or by optimized length and flexibility of the longer Ad5 shaft which have been shown to be of importance for efficient virus entry (Shayakhmetov and Lieber, 2000; Wu et al. 2003). I also explored that ectopic expression of Ad5 fiber by the host cell could not restore the negative effect of the chimeric fiber F5/41s on viral assembly and spread levels (data not shown). This result suggests that this negative effect could not be compensated by Ad5 fiber protein provided from the host cell.

In summary, Ad5/41sHI-RGD showed an early onset of gene expression, but lower infectious particles production in comparison to Ad5HI-RGD. I conclude that the fiber format and the precise position of the model peptide RGD in the knob domain of the serotype 41 short fiber, as well as the position of the modified knob in the virus capsid, and the length or structure of the fiber shaft, are critical determinants for the efficiency of the viral life cycle, especially for receptor binding capacity and virus assembly.

6.2.2 Targeting of F41s-pseudotyped recombinant oncolytic adenovirus by insertion of an EphA2 peptide ligand

During malignant transformation, many cells start to express surface antigens which are normally absent or down-regulated on normal cells. Tumour antigens can either be tumour-specific like MAGE (Farina and van der Bruggen 1996) or tissue-specific like prostate-specific membrane antigen (Israeli et al. 1994). These tumour-specific cell markers are the most attractive targets in cancer therapy. One advantage of tumour-specific markers is that their use has already been proven to be safe in

clinical trials. For example, therapy of breast cancer with HER2/neu antibodies resulted in a positive antitumor response in patient (Weiner et al. 1999).

I suggested recombinant adenoviruses carrying the chimeric fiber F5/41s which consists of the Ad5 tail, Ad41s fiber shaft and knob domain as a novel platform for genetic targeting of adenovirus cell entry. This platform can be exploited for insertion of targeting peptides into the HI loop of the Ad41s fiber knob. My used model peptide RGD which binds to cellular integrins is a well known example to overcome the poor transduction of human cancers caused by low expression of CAR (Wesseling et al. 2001; Sandovici et al. 2006; Dmitriev et al. 1998). Although integrins are highly expressed on cancer cells, the specificity of RGD targeting *in vivo* is questionable because of integrin expression on other tissues.
Previous studies showed that a small, highly affinitive peptide that binds the EphA2 receptor could be a promising candidate peptide ligand used for transductional targeting of cancer therapy. EphA2 is a receptor tyrosine kinase, which is over expressed on a number of solid tumours (Abraham et al. 2006, Muldali et al. 2006, Miyazaki et al. 2003, Zelinski et al. 2001) and is often associated with poor prognosis. Targeting EphA2 showed decrease of tumour growth in multiple preclinical models of ovarian, breast and pancreatic cancer (Landen et al. 2005). Based on these studies, I inserted a small EphA2 peptide ligand into the HI loop of the chimeric fiber F5/41s virus. Since ablation of the native binding reduces liver transduction and improves specific targeting *in vivo* (Einfeld et al. 2001), I combined EphA2-targeting with deletion of the natural cell binding properties. Previously, ablation of the native tropism by chimeric fiber F5/41s via transduction experiments in melanoma cells was shown (Hesse et al. 2007). Consequently, I used the recombinant adenovirus carrying the chimeric fiber F5/41s as platform for detargeting the native tropism. I hypothesized that cell entry of Ad5/41s might be also mediated by binding of the penton base via its RGD motif to cellular integrins. Thus, to further improve the detargeting of the Ad5-derived Ad5/41s viruses the penton base RGD motif was mutated. The recombinant oncolytic EphA2-targeted viruses showed no cell lysis and viral spread by F5/41s-detargeting in melanoma cells that do not express the EphA2 receptor. In addition, I could not detect expression of late viral gene by immunoblot after infection of EphA2-negative cells. Furthermore, the lytic potency of the Ad5/41s was restored by insertion of the EphA2 peptide into the HI loop for EphA2-positive

melanoma cells (Fig. 33). However, the efficiency of cell killing by Ad5/41sHI-ephA2 was reduced in comparison to Ad5/41sHI-RGD. The “double-ablated” Ad5RAE41sHI-ephA2 did not show efficient cell lysis in these cells indicating that penton-integrin interaction is required. In this regard, it was already known that not all retargeted viruses do provide efficient transduction which would explain the lack of cytotoxicity of Ad5RAE41sHI-ephA2. For example, insertion of RGD into a vector in which the CAR binding site was ablated did not result in integrin-mediated uptake (Kritz et al. 2007). However, weak expression of the late fiber protein by Ad5RAE41sHI-ephA2 was shown in targeted cells (Fig. 34). Thus, it seems that the cell binding and entry of the “double-ablated” virus is not completely inefficient. Nevertheless, this virus is not able to lyse EphA2-positive cells and spread (Fig. 33). My data shows that the CAR-negative cell line C8161 was lysed by Ad5wt and Ad5Δ24E3- which is a novel and striking finding. It might be explained by a CAR independent uptake of the virus which could occur during the extended time interval of approximately three weeks of the cytotoxicity assay. In contrast, fiber gene expression was not detectable for Ad5wt and Ad5Δ24E3- in C8161 by immunoblot (Fig. 34), which was performed 40 h post infection, indicating a reduced cell entry of these viruses.

An important result of this study was that I generated a virus ablated for CAR-binding which targets EphA2 receptor expressing melanoma cells via an inserted EphA2 peptide ligand and shows specific cell lysis. However, the lytic potency of the chimeric fiber F5/41s viruses, especially the EphA2-trageted virus, was significantly reduced referring to Ad5HI-RGD. In consequence, for future application of these adenoviruses the loss in lytic activity by chimeric fiber F5/41s has to be complemented without sacrificing their specificity. Notably, this work established the basis for entry-targeted oncolytic adenoviruses and open new ways for future development of improved recombinant adenoviruses for targeted viral oncolysis.

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8 ABBREVIATIONS

aa	aminoacid
Ad	adenovirus
Ad3	adenovirus serotype 3
Ad5	adenovirus serotype 5
amp	ampicillin
approx	approximately
APS	ammonium persulfate
AT	annealing temperature
bp	basepair
β	beta
β-gal	β-galactosidase
β-me	β-mercaptoethanol
BSA	bovine serum albumin
C°	degree celcius
cDNA	complementary DNA
CMV	cytomegalovirus
CsCl	caesium chloride
CRAd	conditionally replicative Adenovirus
Δ	delta
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
dNTP	deoxyribonucleic acid triphosphate
DTT	1,4 dithiothreitol
ECL	enhanced chemiluminescence
E.coli	Escherichia coli
EDTA	ethylendiamin N,N,N,N-tetraacetat
EtBr	ethidiumbromide
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
fig	figure

FCS	fetal calf serum
for	forward
g	gram
G	glutamine
h	hour
HRP	horseradish peroxidase
IgG	immunoglobulin G
k	kilo
kan	kanamycin
kD	kilo dalton
l	liter
LB	Luria-Broth
m	milli
M	molar
mA	milli ampere
MCS	multiple cloning site
min	minute
MMP	powdered milk
MoAb	monoclonal antibody
MOI	multiplicity of infection
n	nano
NEAA	non essential amino acids
OD	optical density
P	penicillin
PBS	Phosphate buffer saline
PCR	polymerase chain reaction
PE	phycoerythrin
pH	potentia hydrogen
PMSF	Phenyl-methyl-sulfonyl-fluorid
rdAd	Replication-deficient adenovirus
rev	reverse
RLU	relative light unit
RNA	ribonucleic acid

rpm	rounds per minute
RT	room temperature
RT-PCR	Reverse transcriptase-PCR
S	streptomycin
s.c.	subcutane
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second
SV40	Simian virus 40
$TCID_{50}$	tissue culture infectious dose 50
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tyr	tyrosinase
U	unit
V	volt
vp	virus particle
WT	wild type

9 PUBLICATIONS

Submitted 05/2010

Quirin C, Rohmer S, Hesse A, Engelhardt S, Behr M, Erbs P, Enk AH, Nettelbeck DM.

Selectivity and Efficiency of Late Transgene Expression by Transcriptionally Targeted Oncolytic Adenoviruses are Dependend on the Trannsgene Insertion Strategy.

Manuscript

Rohmer S, Quirin C, Hesse A, Sandmann S, Bayer W, Herold-Mende C, Haviv YS, Wildner O, Enk AH, Nettelbeck DM.

Transgene Expression by Oncolytic Adenoviruses is Modulated by *E1B19K*-Deletion in a Cell Type-Dependent Manner.

Virology. 2009 Dec 20;395(2):243-54.

Quirin C, Mainka A, Hesse A, Nettelbeck DM.

Combining Adenoviral Oncolysis with Temozolomide improves Cell Killing of Melanoma Cells.

Int J Cancer. 2007 Dec 15;121(12):2801-7.

10 ACKNOWLEDGEMENTS

Es war schön mit Euch zu leben, zu arbeiten, zu experimentieren,
zu diskutieren, zu lernen, zu feiern, zu lachen, zu reifen
und neue Erfahrungen zu sammeln…

DANKE
Tina

Dirk Nettelbeck / Suat Özbek / Anne Regnier-Vigouroux / Stephan Herzig / Mira Rohmer / Kai Zanzinger / Anne Krause / Alex Roller / Stefan Langenberger / Ines Ulibarri / Matthias Sobel / Tanja und Stefan Jutzi / Johanna Kaufmann / Steffi und Markus Sandmann / Sarah Engelhardt / F110 / 1.OG Ost / Jean Rommeleare / Tim Kees / Thomas Holz / meine Eltern