Novel Strategies to Improve the Efficiency of Therapeutic Adenoviruses for the Treatment of Cancer

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zur
Erlangung des Doktorgrades

vorgelegt von
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aus Nürnberg
Die Neugier steht immer an erster Stelle eines Problems, das gelöst werden will

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# Table of Contents

1 **Summary** .................................................................................................................. 1

2 **Introduction** ............................................................................................................ 3

2.1 **Cancer and Cancer Therapy of the Last Centuries** .............................................. 3

2.2 **Gene Therapy for Cancer Treatment** .................................................................... 8

2.3 **Virotherapy for Cancer Treatment** ........................................................................ 12

2.4 **Adenoviruses and their Use as Gene Therapy Vector or Oncolytic Virus** .......... 14

2.4.1 Adenoviruses: Virion Structure, Cell Entry and Genome Organization .......... 15

2.4.1.1 Serotypes and Virus Structure .............................................................................. 15

2.4.1.2 Cell Binding and Entry .......................................................................................... 16

2.4.1.3 Genome Organization and Viral Replication ......................................................... 17

2.4.2 Adenoviral Vectors ................................................................................................. 20

2.4.2.1 First-Generation Vectors ....................................................................................... 20

2.4.2.2 Second-Generation Vectors ................................................................................... 20

2.4.2.3 Helper-Dependent Vectors .................................................................................... 21

2.4.2.4 Conditionally Replication-Competent/Oncolytic Adenoviruses ......................... 21

2.4.3 Oncolytic Adenoviruses ......................................................................................... 22

2.4.3.1 Tumor-selective Replication and Lysis of Oncolytic Adenoviruses by Viral Gene Mutations ........................................................................................................... 23

2.4.3.2 Tumor-selective Replication and Lysis of Oncolytic Adenoviruses by Using Tissue-or Tumor-Selective Promoters ................................................................................................. 24

2.4.3.3 Genetic Modification of the Virus Capsid for Efficient Cell Entry of Oncolytic Adenoviruses ......................................................................................................................... 25

2.4.3.4 Potential Hurdles Limiting Oncolytic Adenovirus Efficacy ................................. 27

2.4.3.5 Strategies to Improve the Therapeutic Efficacy of Oncolytic Adenoviruses ......... 28

3 **Objectives of the study** ......................................................................................... 31

4 **Materials and methods** ......................................................................................... 32

4.1 **Materials** .............................................................................................................. 32

4.1.1 Chemicals, filters and enzymes .............................................................................. 32

4.1.2 Buffers and solutions .............................................................................................. 32

4.1.2.1 Buffers and solutions for gel electrophoresis ...................................................... 32

4.1.2.1.1 Electrophoresis of nucleic acids ......................................................................... 32

4.1.2.1.2 Electrophoresis of proteins ................................................................................. 32

4.1.2.2 Buffers and solutions for western blot analysis ................................................. 33

4.1.2.3 Buffers and solutions for flow cytometry ........................................................... 33

4.1.2.4 Buffers and solutions for viral lysis .................................................................... 33

4.1.2.5 Buffers and solutions for production of transformation competent bacteria ....... 33

4.1.2.6 Buffers and solutions for DNA precipitation ..................................................... 33

4.1.2.7 Buffers and solutions for caesium chloride equilibrium density ultracentrifugation.... 33

4.1.3 Media .................................................................................................................... 34

4.1.3.1 Media for bacterial culture .................................................................................... 34

4.1.3.2 Media and solutions for cell culture .................................................................... 34
**4.1.4 Cells and Bacteria Strains** .................................................................................................................. 34
  4.1.4.1 Bacteria strains ................................................................................................................................. 34
  4.1.4.2 Human cells lines ............................................................................................................................. 35

**4.1.5 Adenoviruses** ................................................................................................................................... 36

**4.1.6 Nucleic acids** ................................................................................................................................... 36
  4.1.6.1 Oligonucleotides ............................................................................................................................. 36
    4.1.6.1.1 Oligonucleotides for PCR cloning ............................................................................................. 36
    4.1.6.1.2 Oligonucleotides for controlling recombinant modified Ad genomes ........................................... 37
    4.1.6.1.3 Oligonucleotides for sequencing ............................................................................................... 38
    4.1.6.1.4 Oligonucleotides for quantitative real time PCR (qPCR) .......................................................... 38
  4.1.6.2 Plasmids ........................................................................................................................................... 39
  4.1.6.3 Antibodies ....................................................................................................................................... 40
    4.1.6.3.1 Antibodies for western blot analysis .......................................................................................... 40

**4.2 Methods** .............................................................................................................................................. 41

**4.2.1 Nucleic acid methods** ....................................................................................................................... 41
  4.2.1.1 DNA cloning ................................................................................................................................... 41
    4.2.1.1.1 Production of transformation-competent bacteria and transformation ...................................... 41
    4.2.1.1.2 Production of chemical-competent bacteria and transformation by heat shock ............................ 41
  4.2.1.2 Production of recombinant adenovirus ............................................................................................ 41
    4.2.1.2.1 Production of electro-competent bacteria and transformation by electroporation .................... 41
    4.2.1.2.2 Homologous recombination for the generation of recombinant adenoviral genomes ................. 42
  4.2.1.3 Preparation of DNA and RNA ......................................................................................................... 43
    4.2.1.3.1 Analytical isolation of plasmid DNA (mini lysate) .................................................................... 43
    4.2.1.3.2 Quantitative isolation of plasmid DNA (midi lysate) ................................................................. 43
    4.2.1.3.3 DNA isolation from infected human cell cultures ..................................................................... 44
    4.2.1.3.4 RNA isolation ............................................................................................................................. 44
  4.2.1.4 PCR (polymerase chain reaction) ...................................................................................................... 44
    4.2.1.4.1 Quantitative real time PCR (qPCR) ............................................................................................. 45
  4.2.1.5 Protein biochemical and immunological methods ............................................................................... 46
    4.2.1.5.1 Preparation of total cell lysates .................................................................................................. 46
    4.2.1.5.2 Determination of total protein concentration ................................................................................ 46
    4.2.1.5.3 Discontinuous SDS-Polyacrylamide Electrophoresis (SDS-Page) ............................................... 46
    4.2.1.5.4 Western Transfer ....................................................................................................................... 46
    4.2.1.5.5 Immunoblot ............................................................................................................................... 47
    4.2.1.5.6 Flow cytometry for the detection of cell death ............................................................................ 47
  4.2.1.6 Cell culture ....................................................................................................................................... 48
    4.2.1.6.1 Passaging, freezing and thawing cell culture cells ....................................................................... 48
    4.2.1.6.2 Transient transfections and reporter assays ................................................................................ 48
      4.2.1.6.2.1 Transient transfection for the analysis of promoter activities ............................................... 48
      4.2.1.6.2.2 Luciferase reporter assay ....................................................................................................... 49
    4.2.1.6.3 Recombinant adenovirus ........................................................................................................... 49
      4.2.1.6.3.1 Generation of recombinant adenovirus .................................................................................. 49
      4.2.1.6.3.2 Caesium chloride gradient equilibrium density ultracentrifugation for the purification of viral particles .................................................................................................................. 50
    4.2.1.7 Determination of viral particle concentration .................................................................................. 51
      4.2.1.7.1 Determination of infectious particle concentration using the Tissue Culture Infectious Dose 50 (TCID₅₀)- assay ........................................................................................................ 51
      4.2.1.7.2 Determination of physical viral particles by reading optical density ....................................... 52
      4.2.1.7.3 Verification of recombinant adenoviral genomes ..................................................................... 52
    4.2.1.8 Transduction and infections of cells with recombinant adenovirus ................................................. 52
      4.2.1.8.1 Transduction with replication-deficient adenovirus for the analysis of luciferase activities ....... 52
5 Results .................................................................................. 55

5.1 Heat-regulated transgene expression from either replication-deficient or replication-competent adenoviral vectors .......... 55

5.1.1 Analysis of heat-inducibility of promoter fragments of stress-inducible genes ................................................................. 55

5.1.2 Influence of adenovirus co-infection and replication on hsp70B' promoter activity in a reporter plasmid ................................................................. 57

5.1.3 Regulation of transgene expression by the hsp70B' promoter in a replication-deficient adenovirus vector ................................................................. 58

5.1.4 Activity and regulation of the hsp70B' promoter in an oncolytic adenovirus ................................................................. 60

5.1.5 Insulator elements as tools for improved regulation of the hsp70B' promoter in replication-deficient but not replicating adenoviral vectors ................. 67

5.2 Combining Rational Mutagenesis and Transgene Expression for Improving Efficacy of Oncolytic Adenoviruses ....................... 70

5.2.1 Spread of E1B19K-deleted, transgene-encoding oncolytic adenovirus in tumor cell cultures ................................................................. 70

5.2.2 Apoptosis induction and cell type-dependent modulation of the adenovirus replication cycle by the E1B19K-deletion ................................................................. 72

5.2.3 Modulation of the expression of transgenes inserted into oncolytic adenoviruses by E1B19K deletion ................................................................. 79

6 Discussion .................................................................................. 82

6.1 Insulated hsp70B' promoter: stringent heat-inducible activity in replication-deficient, but not replication-competent adenoviruses 82

6.1.1 Feasibility of the hsp70B' promoter fragment for heat-inducible transgene expression and its analysis in the context of a replication-deficient adenoviral vector ................................................................. 82

6.1.2 Analysis of heat-directed transgene expression in the context of a replication-competent/oncolytic adenoviral vector ................................................................. 87

6.2 Transgene Expression by Oncolytic Adenoviruses is Modulated by E1B19K-Deletion in a Cell Type-Dependent Manner .......... 91

7 References .................................................................................. 95
Table of Contents

8 Abbreviations ................................................................. 109
9 Curriculum Vitae .......................................................... 112
10 Acknowledgements/Danksagung................................. 113
11 Publications.................................................................. 114
1 ZUSAMMENFASSUNG


SUMMARY
The resistance of cancers to conventional therapies has inspired the search for novel anti-cancer strategies. Two such promising approaches are gene therapy and virotherapy. Gene therapy involves the delivery of therapeutic transgenes, frequently by means of viral vectors, to tumor tissues to selectively kill them. In contrast, virotherapy is defined as killing of cancer cells by specific virus infection, replication, cell lysis and virus spread by so-called oncolytic viruses. Successful implementation of gene therapy requires efficient and targeted gene transfer vectors. Therapeutic gene transfer by replication-deficient or more recently by replication-competent/oncolytic adenoviruses (OAds) has shown much promise. However, for specific applications an external control of transgene expression might be crucial. The 1st project, for the first time, investigated the feasibility of a 245bp fragment of the human hsp70B’ promoter for heat-regulated transgene expression in combination with either replication-deficient or oncolytic adenoviruses. In transient transfection assays this promoter demonstrated low basal activities with induction ratios up to 3741-fold. In context of a replication-deficient Ad, heat-stimulus resulted in 586-fold increased luciferase activity for SK-MEL-28 cells, but was only 44-fold for HeLa cells due to higher basal activities. To improve the heat-inducibility, two novel Ad vectors with an insulated hsp70B’ promoter were developed that feature stringent heat-inducible gene expression with induction ratios up to 8000-fold. However, in a melanoma-targeted OAd regulation of the hsp70B’ promoter was lost specifically during late replication in permissive cells and could not be restored by insulators. In summary, this study revealed that Ad vectors with insulated hsp70B’ promoter have potential for gene therapy applications that require external control of transgenes. Moreover, it shows that vector replication can deregulate inserted promoters, which needs to be considered for the development of transcriptionally regulated oncolytic gene transfer vectors.

Adenoviruses have been intensively exploited for virotherapy approaches. Clinical trials have demonstrated proof of principle and a favourable safety profile, but insufficient therapeutic efficacy for these viruses. The 2nd project was designed to increase the therapeutic potency of OAds by combining two strategies: Deletion of the anti-apoptotic early viral gene E1B19K aimed at accelerated virus spread by early viral release from tumor cells, and was coupled with strategies for insertion of transgenes into the late viral transcription unit. Here the goal was the replication-dependent expression of therapeutic genes to work in concert with viral oncolysis (“armed” OAd). However, it remained to be demonstrated whether early lysis of cancer cells aborts efficient transgene expression or better spread might increase it. Deletion of E1B19K was analyzed in a panel of tumor cell cultures that resulted either in increased early viral release and enhanced cell lysis or in reduced virus replication and oncolysis dependent on the cell type. It could be shown that these opposing phenotypes do not result from differences in apoptosis induction. Deletion of E1B19K rather affected virus replication and release distinctly. Nevertheless, reporter gene assays revealed that E1B19K deletion, despite of earlier induction of apoptosis during viral replication, in cells susceptible for early lysis did not interfere with late transgene expression; luciferase expression by the E1B19K- virus was even superior over time compared to the E1B19K+ virus due to better virus spread. Thus, the E1B19K- virus showed >3 orders of magnitude increased oncolysis and transgene expression 10 days post-infection. Importantly, this study shows that early virus release accelerates spread and tumor cell killing and even dramatically increases expression of transgenes inserted into the viral genome in several tumor types. Considering the clearance of virus by anti-viral immunity such accelerated phenotype has the potential to improve “armed” OAds in cancer therapy.
2 INTRODUCTION

2.1 Cancer and Cancer Therapy of the Last Centuries
Cancer is defined as an abnormal growth of cells, which tend to proliferate in an uncontrolled way leading to serious adverse effects on the host through invasive growth (destruction of adjacent tissues) and metastases (dissemination of cancer cells throughout the organism via lymph or blood). In fact, cancer can involve all living organisms (humans, animals, plants) and every tissue, simply because they are composed of potentially dividing cells. Hence, it is not remarkable that human cancer is most probably as old as the human species. The world’s oldest documented case of human cancer traces back to ancient Egyptian papyri written between 3000-1500 B.C., documenting eight cases of tumors occurring on the breast. But even though medicine progressed and flourished in some ancient civilizations, there was no appreciable development in cancer treatment.

It took until the eighteenth century, when John Hunter (1728-1793), one of the most outstanding scientists and surgeons of his day, suggested, that some cancers might be cured by surgery and described methods to distinguish the surgically removable tumors (Androutsos, Vladimiros et al. 2007). Together with the introduction of anesthesia in 1846 (Stoeckel and Schulte am Esch 1996) many great surgeons like William Stewart Halsted and Theodor Billroth emerged, who carried out the first operations removing the cancerous part and the surrounding lymph nodes. In the last decade of the 19th century Halsted then developed the radical mastectomy (Sakorafas 2008), whereas Billroth performed the first esophagectomy (1872), laryngectomy (1873), partial gastrectomy (1881) and pancreatectomy (1894) to remove a cancerous tumor (Polak and Vojtisek 1959; Komorowski, Wysocki et al. 2006). Hence surgery became the predominant form of cancer treatment, however complete cures of cancer by surgery depended greatly upon a tumor being quickly diagnosed and somewhat readily accessible. The first non-surgical cancer treatment arised with the discovery of X-rays by Wilhelm Conrad Roentgen in 1895 (Roentgen 1959; Gunderson and Tepper 2000) and with the discovery of natural radioactivity a few months later by the french physicist Henry Becquerel (Myers 1976). These two outstanding breakthroughs were the beginning of a new era in science, but also in cancer treatment, because it was quickly recognized that radiation is a powerful and effective tool to kill cancer. Only three months after Roentgen had published his report on X-rays, clinical radiotherapy was born, when Emil Grubbé treated an advanced ulcerated breast cancer with X-rays (Hodges 1964). Few years later in
Introduction

1903 the first cured case of cervical cancer by X-rays was registered. Three years after the introduction of X-rays the French physicists Marie and Pierre Curie discovered the radioactive elements polonium and radium, which led to a further advance in radiation application (Curie P, Curie M, 1898; [link](http://www.academie-sciences.fr/membres/in_memoriam/Curie/Curie_pdf/CR1898_p1215_1217.pdf)). For twenty years radium was the only source of natural high-energy photons or γ-rays for cancer therapy until cobalt and caesium units came into use (Green and Ferrington 1952; Eastwood 1960). The use of such high-energy photons was important for the treatment of deep-seated tumors. Throughout the first four decades of the twentieth century, scientists were mainly focused on improving the quality and quantity of radiation that could be delivered to a tumor. Indeed such treatments frequently led to impressive responses, however by the 1940s much of the initial enthusiasm for radiotherapy had diminished, since the median survival of patients had only changed little.

At the same time began the era of cancer chemotherapy with the discovery of nitrogen mustard, a chemical warfare agent. Autopsy observations of naval personnel who were exposed to mustard gas during the World War II revealed severe lymphoid and myeloid suppression. Therefore two pharmacologists, Louis S. Goodman and Alfred Gilman reasoned that this agent could be used to treat lymphoma (Goodman, Wintrobe et al. 1984). For that purpose they established lymphomas in mice and demonstrated that they could treat them with mustard agents. Next, in collaboration with a thoracic surgeon, Gustav Linskog, they injected a related agent mustine into a patient with non-Hodgkin’s lymphoma and observed a dramatic reduction of the patient’s tumor mass (Thurston 2007). Although this effect lasted only for a few weeks, this was the first step to the realization that cancer could be treated by pharmacological agents. In 1948 Sidney Farber demonstrated that the folic acid antagonist aminopterin led to remission in acute leukemia in children by blocking a critical chemical reaction needed for DNA replication (Patlak 2002). This anticancer agent was the predecessor of methotrexate, a commonly used chemotherapeutic today. Although hematologic malignancies appeared susceptible to many chemotherapeutic drugs, solid tumors were only little affected. That is why by the middle of the 1950s there was uncertainty that chemotherapy as a single agent would be efficient enough to cure every tumor. And even the combination of the abovementioned modalities proved to be insufficient, as they were not applicable to all tumors at all stages.
These circumstances started a new wave of increased investigations in order to get a better understanding of the biology of cancer. An important milestone of this era was made when Alfred Hershey and Martha Chase in the Hershey-Chase experiment showed that DNA is the carrier of the genetic information (Hershey and Chase 1952). Only one year later, another major breakthrough in science took place when James Watson and Francis Crick discovered the exact chemical structure of DNA (Fig.1), for which they later received the Nobel Prize (Watson and Crick 1953). Quickly after these discoveries Marshall Warren Nirenberg, Har Gobind Khorana and Robert W. Holley described the genetic code and how it operates in protein synthesis (Sulek 1969).

At this time point, researchers already hypothesized that chemicals, radiation, heredity and viruses could play an important role in the development of cancer. But the better knowledge of DNA and genes revealed that it was the alteration of the DNA by chemicals and radiation or the introduction of foreign DNA sequences by viruses, which often caused cancer. From the early 1970s on, genetic engineering techniques became available which not only revolutionized the field of genetics but also every other field of biological research. The molecular basis for these novel
techniques were established by Werner Arber, Hamilton Smith and Daniel Nathans who discovered, isolated and implemented the use of restriction endonucleases for which they received the Nobel Prize in Medicine in 1978 (Raju 1999). By means of these restriction endonucleases, which are enzymes that cut double-stranded DNA at specific recognition nucleotide sequences also known as restriction sites, Daniel Nathans was able to map the simian virus 40 (SV40) genome and to locate the origin of replication (Danna, Sack et al. 1973). All these major breakthroughs finally heralded the new era of recombinant DNA technology. Peter Lobban was the first to propose this novel technique, which made it possible to combine two DNAs of different origin, thus producing new combinations of genes that had never appeared in the natural environment. In 1972, Jackson, Symons and Berg then first realized the recombinant DNA technique by inserting a DNA segment of lambda phage and the galactose operon of E.coli into the SV40 genome (Jackson, Symons et al. 1972). Only two years later the first eukaryotic gene from the South African clawed frog *Xenopus laevis* was cloned into a bacterial plasmid (Morrow, Cohen et al. 1974).

Since these days recombinant DNA techniques revolutionized the academic field of molecular biology, providing a means to easily isolate, grow, and manipulate DNA of interest in order to study gene structure and function. In the following years, this technology underwent rapid development that produced an explosion of data worldwide and ultimately helped to understand by which mechanisms genes are expressed and how that expression is modulated during the development or oncogenesis.

To date, carcinogenesis is considered to be a multistep process that involves several genetic alterations in growth-regulatory genes leading to the transformation of normal human cells into highly malignant derivatives. There exist two classes of cellular genes that are altered in cancer: the oncogenes, which function as positive growth regulators and the tumor-suppressor genes, which function as negative growth regulators. In tumorigenesis oncogenes induce aberrant cell growth through a gain of function, whereas tumor suppressor genes contribute to carcinogenesis through a loss of function. In general, DNA alterations in both types of genes are required for cancer to occur. In this regard, Hanahan and Weinberg proposed that most if not all cancers acquire the same type of alterations that collectively dictate malignant growth (Hanahan and Weinberg 2000). These hallmarks of cancer include: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of
programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

These new insights into carcinogenesis and tumor progression built the foundation for significant improvements in all aspects of combating cancer, including ameliorated techniques in early diagnosis, advances in surgery, improved chemotherapy and radiation therapy. For example the introduction of computed tomography (CT) (Ambrose 1973) and nuclear magnetic-resonance imaging (NMRI) (Mansfield and Maudsley 1977) enabled radiation oncologists to visualize the treatment area. The development of intensity-modulated radiotherapy (IMRT) (Bucci, Bevan et al. 2005) in the early 1980s made it possible to deliver therapy shaped to the contours of the tumor with a high dose volume. Further, the introduction of multileaf collimators (LoSasso, Chui et al. 1993) allowed for more homogenous irradiation of the tumor and protection of normal tissues. In case of chemotherapy research of this day mainly focuses on the development of therapeutics with greater specificity, less toxicity and higher therapeutic indices. Especially the field of pharmacogenetics aims to increase drug efficacy and reduce adverse side effects by discovering new therapeutic targets and genetic polymorphisms that affect drug specificity and toxicity (Smith and O'Donnell 2006; Kirchheiner 2007). To date, also radiation-surgery combinations (McLeod and Thrall 1989), either in a pre- or post-operative setting, have shown to be efficacious. Once the malignant tumor is resected, radiotherapy can be used to kill residual tumor cells. Radiation-chemotherapy (Halperin, Perez et al. 2008) combinations have also yielded encouraging results leading to radiosensitizing effects for low doses of drugs and supra-additive effects for full doses of drugs.

Despite the progress made in radiotherapy and chemotherapy survival rates for many tumor types still remain extremely low. Even cancer types that are highly amenable to treatment still respond poorly when detected at an advanced stage. Furthermore, the traditional therapies are, in general, non-specific and often result in serious or even fatal toxicities and can also include an increased risk of developing other types of cancer. Based on these recognitions, it is apparent that novel therapeutic strategies are required to obtain significant increases in clinical survival. In this regard, the development and application of immunotherapy, gene therapy and virotherapy for treating cancer has attracted great attention in the past twenty years. Such innovative strategies were mainly facilitated by the better understanding of
tumor formation, which in turn resulted from the rapid application of the recombinant DNA technology, underscoring the great potential of this technique. The concept of immunotherapy is based on the stimulation of the patient’s natural immune system, via a variety of reagents such as vaccines, infusion of T-cells or cytokines, to attack the malignant tumor cells. These reagents aim to (a) stimulate the antitumor response, either by increasing the number of effector cells or by producing one or more soluble mediators such as lymphokines; (b) decrease suppressor mechanisms; (c) alter tumor cells to increase their immunogenicity and make them more susceptible to immunologic defenses; (d) improve tolerance of cytotoxic drugs or radiotherapy. The concept of gene therapy and virotherapy for the treatment of cancer will be described in the following chapters.

2.2 Gene Therapy for Cancer Treatment
Gene therapy was originally designed to treat inherited diseases at the molecular level, that appear as a result of abnormal genes, by replacing a defective function with a normal gene thus restoring the lost gene function in the patient's body. For that purpose, DNA segments of the gene of interest are cloned into a so-called vector, which is able to deliver the foreign gene into the target cell (non-viral gene transfer = transfection; viral gene transfer = transduction). The therapeutic gene can be delivered either ex vivo – where cells from a selected tissue of the patient are removed, exposed to the gene transfer vector and after selection the genetically corrected cells are reintroduced into the patient’s body; or in vivo where the vector is injected directly into the tissue to be treated. For the gene transfer itself various methods have been developed such as microinjection, electroporation, liposome formulations, gene gun and the use of viral vectors. To date, the most common and most efficient method of gene transfer, particularly for in vivo applications, is by means of viral vectors that have been genetically modified carrying therapeutic genes. In course of time viruses have evolved very efficient strategies to enter the cell, introduce their own genetic information and usurp the cellular machinery to make its own viral proteins. Scientist have tried to take advantage of this capability and manipulated the virus genome by replacing essential viral genes with therapeutic transgenes, which renders the virus replication-deficient.

Rapidly after the introduction of gene therapy in 1990 when W.F. Anderson was the first to successfully treat severe combined immune deficiency (SCID) with virus-based gene therapy by delivering the adenosine-deaminase (ADA) gene into patients (Beardsley 1990; Culver, Anderson et al. 1991), researchers tried to apply it to a
broad range of diseases such as infections, degenerative disorders and also cancer. Many cancer gene therapy clinical trials aim to compensate mutations of either tumor suppressor genes or oncogenes. The best-known tumor suppressor genes that are often mutated in cancer are p53, retinoblastoma (RB), BrEaST CaNcer 1 (BRCA1), adenomatous polyposis coli (APC) and p16$^{INK4a}$, whereas oncogenes like ErbB2, bcl-2 or c-myc are frequently overexpressed. One of the most promising viral vectors is a replication incompetent adenovirus delivering the p53 gene to cancer cells resulting in inhibition of angiogenesis, tumor cell invasion, metastasis and apoptosis through the induction of Fas/CD95 (Merritt, Roth et al. 2001; Swisher, Roth et al. 2003).

Based on the observation that dysregulated apoptosis plays a key role in the pathogenesis and progression of cancer, apoptotic pathways represent an attractive therapeutic target in the treatment of cancer. Apoptosis, which is a built-in cell suicide program, serves to eliminate cells in the organism that are no longer needed or have sustained severe damage. Thus, this mechanism is essential for maintaining the cellular homeostasis to ensure correct development and function of multi-cellular organisms. During tumorigenesis, cancer cells often develop defects in the function of pro-apoptotic molecules, which ultimately leads to inappropriate cell survival and malignant progression. Therefore many cancer gene therapy approaches try to stimulate apoptotic pathways by delivering pro-apoptotic transgenes such as Bax (Arafat, Gomez-Navarro et al. 2000; Norris, Hyer et al. 2001) or death ligands like FasL (Sipo, Hurtado Pico et al. 2006). Another very promising death ligand, which has motivated extensive research efforts, is tumor necrosis factor related apoptosis-inducing ligand (TRAIL). TRAIL has been shown to trigger apoptosis preferentially in tumor cells, whereas many normal cells showed no or less response (Walczak, Miller et al. 1999; LeBlanc and Ashkenazi 2003; Yagita, Takeda et al. 2004). In this regard, Kim and co-workers engineered an adenoviral vector coding for a secretable trimeric TRAIL, which resulted in significant anti-tumor and bystander effects in vitro and in vivo without normal tissue toxicity (Kim, Jeong et al. 2006).

Another approach for cancer gene therapy is the delivery of so-called suicide genes, which encode enzymes, needed for the metabolic conversion of non-toxic pro-drugs into toxic products capable of killing tumor cells. The most frequently used enzyme/pro-drug combinations are thymidine kinase (TK) from the Herpes simplex virus (HSV-TK) and ganciclovir (Song and Kim 2004) and cytosine deaminase (CD) from Escherichia coli (E.coli) or yeast and 5-fluorocytosine (Huang, Zhang et al. 2002; Nyati, Sreekumar et al. 2002).
Other strategies attempt to augment the anti-tumor immune response in order to enhance the patient’s capacity for recognizing cancer cells. Therefore, cancer cells are transduced with immunostimulatory genes, for example cytokines, which produce proteins that unmask the cells from immune evasion and encourage the development of antitumor immune responses, usually cytotoxic T-lymphocytes (CTLs), which are able to destroy the tumor cells (Nawrocki, Wysocki et al. 2001; Henderson, Mossman et al. 2005). For instance, in a phase I trial the adenovirus-mediated delivery of the cytokine IL-2 in high-risk localized prostate cancer demonstrated to cause an inflammatory response consisting predominantly of CD3+CD8+ T lymphocytes (Trudel, Trachtenberg et al. 2003).

A key requirement of cancer gene therapy is that the appropriate genes must be delivered to and expressed in target cells, without harming non-target cells in order to become cancer specific, and thus safer. Despite the high transduction efficiency of viral vectors, their tropism frequently does not match the therapeutic need. Therefore, many researchers focused on modifying viral vectors to target them to the diseased tissue or cells at which the therapeutic gene transfer is aimed. Selective cell entry (=transductional targeting) and selective gene expression (=transcriptional targeting) are tools for directing gene delivery. Transductional targeting has, for example, been implemented by genetic engineering of the virus capsid (Glasgow, Everts et al. 2006) (for more details see chapter 2.4.3.3), whereas transcriptional targeting has been realized by inserting promoter fragments or optimized promoter constructs into the viral genome for cell-type-selective expression of therapeutic genes (Nettelbeck, Jerome et al. 2000). These include the use of tissue-specific promoters like the prostate-specific antigen (PSA) promoter (Pang, Taneja et al. 1995; Lee, Liu et al. 1996; Martiniello-Wilks, Garcia-Aragon et al. 1998) for prostate cancer or the tyrosinase promoter for melanoma (Vile and Hart 1993; Hughes, Wells et al. 1995). Others implemented the use of tumor-selective promoters that are overactive in certain tumor types, like the α-fetoprotein promoter (AFP) (Huber, Richards et al. 1991; Ido, Nakata et al. 1995; Kaneko, Hallenbeck et al. 1995) that is specifically active in the foetal liver, but becomes reactivated in hepatoma cells.

Besides cell-type specificity, external control of drug activity can be advantageous or even critically required, for example, when considering the application of cytotoxic transgenes like tumor necrosis factor-α (TNF-α). Regulatable transgene expression systems can represent a (a) safety feature; (b) a tool for fine-tuned dosing of gene products in order to maintain therapeutic concentrations or to respond to the course
Introduction

of disease/therapeutic intervention; (c) a means for spatial restriction of gene expression; or (d) a means to adjust to therapeutic regimens such as combination therapies. Inducible transcription in gene therapy can be achieved by two major strategies. The first is drug-inducible transcription, which requires expression of a drug-inducible transcription factor that regulates therapeutic gene expression via corresponding responsive promoter elements. Examples are tetracycline-, rapamycin-, or hormone-responsive systems (Toniatti, Bujard et al. 2004) (Tet-on/off system: (Pitzer, Schindowski et al. 1999; Rubinchik, Wang et al. 2001), rapamycin: (Rivera, Ye et al. 1999; Chong, Ruchatz et al. 2002), hormone-inducible: (ecdysone: (Christopherson, Mark et al. 1992; No, Yao et al. 1996)). The second strategy represents promoters induced by endogenous factors that respond to chemical (oxygen pressure: (Ido, Uto et al. 2001; Shibata, Giaccia et al. 2002); chemotherapeutic drugs: (Walther, Stein et al. 2000; Walther, Stein et al. 2002)) or physical stimulation (radiation: Egr-1: (Hallahan, Mauceri et al. 1995; Manome, Kunieda et al. 1998; Kawashita, Ohtsuru et al. 1999); WAF-1: (Worthington, Robson et al. 2002), (heat: Gadd 153: (Ito, Shinkai et al. 2001), Hsp70B: (Brade, Ngo et al. 2000; Braiden, Ohtsuru et al. 2000; Huang, Hu et al. 2000; Lohr, Hu et al. 2000; Lee, Galoforo et al. 2001)).

In this regard, the heat-shock protein promoters, particularly hsp70, have attracted great attention as possible tools for gene therapy. This is because the heat-shock response was rapidly identified as the most dramatic example of selective and inducible transcriptional regulation known in any eukaryotic cell (Voellmy 1994). The heat shock response is characterized by the rapid induction of heat shock protein (Hsp) expression in response to supra-physiological temperatures. In humans, four inducible hsp70 genes have been described: hsp70(A)-1 (HSPA1A locus), hsp70(A)-2 (HSPA1B), hsp70B’ (HSPA6), and hsp70B (HSPA7). Importantly, small and simply structured promoter fragments were found to control heat-inducible expression of the inducible human hsp70 genes by several groups (Rome, Couillaud et al. 2005). These promoters are regulated by the heat shock transcription factor-1 (HSF-1), which undergoes nuclear translocation, trimerization and phosphorylation by stress stimuli. In the nucleus it binds to heat shock elements (HSEs) of the hsp70 promoter, which results in transcriptional activation of the heat-shock genes (Fig.2). The hsp70B promoter as well as the hsp70(A)-1 promoter have frequently been used in vitro and in vivo for the expression of either reporter (Huang, Hu et al. 2000; Vekris, Maurange et al. 2000; Smith, Machluf et al. 2002) or therapeutic transgenes
Introduction

(Dreano, Brochot et al. 1986; Braiden, Ohtsuru et al. 2000; Huang, Hu et al. 2000) because besides their high efficiency they provide a high level of control over transgene expression.

Fig. 2: Proposed mechanism of stress-induced heat-shock response in human cells. In unstressed cells heat-shock proteins (HSPs) are bound to heat-shock factors (HSFs) that reside in the cytosol. Cells that are exposed to elevated temperature or other stress stimuli lead to a strong increase of misfolded proteins. In response to this stress, HSFs are separated from the HSPs and are translocated to the nucleus. In the nucleus they form a homotrimeric structure. These trimers bind to heat-shock elements (HSEs) within the promoter of the heat-shock genes. Phosphorylation of the HSFs then results in transcriptional activation of the heat-shock genes. The newly synthesized HSPs then assist the denatured proteins in refolding. In addition, they bind HSFs to prevent further synthesis of HSPs.

Another major advantage of using heat-inducible promoters is that hyperthermia can be applied to well-defined tissue volumes, so that only transduced cells will be activated, and cells located distantly from the heating site would not express the therapeutic gene product. To date, several studies demonstrated the use of local hyperthermia to control the spatial expression of transgenes (Brade, Ngo et al. 2000; Guilhon, Voisin et al. 2003; Xu, Zhao et al. 2004). Furthermore, by coupling focused ultrasound (FUS), that enable scientists to heat deep-laying soft tissues, with magnetic resonance (MR) temperature mapping, the thermal dose can be controlled allowing precise control of transgene expression based on temperature (Guilhon, Quesson et al. 2003).

2.3 Virotherapy for Cancer Treatment

Another growing research area for the treatment of cancer is virotherapy, the use of oncolytic viruses where tumor cell killing is achieved by the direct consequence of viral replication followed by cell lysis to release the progeny virions. The new
generation of oncolytic viruses can spread through the tumor mass and start a new cycle of infection, replication and tumor cell killing. However, the idea of using oncolytic viruses in the treatment of cancer is not new. Already at the end of the 19th century observations of tumor regressions were made that coincided with natural virus infections. In 1896, Dock G. described a 42-year-old woman with myelogenous leukemia that showed remission after a presumed influenza infection – 37 years before it was determined that influenza was a virus infection. More recent clinical reports have described the regression of leukemia (Gross 1971; Pasquinucci 1971), Hodgkin’s disease (Zygiert 1971; Taqi, Abdurrahman et al. 1981), and Burkitt’s lymphoma (Bluming and Ziegler 1971) concomitantly with measles virus infection. Therefore it was concluded, that under certain circumstances, viruses are able to replicate and spread selectively in tumor deposits and even lyse tumor cells and thus could be used to treat cancer. The reasons why cancer cells are such good hosts for viruses are poorly understood, but are likely to be found in the genetic and physiological features specific for malignant cells. Cancer cells have undergone a mini-evolution, involving several point-mutations as well as larger chromosomal alterations or even translocations, which provides them with selective growth advantages over normal cells (see also chapter 2.1) and thus become fertile ground for the replication of viruses. For example, many cancer cells cannot produce interferon (IFN) or respond to IFN stimulation (Linge, Gewert et al. 1995; Ralph, Wines et al. 1995; Matin, Rackley et al. 2001). Interferons are natural cell-signalling proteins produced by the cells of the immune system in response to challenges such as viruses and parasites. Hence, such abnormalities make cancer cells highly susceptible to virus infection. Nevertheless, such naturally occurring, virus-induced remissions have generally been rare, short-lived and incomplete. In 1948, when ex vivo culture of human cells and the implantation of these cells into laboratory rodents became possible, researcher of these days started to investigate a panel of human viruses for their oncolytic activity, which included dengue, yellow fever, West Nile virus (Moore 1952; Southam and Moore 1952), Semliki forest virus, mumps, vaccinia (Newman and Southam 1954) and adenovirus (Huebner, Rowe et al. 1956). These viruses appeared to have tremendous potential, however, in clinical trials they rarely caused complete cures (Sinkovics and Horvath 1993) and often resulted in severe side effects (Kelly and Russell 2007). Thereupon it was recognized that viruses needed manipulation to be targeted more specifically to cancer cells. But direct manipulation
of viral genomes was not possible until the 1968s when polynucleotides were added to the tobacco mosaic virus genome (Rogers and Pfuderer 1968) leading to expression of polylysine. From that time, recombinant DNA technology became standard and in 1990 the first virus-based gene therapy for severe combined immunodeficiency entered the clinics (see also chapter 2.2). This was followed by the engineering of a mutant herpes simplex virus (HSV) for the treatment of malignant glioma by Martuza and coworkers. This mutant virus is deleted for thymidine kinase so that it can only replicate in dividing cells, whereas the non-proliferating normal brain tissue is spared, thus achieving some level of tumor specificity (Martuza, Malick et al. 1991). To date, two HSV-1 mutants, OncoVex\textsuperscript{GM-CSF} for melanoma and mutant 1716 for brain tumors, are tested in a phase II and phase III trial, respectively. After Martuza’s breakthrough research focused on the engineering of adeno- (Alemany, Balague et al. 2000; Mathis, Stoff-Khalili et al. 2005), paramyxo- (Nakamura and Russell 2004; Fielding 2005), herpes- (Latchman 2005), picorna- and poxviruses (McFadden 2005; Shen and Nemunaitis 2005). Currently, virotherapy aims to genetically engineer these viruses in order to achieve tumor specificity but without destroying their oncolytic potency.

2.4 Adenoviruses and their Use as Gene Therapy Vector or Oncolytic Virus

One very promising gene transfer vector and oncolytic virus is based on adenovirus (Ad) a non-enveloped, icosahedral virus with a double-stranded DNA genome, which has been investigated extensively since its initial description in the early 1950s. Therefore, the adenoviral genome structure and organization, capsid composition, and infection pathway are known in detail (Zhang 1999; Russell 2009). Besides this detailed knowledge of the adenoviral life cycle, Ads possess several other features that make them well suited for gene delivery and virotherapy, including the high capacity of Ad for transgene insertion (up to 36kb), their ability to efficiently transduce both non-dividing and actively dividing cells, as well as possessing high \textit{in vivo} stability. Adenoviruses can also easily be produced at high titers and purified, which is essential for clinical utility. Furthermore, the life cycle of Ad does not require integration into the host cell genome, thus foreign genes are expressed episomally thereby having low genotoxicity if applied \textit{in vivo}. Finally, the development of many recombinant DNA techniques for manipulating the viral genome, have made Ad a popular choice as a gene transfer vector. This is evidenced by the fact, that in 2008
Ads were being used in 24.9% of all gene therapy clinical trials (*The Journal of Gene Medicine*, 2008; http://www.wiley.co.uk/genetherapy/clinical/).

### 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 subgrouped 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).

![Schematic diagram of the Ad5 virion.](http://biomarker.cdc.go.kr:8080/index.jsp)

Adenoviruses are non-enveloped, icosahedral 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.3). 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. These complexes act 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 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 stabilizing the trimeric configuration of the fiber shaft, 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 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) bind to the plasma membrane protein CD46 expressed on hematopoietic and dendritic cells (Zhang and Bergelson 2005).

![Cell binding mechanism of Ad5 virion](image)

Fig. 4: **Cell binding mechanism of Ad5 virion.** Ad5 binds to its receptor CAR through the fiber knob domain, which is composed 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.
Introduction

Cell infection for most non-group B viruses starts with high-affinity binding to CAR via the fiber knob domain (Fig.4). 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 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.5).

![Fig.5: Schematic representation of adenovirus genome and transcription units](image)

The first viral transcription unit to be expressed is E1A, which activates transcription of the other 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 tumor 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
Introduction

genes involved in cell cycle entry and DNA replication. Such a cell cycle deregulation would normally lead to the accumulation of the tumor suppressor p53, followed by apoptosis. Expression of the adenoviral E1B genes prevent 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.

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).

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 host cell shutoff, which means 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 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 and results in the production of approximately $10^4$ new viral particles per cell. At approximately 30-40h (dependent on the cell type) after virus entry the new virus generation is released by cell lysis (Büchen-Osmond, 2003; http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.001.0.01.010.htm). 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

2.4.2.1 First-Generation Vectors
The initial strategy to design Ad vectors for gene therapy was to substitute the viral E1 region by transgenes. As described above this transcription unit encodes genes, which are necessary for the activation of viral promoters and the expression of both early and late genes. Thus, removal of the E1 coding sequence renders the vectors replication-defective and simultaneously reduces the toxicity to the transduced cell. Nevertheless, E1 genes are essential for viral replication and for the production of progeny viruses they therefore have to be provided in trans. For this purpose Ad-helper cell lines that complement E1 functions had been developed. One of the first Ad producer cell lines, a human embryonic kidney-derived cell line, 293, had been transformed by the Ad E1 region and therefore constitutively expresses E1 proteins (Graham, Smiley et al. 1977; Fallaux, Bout et al. 1998; Schiedner, Hertel et al. 2000). Although first generation Ad vectors have proven to be highly promising as vehicles for gene therapy, disadvantages do exist. One problem of the first generation Ad vectors is, that E1 deletion provides only 5.1 kb for insertion of therapeutic transgenes and sometimes higher transgene capacity is needed. To overcome this, Ad vectors with an additional E3 deletion have been developed. The entirely E3 unit is dispensable for virus growth in vitro and in combination with the E1 deletion the resulting vectors can accommodate up to 8.2 kb of foreign DNA. Another major impediment of the first generation vectors is, that in spite of the absence of E1 genes, these vectors express early and late genes at very low levels, causing direct toxicity and immunogenicity by the viral gene products. This can induce a cytotoxic T lymphocyte-mediated immune response that often leads to the clearance of vector-transduced cells and to transient transgene expression. Although this might preclude the use of first-generation vectors in some settings they still remain promising for applications that require short-term expression such as, many approaches of cancer gene therapy and vaccination.

2.4.2.2 Second-Generation Vectors
This generation of Ad vectors is characterized by various deletions of E1, E2 (Amalfitano, Hauser et al. 1998; Lusky, Christ et al. 1998) and E4 (Armentano, Sookdeo et al. 1995) genes. The E2 region encodes several proteins that are required for viral DNA replication, thus requires the expression of E2 genes in trans. However, the development of complementing cell lines that stably express E2
proteins has proved to be difficult due to the toxicity of the E2 proteins. Therefore, instead of deleting the entire E2 region only individual E2 genes were removed. The E4-deleted vectors were supposed to produce fewer late gene products compared to E1-only-deleted vectors, thus reducing the induction of vector-specific immune responses and leading to prolonged transgene expression (Armentano, Sookdeo et al. 1995; Gao, Yang et al. 1996; Wang, Greenburg et al. 1997). Nevertheless, E4-encoded proteins have a wide variety of functions thus deletion of the entire E4 region results in significant reduced viral yield. To overcome this, the E4 region was only partially deleted. For propagation of E4-deleted vectors complementing cell lines, like W162, had been generated that express the E4 proteins. It is noteworthy that E4orf6 alone is sufficient for replication in vitro, thus only E4orf6 needs to be expressed from such cell lines. In some studies these second-generation vectors indeed led to reduced inflammatory responses and improved transgene persistence; however other studies failed to demonstrate similar results, so these effects remain controversial (Gao, Yang et al. 1996; Lusky, Christ et al. 1998).

2.4.2.3 Helper-Dependent Vectors
This type of Ad vector, which holds the most promise for long-term transgene expression, is also called “high capacity” or “gutless” vector due to the lack of all viral genes. In this strategy all of the viral genes are deleted, except the two ITRs and the packaging signal, which allows up to 36 kb for transgene insertion. The propagation of such a vector needs the presence of a helper virus that provides the functions required for replication and assembly of the virions. Until now it has been difficult to completely separate virions containing the helper-dependent chromosomes from those containing the helper virus genome (Steinwaerder, Carlson et al. 1999; Sandig, Youil et al. 2000).

2.4.2.4 Conditionally Replication-Competent/Oncolytic Adenoviruses
Compared to the abovementioned Ad vectors the conditionally, replicative / oncolytic adenoviruses (OAds) are intended to replicate and spread exclusively in tumor cells with the aim to eliminate these cells through viral cytotoxicity (see also chapter 2.4.3). Hence, OAds are not just vehicles for the transfer of therapeutic genes, their replication itself is a therapeutic mechanism. Their ability to replicate provides the additional benefit of local amplification, but needs to be restricted to tumor cells.
Introduction

Strategies how the replication of OAds can be restricted to tumor cells will be presented in the following chapters.

2.4.3 Oncolytic Adenoviruses

In the last two decades the development of the abovementioned oncolytic adenoviruses (OAds) for the treatment of cancer has attracted great attention. This strategy exploits the lytic property of virus replication to kill tumor cells (see also chapter before) without causing excessive damage to normal non-cancerous tissues (Fig.6). However, when used as anticancer drugs, they must meet stringent criteria for safety and efficacy and be amenable to pharmacological study in human subjects. Specificity for cancerous tissue is the key to safety, and this goal can be achieved through a variety of virus-engineering strategies that aim to take advantage of tumor specific changes that allow preferential replication of the virus only in target cancer cells.

Fig.6: Concept of adeno viral 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 tumor-specific promoters. Oncolytic adenoviruses infect tumor cells, replicate their genome, assemble new viral particles and kill the host tumor 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 tumor cell killing. Genetic modifications of adenoviruses allow for efficacy enhancement and/or tumor cell restriction at various steps of the viral life cycle, such as viral infection, viral replication and/or virus release/tumor 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.
Tumor-specific adenoviral replication has for example been achieved by the mutation or deletion of viral genes or by placing viral genes that are essential for viral replication under the control of tumor-specific promoters. Other strategies aim to restrict viral replication to tumor cells by selective cell entry. The specificity of these strategies should prevent normal tissue toxicity and thereby provide improved targeting of metastatic tumors and allow systemic delivery.

2.4.3.1 Tumor-Selective Replication and Lysis of Oncolytic Adenoviruses by Viral Gene Mutations

Replication of the adenovirus genome after infection depends on the ability of the virus to effectively take over the biosynthesis machinery of the host cell to favor the synthesis of viral proteins. Studies in recent years have led to the generation of modified adenoviruses that contain gene mutations in key regulators of viral replication, whose products are essential for virus propagation in normal cells but not in tumor cells. In this regard, most approaches have focused on the genetic engineering of E1 genes, as these are the first viral genes expressed after host cell entry, which are mandatory to induce viral replication. The prototype for oncolytic adenoviral therapy is dl1520 or ONYX-015, which was published by Bischoff and colleagues in 1996, that contains a deletion of E1B55K. In infected cells this protein binds and inactivates the tumor-suppressor protein p53 thereby blocking premature apoptosis after E1A induced S-phase entry (Bischoff, Kirn et al. 1996). Thus, the presumption was that ONYX-015 can replicate only in p53-deficient tumor cells, but is unable to replicate in normal cells where wild-type p53 is expressed. Even though initial results obtained with ONYX-015 showed that it replicates better in tumor than in normal cells, it turned out that ONYX-015 could replicate in some tumor cells that retain wild-type p53. Subsequently, it was shown that loss of E1B55K-mediated late viral RNA export, rather than p53 degradation, restricts ONYX-015 replication to tumor cells (O'Shea, Johnson et al. 2004; O'Shea 2005). In contrast, tumor cells that support ONYX-015 replication provide the RNA export function of E1B55K (O'Shea, Johnson et al. 2004). However, it turned out that ONYX-015 was strongly attenuated even in tumor cells, which is probably due to the lack of E1B55K functions different from the export of late viral RNAs.

Another well-known OAd is Ad5Δ24, which has a 24bp deletion in the E1A region responsible for binding the Rb protein (see also chapter 2.4.1.3). This strategy aims to ablate S phase induction by the OAd, which is dispensable in proliferating (tumor)
Introduction

cells, but retain E1A transactivation functions that are crucial for viral gene expression and thus replication.

2.4.3.2 Tumor-Selective Replication and Lysis of Oncolytic Adenoviruses by Using Tissue-or Tumor-Selective Promoters

Selective viral replication can also be achieved by controlled viral gene expression at the transcriptional level. In this approach, key virus genes are placed under the control of tissue- or cancer-specific promoters, such that they are preferentially, if not exclusively, expressed in tumor cells, thus restricting replication to these cells. Critical for the development of such OAds is the availability of promoter fragments with specific activity and the retention of specific promoter activity after introduction into the adenoviral genome and during adenoviral replication. Rodriguez and co-workers (1997) were the first to create a prostate-specific OAd by inserting sequences from the prostate-specific antigen (PSA) promoter into the adenovirus genome for controlled E1A expression. It was demonstrated that the E1A expression of the so-called Calydon CV706 adenovirus indeed was limited to human prostate cancer cells. Moreover the study showed anti-tumor activity against human prostate tumor xenografts (Rodriguez, Schuur et al. 1997). Thereupon a number of OAds have been developed that exploit promoters of the genes encoding AFP (hepatocellular carcinoma) (Hallenbeck, Chang et al. 1999; Li, Yu et al. 2001; Ohashi, Kanai et al. 2001), kallikrein 2 (prostate cancer) (Yu, Sakamoto et al. 1999), probasin (prostate cancer) (Yu, Chen et al. 1999), MUC1 (breast cancer) (Kurihara, Brough et al. 2000) or tyrosinase (melanoma) (Nettelbeck, Rivera et al. 2002; Zhang, Akbulut et al. 2002) and others. Nevertheless, it has been shown that viral sequences, such as the left ITR or the packaging sequence, could interfere with cellular promoters placed in the viral genome allowing non-specific viral replication (Miralles, Cortes et al. 1989; Hatfield and Hearing 1991; Hoffmann, Jogler et al. 2005; Hurtado Pico, Wang et al. 2005). Several different approaches have been pursued to prevent promoter interference by read-through transcription from cryptic initiation sites: One strategy is to insert a poly A transcription termination signal upstream of cellular promoters (Vassaux, Hurst et al. 1999; Martin-Duque, Jezzard et al. 2004). Another strategy is to reverse the promoter E1A expression cassette, which has resulted in improved selectivity but reduced efficiency of expression of the reversed gene (Steinwaerder and Lieber 2000; Yamamoto, Davydova et al. 2003). Other groups implemented the incorporation of insulator sequences, DNA sequence
elements that could block the interactions between promoters and enhancers (West, Gaszner et al. 2002). Another major limitation of OAds with tissue- or tumor-specific promoters is that they are not always active in all tumor types. Even within a tumor, it is unlikely that all of the cells will express a specific tumor marker, because most tumors are heterogeneous. Therefore, researchers had focused on identifying promoters that are more universally active in all tumor types. E2F-1, for instance, which is an important transcription factor, is responsible for the activation of genes that promote the transition from G1 to S phase of the cell cycle. Tumor cells mostly have constitutively high levels of E2F-1 promoter activity due to a disruption of pathways that control its activity. The AdE2F-1 virus, in which the E1A gene is under the control of the E2F-1 promoter, has shown to induce cytolysis selectively in tumor cells, but not in non-proliferating normal cells (Johnson, Shen et al. 2002; Tsukuda, Wiewrodt et al. 2002).

2.4.3.3 Genetic Modification of the Virus Capsid for Efficient Cell Entry of Oncolytic Adenoviruses
Although adenoviruses possess a very high capacity to achieve in vivo gene transfer, the overall efficacy of OAds in patients remained limited. Even with ONYX-015 that demonstrated promising antitumor activity in preclinical studies, only little effect was seen in a phase I trial of 16 women with refractory ovarian cancer that received ONYX-015 intraperitoneally (Vasey, Shulman et al. 2002). One possible reason leading to limited efficacy of OAds in vivo has been shown to result from a paucity of expression of the primary receptor CAR on tumors. Indeed, freshly isolated tumor cells, compared to their cell line counterparts, were shown to be resistant to adenovirus infection due to a lack of CAR expression on their cell surface (Hemmi, Geertsen et al. 1998; Miller, Buchsbaum et al. 1998; Li, Pong et al. 1999). For instance, adenovirus serotype 5, upon which most of the oncolytic adenoviruses are based, has demonstrated only low transduction rates of several primary cancer cells as well as some cancer cell lines, such as squamous cell carcinoma, glioma, bladder cancer cells and melanoma (Hemmi, Geertsen et al. 1998; Miller, Buchsbaum et al. 1998; Li, Pong et al. 1999). Clearly, a lack of CAR expression on tumor cells will restrict not only the efficiency of infection by initially injected virus but also the ability of the virus to spread afterwards.

Based on these observations researchers focused on the genetic modification of the virus capsid to enhance cellular transduction. As already mentioned in chapter
The mechanism of Ad binding and internalization is mediated by the two capsid proteins, fiber and penton base. Whereas the globular knob domain of the Ad5 fiber binds to CAR, the Arg-Gly-Asp (RGD) motifs of the penton base interact with cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Therefore the most obvious approach to achieve efficient or even selective cell entry is to alter the tropism of the Ad via genetic modifications of the fiber proteins. First efforts focused on the incorporation of peptide ligands into the C-terminus of the fiber protein. For example, the incorporation of polylysine residues, which target heparan sulfates, has been shown to result in increased transduction efficiency for fibroblasts, T cells, glioma cells, myeloma cells, skeletal muscle cells and others ((Wickham, Tzeng et al. 1997; Yoshida, Sadata et al. 1998; Gonzalez, Vereecque et al. 1999; Hidaka, Milano et al. 1999). Augmented transduction efficiency was also seen for endothelial and smooth muscle cells after introduction of a RGD motif into the C-terminus (Wickham, Tzeng et al. 1997; McDonald, Zhu et al. 1999).

Another locale for the insertion of peptides was identified after the crystal structure of the Ad5 fiber was revealed by X-ray crystallography. The HI loop, which is exposed on the fiber knob, can accommodate insertions of up to 63 amino acids (Krasnykh, unpublished data), without disturbing proper fiber folding. Krasnykh and co-workers were the first to incorporate a FLAG octapeptide into the HI loop, demonstrating that a genetically modified fiber protein retained its ability to trimerize and to bind and infect CAR-positive cells, indicating that native tropism had not been abolished (Krasnykh, Dmitriev et al. 1998). Subsequently, Dmitriev and co-workers inserted the targeting peptide RGD-4C into the HI loop of an Ad (Dmitriev, Krasnykh et al. 1998). This resulted in an expanded tropism for the Ad, since on one hand it retained the ability to recognize CAR, but on the other hand was able to bind cells via the penton bases thus entry occurred through the $\alpha_v$ integrin-mediated pathway. Ads containing the RGD motif in the HI loop showed enhanced transduction efficacy for primary and established ovarian cancer cells (Vanderkwaak, Wang et al. 1999; Biermann, Volpers et al. 2001), squamous cell carcinoma of the head and neck (Kasono, Blackwell et al. 1999), glioma (Grill, Van Beusechem et al. 2001; Mizuguchi, Koizumi et al. 2001), pancreatic cancer cells (Wesseling, Bosma et al. 2001), as well as dendritic cells (Dcs) (Asada-Mikami, Heike et al. 2001) primary endothelial cells (Biermann, Volpers et al. 2001) and others.

Another mechanism to achieve tropism modification of adenoviruses is to replace the complete knob domain with those from other serotypes, also termed fiber
pseudotyping. Initially, the Ad5 fiber knob domain was substituted with that of Ad3 fiber (Krasnykh, Mikheeva et al. 1996), which resulted in enhanced cytotoxicity of oncolytic adenoviruses for primary melanoma cells (Rivera, Davydova et al. 2004), 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). Several groups have also used Ad35 for fiber substitutions and have demonstrated higher transduction efficiency of adenoviral vectors into human CD34(+) stem cells and dendritic cells (Shayakhmetov, Papayannopoulou et al. 2000; Gao, Robbins et al. 2003; Sakurai, Mizuguchi et al. 2003; Sakurai, Mizuguchi et al. 2003). Furthermore, biodistribution studies in mice showed extremely low levels of Ad35 uptake in the liver, lung, spleen and bone marrow (Seshidhar Reddy, Ganesh et al. 2003).

2.4.3.4 Potential Hurdles Limiting Oncolytic Adenovirus Efficacy

Pre-clinical and clinical studies have clearly shown that adenoviral oncolysis is feasible as a new modality to treat cancer. Likewise, they revealed that the therapeutic efficiency of OAds is limited and their ability to completely eradicate a tumor has not been reached so far thus further optimization is needed. One major impediment is that the pattern of infection within a solid tumor mass is often highly heterogeneous and incomplete (Heise, Williams et al. 1999). This is probably due to physical barriers such as interstitial pressure (Tanaka, Yamanaka et al. 1997), necrotic areas, normal stroma cells, extracellular matrix or the presence of the basal membrane that hinder the virus to spread insight the entire tumor mass. In fact, mathematical models of viral replication demonstrated that inoculation of the tumor must be diffuse throughout the tumor in order to control it and for the activation of a self-perpetuating process of intratumoral viral replication (Wein, Wu et al. 2003). Furthermore, the use of OAds has been considered not only for locoregional tumors, but also for disseminated tumors that require systemic delivery. But already in 1952, Chester Southam and Alice Moore identified the immune response as the first obstacle in the use of viruses for cancer therapy (Southam and Moore 1952). Indeed, systemically given Ads are rapidly cleared by neutralizing antibodies (Green and Seymour 2002), due to pre-existing or de novo cellular immunity. Large proportion of viral particles is also sequestered by the liver where they may cause significant toxicity which in turn limits their use in systemic virotherapy (Fechner, Haack et al. 1999; Bernt, Ni et al. 2003). Further, the uptake of Ads by liver Kupffer cells and
other macrophages result in activation of innate immune responses, which lead to
the release of large quantities of inflammatory cytokines (Liu and Muruve 2003).
Another obstacle that limits the efficacy of oncolytic adenoviruses is the insufficient
expression of viral receptors on target tumors, which has already been described in
the previous chapter. Hence it appears that next generations of oncolytic Ads need
further optimization.

2.4.3.5 Strategies to Improve the Therapeutic Efficacy of Oncolytic
Adenoviruses
In order to overcome the described roadblocks for OAd efficacy two major strategies
are being pursued currently. The first strategy aims to improve the lytic activity of
oncolytic Ads. As already mentioned in the previous chapter viral oncolysis can be
hampered by physical barriers, immune responses and/or by insufficient expression
of viral receptors on target tumors (see chapter 2.4.3.3). To circumvent physical
barriers a pre-treatment with matrix-modifying agents (bacterial collagenase,
hyaluronidase) had been developed that was supposed to alter the extracellular
matrix thus facilitating virus penetration (McKee, Grandi et al. 2006; Mok, Boucher et
al. 2007). Others used vaso-active compounds, such as bevacizumab, bradykynin or
low-dose paclitaxel to increase the blood-tumor permeability (Bilbao, Bustos et al.
2000).
Methods to reduce unmeant immune responses upon oncolytic adenovirus
inoculation have also been proposed. For instance, the administration of
cyclophosphamid, which is able to inhibit the induction of neutralizing antibodies,
macrophages, regulatory T cells and intratumoral interferon-γ production (Di Paolo,
Tuve et al. 2006; Fulci, Breymann et al. 2006; Lamfers, Fulci et al. 2006), which
otherwise prevent adenoviral replication.
Another strategy to enhance lytic activity includes the modification of adenoviral
genes regulating cell death. The E1B19K gene is the most prominent adenoviral
gene that exerts potent anti-apoptotic function by several mechanisms. Early studies
showed that E1B19K has sequence homology with members of the Bcl-2 family and
interacts and inhibits the pro-apoptotic function of Bax, Bak and Nbk/Bik ((Chiou,
Tseng et al. 1994; Chen, Branton et al. 1996; Han, Sabbatini et al. 1996; Han,
Sabbatini et al. 1996). Additionally, the E1B19K protein is able to block Fas and
tumor necrosis factor receptor I-mediated apoptosis, by interacting with the protein
FLASH (Imai, Kimura et al. 1999). Notably, in the early 1980s Chinnadurai and
colleagues analyzed Ad2 and Ad5 mutants that showed a large plaque phenotype in tumor cells, indicative of accelerated cell-to-cell spread. After characterization of these mutants it turned out that they were deficient for \textit{E1B19K} (Chinnadurai 1983). Hence, it was suggested that \textit{E1B19K}-deleted adenovirus might have great potential to improve oncolysis. However, the effect of \textit{E1B19K} deletion on viral replication was considered to be of concern, as premature death of the host cell and viral DNA degradation may compromise viral yield (Pilder, Logan et al. 1984; Subramanian, Kuppuswamy et al. 1984; Subramanian, Kuppuswamy et al. 1984; Hu and Hsu 1997). Importantly, in an effort to improve adenoviral oncolysis, Sauthoff and co-workers demonstrated increased cytotoxicity in tumor cells for an \textit{E1B19K}-deleted Ad5 mutant, without reduction in infectious particle production (Sauthoff, Heitner et al. 2000). Indeed, such an accelerated phenotype is of special interest considering the limited therapeutic window for virus activity due to mounting anti-viral immune responses, where rapid virus spread might be crucial for therapeutic success.

Another gene that has been shown to mediate efficient cell lysis and release of progeny viruses from host cells is the adenovirus death protein (ADP) (see also chapter 2.4.1.3). This gene resides in the E3 region of the adenovirus genome and has often been deleted due to size limitations. However, reintroduction or overexpression of the ADP gene caused more rapid cell lysis and enhanced cell-to-cell spread, which significantly increased anti-tumor activity (Doronin, Toth et al. 2000; Kuppuswamy, Spencer et al. 2005).

An alternative perspective to increase the efficiency of oncolytic Ads is to employ additional cytotoxic mechanisms beyond the direct lytic properties of the virus. This can be achieved by “arming” these viruses with therapeutic transgenes, such as, cytokine, cytotoxic, suicide, immunoregulatory or siRNA genes, which result in a potent bystander effect capable of eliminating tumor cells that the virus cannot reach. Thus, the goal here is to combine viral oncolysis with different gene therapy approaches (see chapter 2.2).

Previously, it has also been shown that oncolytic viruses expressing cytotoxic fusogenic membrane glycoproteins of measles virus can kill tumor cells by fusing them into lethal multi-nucleated syncytia (Horn, Vongpunsawad et al. 2005).

The suicide gene therapy involves the transfer and expression of genes encoding enzymes that convert non-toxic prodrugs, which can be administered systemically at high doses, into a toxic metabolite (Springer and Niculescu-Duvaz 2000). The most advanced prodrug-based therapies exploited in OAds thus far are thymidine kinase
(TK) and cytosine deaminase (CD) and their respective prodrugs ganciclovir (GCV) and 5-fluorocytosine (5-FC), which have shown increased therapeutic activity (Kirn, Niculescu-Duvaz et al. 2002; van Dillen, Mulder et al. 2002). A further improvement of this strategy was realized by the CD/TK fusion enzyme engineered into an oncolytic adenovirus. This has shown promising results in a clinical prostate cancer study that resulted in two complete tumor regressions and four partial regressions out of 14 patients (Freytag, Khil et al. 2002).
3 Objectives of the Study

The aim of the first project was the development of adenoviral vectors, both replication-deficient and replication-competent, that feature stringent heat-inducible transgene expression. Inducible gene expression systems in context of adenoviral vectors that allow for temporal and even spatial control are highly desired and have been implemented by several groups before. However, so far these approaches failed to show the required efficacy. For that purpose, the first project aimed to analyze the feasibility of a promoter fragment of the human hsp70B' gene (hsp70B'p), that features unique stringency, for heat-regulated transgene expression by replication-deficient or oncolytic adenoviruses (OAds). First, the activity of the hsp70B'p with and without heat-shock should be analyzed in transient transfection assays by determining reporter gene activity in a panel of different tumor cell lines. This included the comparison to constructs containing either no promoter or constitutively active promoters. So far the hsp70B'p had not been analyzed in context of an Ad genome. Therefore, the hsp70B' promoter-reporter cassette should be incorporated into replication-deficient and OAd to evaluate the effects of Ad gene transfer and Ad genome replication with and without heat stimulus on this promoter.

Finally, it should be investigated whether optimized insulator elements could improve heat-inducible gene expression in the context of replication-deficient and replicating Ads.

The second project was designed to improve the therapeutic potency of OAds by coupling two strategies: First, accelerated virus release and spread should be accomplished by mutating viral genes. Second, insertion of transgenes into the genome of OAds should aim at the killing of neighbouring uninfected cancer cells by transgene-encoded therapeutic proteins, thereby generating so-called “armed” OAds. However, it was unclear whether early lysis of cancer cells and efficient transgene expression by the same cell are compatible. Therefore, an OAd should be generated that features a deleted anti-apoptotic viral gene E1B19K and an insertion of the reporter gene luciferase via an IRES into the late transcription unit. By performing cytotoxicity assays in a panel of tumor cell cultures the effect of E1B19K-deletion on cell lysis and spread should be investigated and compared to the E1B19K-positive counterpart. Considering the anti-apoptotic activity of E1B19K it should be analyzed by which mechanism cells die after infection with the E1B19K- virus in comparison to E1B19K+ virus. To determine whether E1B19K-deletion affects virus replication, infectious viral particle production and real-time PCRs to quantify Ad genomes and mRNA expression of adenoviral late genes should be performed. Finally, reporter gene assays should reveal whether the E1B19K deletion modulates transgene expression during both, a virus replication cycle and prolonged virus infection and spread.
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$_2$HPO$_4$; 1.4mM KH$_2$PO$_4$; 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 flow cytometry
10x Annexin V Binding Buffer (BD Biosciences, Heidelberg)

4.1.2.4 Buffers and solutions for viral lysis
TE-buffer: 100mM Tris-HCl pH 8.0; 10mM EDTA
VL buffer: 1M TE buffer; 10% SDS

4.1.2.5 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

4.1.2.6 Buffers and solutions for DNA precipitation
Lithium chloride: 4M
EtOH: 100%

4.1.2.7 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 MgSO₄; 10mM MgCl₂; 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 (Lonza, Belgium; 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 PO₄ 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)
Amphotericine B: 250µg/ml (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 Electro Maximum DH5α: genotype: F Φ80ΔlacZΔM15, rec A1, end A1, hsdR17 mcrA, mcrB, mcrC, mrr (Invitrogen, Karlsruhe)
**Materials and Methods**


### 4.1.4.2 Human cells lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Cell Type</th>
<th>Medium</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>Human lung adenocarcinoma epithelial cell line</td>
<td>DMEM/10%FCS/1%P/S/G</td>
<td>ATCC (Manassas, USA)</td>
</tr>
<tr>
<td>C8161</td>
<td>Melanoma cell line</td>
<td>DMEM/10%FCS/1%P/S/G</td>
<td>(Welch et al., 1991)</td>
</tr>
<tr>
<td>Colo829</td>
<td>Melanoma cell line</td>
<td>RPMI/10%FCS/1%P/S/G/0.04% β-Me</td>
<td>J. Banchereau (Texas, USA)</td>
</tr>
<tr>
<td>HaCat</td>
<td>immortalized keratinocyte cell line</td>
<td>DMEM/10%FCS/1%P/S/G</td>
<td>P. Boukamp (Heidelberg, Germany) (Boukamp, Petrussevska et al. 1988)</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervical carcinoma cell line</td>
<td>DMEM/10%FCS/1%P/S/G</td>
<td>ATCC (Manassas, USA)</td>
</tr>
<tr>
<td>HFF</td>
<td>Primary normal foreskin fibroblast</td>
<td>MEM/7.5% FCS/1%G/1% gentamycin</td>
<td>M. Marschall (Erlangen, Germany)</td>
</tr>
<tr>
<td>293</td>
<td>Human embryonic kidney cells (stably transformed with sheared Ad5 DNA)</td>
<td>DMEM/10%FCS/1%P/S/G</td>
<td>G. Fey (Erlangen, Germany)</td>
</tr>
<tr>
<td>Mel624</td>
<td>Melanoma cell line</td>
<td>RPMI/10%FCS/1%P/S/G</td>
<td>J. Schlom (Bethesda, USA)</td>
</tr>
<tr>
<td>Mel888</td>
<td>Melanoma cell line</td>
<td>RPMI/10%FCS/1%P/S/G</td>
<td>J. Schlom (Bethesda, USA)</td>
</tr>
<tr>
<td>NCH82</td>
<td>Low passage glioblastoma</td>
<td>DMEM/10%FCS/1%P/S/G/1% hepes 0.1% amphotericine B</td>
<td>C. Herold-Mende (Heidelberg, Germany)</td>
</tr>
<tr>
<td>NCH89</td>
<td>Low passage glioblastoma</td>
<td>DMEM/10%FCS/1%P/S/G/1% hepes 0.1% amphotericine B</td>
<td>C. Herold-Mende (Heidelberg, Germany)</td>
</tr>
<tr>
<td>NCH468</td>
<td>Low passage glioblastoma</td>
<td>DMEM/10%FCS/1%P/S/G/1% hepes 0.1% amphotericine B</td>
<td>C. Herold-Mende (Heidelberg, Germany)</td>
</tr>
<tr>
<td>PMelA</td>
<td>Low passage melanoma cells</td>
<td>As mentioned above for primary melanoma</td>
<td>D. Dieckmann, M. Lüftl (Erlangen, Germany)</td>
</tr>
<tr>
<td>PMelL</td>
<td>Low passage melanoma cells</td>
<td>As mentioned above for primary melanoma</td>
<td>D. Dieckmann, M. Lüftl (Erlangen, Germany)</td>
</tr>
<tr>
<td>SK-Mel-28</td>
<td>Melanoma cell line</td>
<td>DMEM/10%FCS/1%P/S/G</td>
<td>ATCC (Manassas, USA)</td>
</tr>
</tbody>
</table>

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).

Ad5/3 WT: Ad5 wild type with a chimeric fiber: fiber-knob domain was replaced with the fiber-knob domain of Ad serotype 3; provided by D.Curiel, Birmingham, USA (Davydova, Le et al. 2004).

Ad5/3-luc: serotype 5 derived Ad that contains the fiber-knob domain from adenovirus serotype 3, replication-deficient Ad; a firefly luciferase-transgene cassette was inserted under the control of the CMV immediate early (CMV-IE) promoter in place of the deleted E1 region (Volk, Rivera et al. 2003).

Ad337: Ad serotype 5 with a deletion of E1B19K resulting from a 146bp deletion between nucleotide positions 1770 and 1916. The E1B55K reading frame remains intact under its usual control; provided by Matthias Dobbelstein.

Ad2xTyr: Ad serotype 5 that expresses E1AΔ24 from an optimized human tyrosinase enhancer/promoter (hTyr2E/P) and E4 from a similar murine tyrosinase enhancer/promoter (mTyr2E/P) (Banerjee, Rivera et al. 2004).

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Description</th>
<th>Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp70B’_for</td>
<td>5’-CGC ATG GTA ACA TAT CTT CGG TC-3’</td>
<td>Human hsp70B’ promoter</td>
<td>245bp</td>
</tr>
<tr>
<td>hsp70B’_rev</td>
<td>5’-GTC GAT GCC CAC CGC GAG CTC-3’</td>
<td>245bp</td>
<td></td>
</tr>
<tr>
<td>Gp96p_for_(BglII)</td>
<td>5’-GAT CAG ATC TTT GCA ACC TCT CTC GTG TGT TTC-3’</td>
<td>Human gp96/grp94 promoter</td>
<td>449bp</td>
</tr>
<tr>
<td>Gp96p_rev_(NcoI)</td>
<td>5’-GAT CCC ATG GCG TGC GCG TGG TTA GTC TCA AG-3’</td>
<td>449bp</td>
<td></td>
</tr>
<tr>
<td>19K-5’</td>
<td>5’-CGA GGA CTT GCT TAA CGA GC-3’</td>
<td>Adenoviral E1B19K deletion</td>
<td>504bp</td>
</tr>
<tr>
<td>19K-3’</td>
<td>5’-GGA CGG AAG ACA ACA GGA GC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

4.1.6.1.2 **Oligonucleotides for controlling recombinant modified Ad genomes**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeqITR for</td>
<td>5'-CGG GAA AAC TGA ATA AGA GGA AGT GA-3'</td>
</tr>
<tr>
<td>Ad1124-1100 rev</td>
<td>5'-ATT TTC ACT TAC TGT AGA CAA ACA T-3'</td>
</tr>
<tr>
<td>E1AΔ24 rev</td>
<td>5'-AAA GCC AGC CTC GTG GCA GGT AAG-3'</td>
</tr>
<tr>
<td>pGL3-Seq3' MCS rev</td>
<td>5'-TTA TGC AGT TGC TCT CCA CCG GTT C-3'</td>
</tr>
<tr>
<td>mTyr for</td>
<td>5'-CAT CCA GTA AGT CCA TTA CTC AC-3'</td>
</tr>
<tr>
<td>E3 for</td>
<td>5'-CTG CTA GTT GAG CGG GAC AGG GGA C-3'</td>
</tr>
<tr>
<td>E3 rev</td>
<td>5'-GGC AAG GAG GTG CTG CTG AAT AAA C-3'</td>
</tr>
<tr>
<td>E4-for</td>
<td>5'-ATT GAA GCC AAT ATG ATA ATG AGG G-3'</td>
</tr>
<tr>
<td>E4-rev</td>
<td>5'-CAC AGC GGC AGC CTA ACA GTC-3'</td>
</tr>
<tr>
<td>5knob for</td>
<td>5'-AGG CAG TTT GGC TCC AAT ATC TGG-3'</td>
</tr>
<tr>
<td>3knob for</td>
<td>5'-TTA ATG TAG AAC TAT ACT TTG ATG C-3'</td>
</tr>
<tr>
<td>Seq Mfe fiber rev</td>
<td>5'-TGT ATA AGC TAT GTG GTG GTG GGG-3'</td>
</tr>
<tr>
<td>E1B19K-5'</td>
<td>5'-CGA GGA CTT GCT TAA CGA GC-3'</td>
</tr>
<tr>
<td>E1B19K-3'</td>
<td>5'-GGA CGG AAG ACA ACA GTA GC-3'</td>
</tr>
</tbody>
</table>

Table 3: Oligonucleotides for controlling recombinant modified Ad genomes

<table>
<thead>
<tr>
<th>Combination of oligonucleotides</th>
<th>Ad genome region</th>
<th>DNA fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeqITR for + Ad1124-1100 rev</td>
<td>E1A-region</td>
<td>Ad5 WT; Ad5/3 WT: 844bp, Ad5 2xTyr.hsp70B'pL; Ad5 Δ24 E3-; Ad5/3 Δ24 IL; Ad5/3 Δ24 E1B19K- IL: 820bp</td>
</tr>
<tr>
<td>E1AΔ24 rev + Seq ITR</td>
<td>E1A-region</td>
<td>Ad5 WT: 666bp, AdΔ24: -</td>
</tr>
<tr>
<td>pGL3-Seq3' MCS rev + Seq ITR</td>
<td>E1A-region with luciferase insertion</td>
<td>Ad5-CMV-luc: 1030bp Ad5-SV40-luc: 601bp</td>
</tr>
<tr>
<td>pGL3-Seq3' MCS rev + E4 for</td>
<td>E4-region with luciferase insertion</td>
<td>Ad5 2xTyr.hsp70B'pL: 599bp</td>
</tr>
<tr>
<td>pGL3-Seq3' MCS rev + 5knob for</td>
<td>Fiber with luciferase insertion</td>
<td>Ad5/3 Δ24 fiber IL; Ad5/3 Δ24 E1B19K- IL: 1172bp</td>
</tr>
<tr>
<td>pGL3-Seq3' MCS rev + 3knob for</td>
<td>Ad5 fiber with luciferase insertion and Ad3 knob domain</td>
<td>Ad5/3 D24 fiber IL; Ad5/3 D24 E1B19K- IL: 1140bp</td>
</tr>
</tbody>
</table>
4.1.6.1.3 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL3-for</td>
<td>5'-CTA GCA AAA TAG GCT GTC CCC TA-3'</td>
</tr>
<tr>
<td>pGL-rev</td>
<td>5'-CTT TAT GTT TTT GGC GTC TTC C-3'</td>
</tr>
<tr>
<td>SeqITR</td>
<td>5'-CGG GAA AAC TGA ATA AGA GGA AGT GA-3'</td>
</tr>
<tr>
<td>Seq Mfe fiber rev</td>
<td>5'-TGT ATA AGC TAT GTG GTG GTG GGG-3'</td>
</tr>
<tr>
<td>CMV for</td>
<td>5'-CGC AAA TGG GCG GTA GGC GTG-3'</td>
</tr>
<tr>
<td>19K-for</td>
<td>5'- CGA GGA CTT GCT TAA CGA GC-3'</td>
</tr>
<tr>
<td>19K-rev</td>
<td>5'- CGA GGA CTT GCT TAA CGA GC-3'</td>
</tr>
<tr>
<td>pGL for</td>
<td>GTA TCT TAT GTG GTG GTG GTG GGG-3'</td>
</tr>
<tr>
<td>pS for</td>
<td>GTA TCT TAT GTG GTG GTG GTG GGG-3'</td>
</tr>
<tr>
<td>Seq TPL</td>
<td>5'-CGT AAC CGA GTA AGA TTT GGC-3'</td>
</tr>
</tbody>
</table>

4.1.6.1.4 Oligonucleotides for quantitative real time PCR (qPCR)

<table>
<thead>
<tr>
<th>Human gene</th>
<th>Orientation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>sense</td>
<td>5'-GGT TTA CAT GTT CCA ATA TGA TTC CA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5'-ATG GTA CAA GGA CTT TTT CCC AGA AG-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>sense</td>
<td>5'-TAA GTA GGC GCA CAG TAG GTC TGA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5'-AAA GTG CAA AGA ACA CGG GTA AG-3'</td>
</tr>
</tbody>
</table>
Materials and Methods

### Table 6: Oligonucleotides for quantitative real time PCR

<table>
<thead>
<tr>
<th>Adenovirus gene</th>
<th>Orientation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4</td>
<td>sense</td>
<td>5’-GGT TGA TTC ATC GGT CAG TGC-3’</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5’-TAC GCC TGC GGG TAT GTA TTC-3’</td>
</tr>
<tr>
<td>Fiber</td>
<td>sense</td>
<td>5’-TGA TGT TTG ACG CTA CAG CCA TA-3’</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5’-GAT TTG TGT TTG GTG CAT TAG GTG-3’</td>
</tr>
<tr>
<td>Hexon</td>
<td>sense</td>
<td>5’-ACC TGG GCC AAA ACC TTC TC-3’</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5’-CGT CCA TGG GAT CCA CCT C-3’</td>
</tr>
</tbody>
</table>

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).

**pGL3hsp70B’pL**: luciferase reporter plasmid that contains the hsp70B’ promoter upstream of the luciferase gene (Knippertz, unpublished)

**pGL3gp96**: luciferase reporter plasmid that contains the human gp96/grp94 located upstream of the luciferase gene (Knippertz, unpublished)

**pGL3-hTyr2E/P**: luciferase reporter plasmid that consists of a synthetic construct composed of the 260bp core promoter fused to a dimer of a 200bp distal enhancer, both from the human tyrosinase gene (Nettelbeck et al., 1999; Siders et al., 1996).

**pGL3-mTyr2E/P**: luciferase reporter plasmid that consists of a synthetic construct composed of a 700bp core promoter fused to a dimer of a 200bp distal enhancer, here from the murine tyrosinase gene (Banerjee et al., 2004).

**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**: 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).

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

pSΔ2419K: contains a mutant E1A gene with a 24bp deletion (E1AΔ24) to prevent binding and inactivating pRB. Additionally contains a 146bp deletion of E1B19K, however the E1B55K reading frame remains intact under its usual control.

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

pVK500fiberIL: plasmid containing the Ad5 genome with partial deletion of the fiber gene; additionally the reporter gene luciferase is fused via an IRES to the fiber gene.

### 4.1.6.3 Antibodies

#### Antibodies for western blot analysis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clonality</th>
<th>Species</th>
<th>Dilution</th>
<th>Buffer condition</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-PARP (Poly(ADP-ribose) polymerase)</td>
<td>monoclonal</td>
<td>mouse</td>
<td>1:1000</td>
<td>5%BSA/TBST</td>
<td>BD Biosciences (Heidelberg)</td>
</tr>
<tr>
<td>Active/Cleaved Caspase-3</td>
<td>monoclonal</td>
<td>mouse</td>
<td>1:2000</td>
<td>5%BSA/TBST</td>
<td>Imgenex (San Diego)</td>
</tr>
<tr>
<td>Ad5 fiber tail (monomeric and trimeric)</td>
<td>monoclonal</td>
<td>mouse</td>
<td>1:2000</td>
<td>TBST</td>
<td>Abcam (Cambridge)</td>
</tr>
<tr>
<td>Ad5 hexon</td>
<td>polyclonal</td>
<td>rabbit</td>
<td>1:2000</td>
<td>TBST</td>
<td>Abcam (Cambridge)</td>
</tr>
<tr>
<td>Human β-Actin</td>
<td>monoclonal</td>
<td>mouse</td>
<td>1:2000</td>
<td>TBST</td>
<td>Sigma (Deisenhofen)</td>
</tr>
<tr>
<td>mouse IgG-HRP</td>
<td>polyclonal</td>
<td>goat</td>
<td>1:5000</td>
<td>5%MMP/TBS</td>
<td>Cell Signaling Technology (Danvers)</td>
</tr>
<tr>
<td>Rabbit IgG H&amp;L-HRP</td>
<td>polyclonal</td>
<td>goat</td>
<td>1:10000</td>
<td>5%MMP/TBS</td>
<td>Cell Signaling Technology (Danvers)</td>
</tr>
</tbody>
</table>

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 1:4. 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
Materials and Methods

resuspended in 150ml of ice-cold, sterile 0.1M MgCl₂-solution and incubated on ice for 1 h. Following centrifugation (see above) bacteria were resuspended in 12ml ice-cold, sterile 0.1M CaCl₂-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.

4.2.1.1.2 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 over night culture was added to Erlenmeyer-flasks containing a total volume of 500ml LB-medium. Cells were cultivated until they reached OD₆₀₀nm 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.
**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 pVK500fiberIL) 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.

**Preparation of DNA and RNA**

**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.

**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 150μl 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.

4.2.1.3 PCR (polymerase 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 MgCl₂ 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 MgCl₂, 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:
Annexing 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).

8.2.1.3.1 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 4.1.5.1.3.1 Oligonucleotides for quantitative real time PCR (qPCR)). 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
(10^6, 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. Data were analyzed with the 7300 System SDS
Software (Applied Biosystems) and are presented as Ad genome copy numbers/ ng
Materials and Methods

4.2.1.4 Protein biochemical and immunological methods

4.2.1.4.1 Preparation of total cell lysate
For the preparation of total cell lysate from Ad-infected cells (analysis of PARP and Caspase-3 cleavage), 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 Dc 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 Discontinuous SDS-Polyacrylamidegelectrophoresis (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/cm². Whatman filters (Schleicher &
Materials and Methods

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/NaN₃) 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 10 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 10 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 10 min to 45 min.

4.2.1.4.6 Flow cytometry for the detection of cell death
To analyze cell death after adenoviral infection Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was utilized. Annexin V-FITC is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V-FITC is a calcium-dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas membranes of dead and damaged cells are permeable to PI. Cells that stain only positive for Annexin V-FITC are undergoing apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both are alive and not undergoing measurable apoptosis.
To determine whether adenoviral infection leads to apoptosis induction 600.000 cells were plated in 10 cm dishes (Greiner). The following day cells were infected with the
indicated Ads at 10,000 vp/cell or were mock-infected in 5ml growth medium containing 2% FBS. Two hours post-infection growth medium containing 10% FBS was added. As a positive control cells were either irradiated with UVC (Stratalinker, Stratagene, Waldbronn/Karlsruhe; 80kJ; for PMelL) or UVB (Waldmann UV 181 BL, 3 x 0.5 J/cm²; for A549). 16h (PMelL) or 26h (A549) after irradiation and 48h post-infection cells were harvested by trypsinization, centrifuged for 5min at 1300rpm and afterwards resuspended in 450µl 0.5 x Binding Buffer. 100µl of the solution were transferred to four different FACS-tubes. The first tube was left untreated, the second was stained for Annexin V-FITC alone, the third for propidium iodide alone and the fourth was stained for both Annexin V-FITC and propidium iodide following the manufacturer’s staining protocol.

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% CO₂. 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 Transient transfections and reporter assays
4.2.1.5.2.1 Transient transfection for the analysis of promoter activities
Transient transfection for the analysis of promoter activities was performed using Lipofectamine (Invitrogen) according to the manufacturer’s instruction. Promoter-reporter gene constructs were transfected in triplicates and each experiment was repeated at least thrice including usage of a second plasmid preparation. Fifty-thousand cells/well were seeded into 24-well plates in 0.5ml accordant growth
medium one day prior to transient transfection. The next day, 0.4μg of promoter-reporter gene plasmid was mixed with 1μl of Lipofectamine, adjusted to a volume of 25μl with OptiMEM (Invitrogen) and incubated at RT for 30 min. Meanwhile cells were washed with 200μl OptiMEM. After 30 min of incubation at RT, 50μl of transfection mixture was dispersed in 150μl of OptiMEM onto exponentially growing cells (70-80% confluency). Four hours later transfection-mixtures were removed and replaced with relevant growth medium. Forty-eight hours post transfection cells were harvested for Luciferase reporter assay.

For heat shock experiments, transfected cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ overnight. The following day, heat-shock was carried out by incubating the cells at 42°C for 90 min. Control cells were not heat-shocked. After 90 min heat-shocked cells were subsequently incubated at 37°C for another 4.5 h. Then cells were harvested as described below.

4.2.1.5.2.2 Luciferase reporter assay
For the determination of luciferase activity in transfected (promoter studies) or transduced cell lysates (Ad-luc transductions), 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 (Wallac Victor2 1420 multilabel counter, EG&G, Turku, Finland). For the analysis, background (only lysis buffer) values were subtracted. Values of samples were normalized with readings of CMV or SV40 promoter to standardize for transfection and transduction efficiency, respectively.

4.2.1.6 Recominant 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 pVK500fiberL, see chapter 4.1.6.2. Plasmids). For the generation of replication-competent adenoviruses, A549 (Ad5/3.19K-IL) or Colo-829 (Ad2xTyr.hsp70B’pL) 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 H₂O, 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, two (293) or three (A549) 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 (145cm²) 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
Materials and Methods

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\textsubscript{50})-assay

To determine the concentration of infectious particles of purified recombinant adenovirus, TCID\textsubscript{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\textsuperscript{-5}-10\textsuperscript{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\textsubscript{50}/ml:

\[
\text{Titer} = 10^{1 + \frac{1}{(S-0.5)}} \times 10 \text{TCID50/ ml}
\]

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

vp/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 $\lambda = 260$nm 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 ($1.1 \times 10^{11}$ 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$_2$O and incubated at 95°C for 10 min. Five $\mu$l was used as DNA template for PCR with a total volume of 25$\mu$l to control recombinant modified adenoviral genomes (see chapter 4.1.6.1.2.: Oligonucleotides for controlling recombinant modified Ad genomes and chapter 4.2.1.3.: PCR).

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 500$\mu$l infection medium (RPMI or 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 250$\mu$l infection medium. Following 2 h incubation at 37°C, 500$\mu$l 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 **Ad5wt superinfection after transfection**

For Ad5wt superinfection after transfection, 50,000 cells were seeded in 24-well plates. The following day cells were transfected with 0.4$\mu$g of plasmid using Lipofectamine as described above. After 24 h, cells were infected with Ad5wt at 500 vp/cell in 250$\mu$l of growth media containing 2% FBS. Following 1 h incubation at 37°C, infection media was removed and 500$\mu$l growth medium containing 10% FBS was added. Two or 24 h post-infection cells were harvested and lysed for Luciferase assay.
4.2.1.6.4.3 Co-infection assay
For co-infections, 50,000 cells were seeded in 24-well plates in 500µl growth media containing 10 % FBS. The following day, growth medium was removed and cells were infected for 2 h with the first adenovirus in 250µl growth media containing 2 % FBS. Two hours post-infection, infection media was removed and cells were infected with the second adenovirus in the manner described before.

4.2.1.6.4.4 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 containing 10 % FBS. The following day growth medium was removed and cells were infected with adenoviruses at 500 vp/cell in 250µl growth medium containing 2 % FBS. To inhibit virus genome replication cells were incubated with AraC (Sigma-Aldrich) at 2µM, which was added at 1h and 13h post-infection.

4.2.1.6.4.5 Infection for cytotoxicity assay
For the determination of virus-mediated cytotoxicity by crystal violet staining, 50,000 cells per well were seeded either in 48- or 24-well plates. The following day, cells were infected in 200µl or 250µl of growth medium containing 2% FBS with adenoviruses at 10-fold increasing titers of 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 0.1 TCID\textsubscript{50}/cell, cells were stained with crystal violet (see next chapter).

4.2.1.6.4.6 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% crystal 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.

4.2.1.6.4.7 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
Materials and Methods

10 % FCS). Cells were infected the next day at 10vp/cell for A549 or 1000vp/cell for PMelL 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).

4.2.1.6.4.8 Infection for the analysis of protein-expression
For the detection of apoptosis and adenoviral protein expression 800.000 cells were seeded in 10cm dishes in 5ml of growth medium. The following day cells were infected at 10,000vp/cell (apoptosis) or 1000vp/cell (adenoviral proteins) of adenovirus in 5ml infection medium. Two hours post infection 5ml 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).

4.2.1.6.4.9 Infection for the quantification of infectious particles of oncolytic adenoviruses w/ w/o adding Z-VAD-FMK or Q-VD-OPH
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 in the presence or absence of the caspase-family inhibitors Z-VAD-FMK (PMelL) or Q-VD-OPH (A549) at a concentration of 50µM. 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.
5 Results

5.1 Heat-regulated transgene expression from either replication-deficient or replication-competent adenoviral vectors

5.1.1 Analysis of heat-inducibility of promoter fragments of stress-inducible genes

Towards the goal to develop adenoviral vectors and oncolytic adenoviruses that feature heat-inducible expression of therapeutic genes, the hsp70B’ promoter (hsp70B’p) might be a promising tool because of its dose response effect with regard to temperature. Hsp70B’ mRNA was shown to be greatly induced by cellular stress and hyperthermia (Leung, Rajendran et al. 1990). Furthermore, basal mRNA expression, in the absence of heat stimulus, was reported to be lower than for other hsp70 genes (Leung, Rajendran et al. 1990; Parsian, Sheren et al. 2000).

To analyze the activity of the promoter of the human hsp70B’ gene, the -250/-6 bp fragment of this gene (sequence identical to GenBank accession no. NM002155) was cloned into the pGL3 luciferase reporter vector (Promega), resulting in pGL3hsp70B’p. Likewise the -553/+104 bp fragment of the human stress-inducible gp96/grp94 gene, amplified by PCR from human genomic DNA, and the -132/+7 bp fragment of the human grp78 gene (kindly provided by Mori K, Kyoto University, Kyoto, Japan) were cloned into the pGL3 vector for comparison (nucleotide positions relative to translation start for hsp70B’, relative to transcription start for gp96 and grp78). These plasmids and pGL3basic (promoterless) and pGL3SV40p (containing the constitutive SV40 promoter) were transfected into HeLa cells and various human melanoma cell lines using lipofectamine. Cells were heat-shocked (90 min, 42°C) two days post-transfection and 4.5 hours before detection of luciferase activity. This protocol has been shown to result in maximal heat induction of the hsp70B’ promoter (data not shown). Control cells were transfected, but not heat-shocked (Fig. 7). Without heat-shock, luciferase activities for pGL3hsp70B’p were minimal in transfected HeLa cells and 3- to 50-fold above activities of the promoterless control in melanoma cells. Importantly, the hsp70B’ promoter was dramatically induced by heat shock resulting in 533-fold (HeLa) to 3741-fold (Colo-829) increased luciferase activity. Moreover, in comparison to the SV40 promoter, the induced hsp70B’ promoter resulted in 22-fold (HeLa) to 660-fold (Colo-829) higher luciferase activity (Fig.7A). In contrast, both the grp78 and gp96/grp94 promoters resulted in basal activities higher than for the SV40 promoter and were only minimally enhanced by heat treatment (Fig.7B).
In summary, these results demonstrate a strong and heat-shock-dependent activity for the 245bp human \textit{hsp70B'} promoter fragment.

Fig. 7: \textit{Activity and heat induction of promoters of stress-inducible genes.} (A) HeLa or melanoma cells (SK-MEL-28, Colo-829, Mel888 and Mel624) were transfected in triplicates with a pGL3 luciferase reporter plasmid that contained either no promoter (pGL3basic), the SV40 promoter (pGL3SV40p), or the \textit{hsp70B'} promoter (pGL3hsp70B'p). Two days post-transfection, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5 h later. Columns show mean RLU values; error bars show standard deviations. (B) Colo-829 cells were transfected with pGL3SV40p, pGL3hsp70B'p, or pGL3 luciferase reporter plasmid that contained either the grp78 promoter (pGL3grp78) or the gp96 promoter (pGL3gp96). Data shown as described in figure (A). RLU, relative luminescence units.
5.1.2 Influence of adenovirus co-infection and replication on hsp70B' promoter activity in a reporter plasmid

Essential for the application of heterologous promoters in replication-deficient or oncolytic adenoviruses is that their activity is inert to adenovirus transduction and adenoviral protein activity. To investigate a potential interference of adenovirus infection or adenovirus gene expression with hsp70B' promoter activity, pGL3hsp70B'p, pGL3basic, and pGL3SV40p were transfected into HeLa and SK-MEL-28 cells using lipofectamine, followed by super-infection with wild-type adenovirus serotype 5 (Ad5wt) or mock-infection the next day. The adenovirus titer (500 vp/cell) used in this experiment was chosen to result in approximately 100% infection efficiency for each cell line to ensure that all plasmid-transfected cells were super-infected with Ad5wt. Two hours or one day after virus infection, cells were heat-treated or remained at 37°C and luciferase activity was determined 4.5 h post heat-treatment (Fig.8).

Fig.8: Influence of early and late adenovirus infection on hsp70B' promoter activity after reporter plasmid transfection. HeLa or SK-MEL-28 cells were transfected with a pGL3 luciferase reporter plasmid that contained either no promoter (pGL3basic), the SV40 promoter (pGL3SV40p), or the hsp70B' promoter (pGL3hsp70B'p). At 24 h post-transfection, cells were infected with Ad5wt at 500 vp/cell, resulting in approximately 100% infection efficiency, or were mock-infected. Two or 24 hours post-infection, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5 h later. Columns show mean RLU values of triplicate experiments; error bars show standard deviations. RLU, relative luminescence units.
Results

These settings were chosen to assess the influence of early (before virus genome replication) or late (post-replication) virus infection on promoter activity, respectively. For pGL3SV40p and pGL3basic no significant influence of adenovirus infection on luciferase activities was observed for SK-MEL-28 cells (2h, 24h) or for HeLa cells (2h). In HeLa cells, late adenovirus infection resulted in increased luciferase activity for both plasmids, with or without heat shock (2.6- to 5.7-fold). For the hsp70B’ promoter no significant influence of early adenovirus infection, with or without heat shock, was observed in both Hela and SK-MEL-28 cells. Late adenovirus infection resulted in an increase in hsp70B’ promoter activity without heat shock (20.0-fold for HeLa, 6.7-fold for SK-MEL-28) and less so with heat shock (2.0-fold for HeLa, 2.1-fold for SK-MEL-28). Note that for HeLa cells late adenovirus infection also increased luciferase activity for pGL3SV40p and pGL3basic. The activity of the hsp70B’ promoter was not influenced by super-infection with non-replicating adenovirus vectors (data not shown).

In conclusion, heat-inducibility of the hsp70B’ promoter was largely preserved in the presence of adenovirus infection. However, a modest increase in hsp70B’ promoter activity was observed during late adenovirus replication.

5.1.3 Regulation of transgene expression by the hsp70B’ promoter in a replication-deficient adenovirus vector

Next a first generation, E1- and E3-deleted adenovirus vector, Adhsp70B’pL, was generated to investigate the feasibility of the hsp70B’ promoter for transcriptional control of transgene expression by adenoviral gene transfer vectors. Adhsp70B’pL contained the polyA-hsp70B’ promoter-luciferase-polyA cassette of pGL3hsp70B’p. A polyA transcription termination sequence was included upstream of the hsp70B’ promoter to prevent promoter-deregulation by read-through transcription from the left terminus of the Ad5 genome (Fig. 9). HeLa and SK-MEL-28 cells were transduced with Adhsp70B’pL and AdTL at 10 TCID$_{50}$ per cell. AdTL contains the identical luciferase expression cassette like Adhsp70B’pL, but under control of the strong and constitutive CMV promoter/enhancer and was used to normalize for differences in transduction efficiency and for hsp70B’ promoter-independent effects of heat treatment. Transduced cells were either heat-shocked 2 days post-transduction or remained at 37°C and luciferase activity was determined 4.5 h after heat-shock (Fig.10).
Fig. 9: Adenovirus constructs used in this experiment. Schematic outline of replication-deficient adenovirus vectors (AdTL, Adhsp70B’pL) with identical luciferase expression cassettes genes (derived from pGL3; Promega, Mannheim, Germany) but either under control of the strong CMV- or hsp70B’ promoter and of the replication-competent parental wild type adenovirus serotype 5 (Ad5wt). ΔE1, E1 region deleted; ΔE3, E3 region deleted; E1A and E4, early adenoviral genes; E1Ap and E4p, promoters of E1A and E4 genes; LITR, left inverted terminal repeat; pA, polyadenylation signal; RITR, right inverted terminal repeat; Ψ, packaging signal.

For both cell lines, hsp70B’ promoter activity was strongly induced by heat shock. However, clear differences between the cell types were observed: After adenoviral gene transfer, as also observed after transient transfection, heat-induction of the hsp70B’ promoter was stronger for SK-MEL-28 cells (586-fold) compared to HeLa cells (44-fold). Still, the hsp70B’ promoter showed considerable basal activity after adenoviral gene transfer, especially in HeLa cells. Thus, for the hsp70B’ promoter (Adhsp70B’pL) luciferase activities were 0.3% (SK-MEL-28) or 22.9% (HeLa) of those resulting from the strong CMV promoter/enhancer (AdTL).

Fig. 10: Activity of the hsp70B’ promoter after gene transfer with a replication-deficient adenoviral vector. HeLa or SK-MEL-28 cells were transduced with Adhsp70B’pL or AdTL (luciferase expressed from constitutive CMV promoter) at 10 TCID_{50} per cell. Two days post-transduction, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5h later. Columns show mean RLU values after standardization with readings for AdTL of triplicate experiments; error bars show standard deviations. RLU, relative luminescence units.
In summary, the \textit{hsp70B'} promoter allowed for strongly heat-inducible transgene expression by replication-deficient adenoviral vectors; however, it showed a considerable to strong activity at normal temperatures already.

5.1.4 Activity and regulation of the \textit{hsp70B'} promoter in an oncolytic adenovirus

Previously Rivera et al. (2004) showed that transgene expression by replication competent, oncolytic adenoviruses is dramatically increased in comparison to replication-deficient adenovirus vectors. In light of these results, it was analyzed whether the \textit{hsp70B'} promoter facilitates external control of transgene expression from an amplified viral genome. Such a tool would clearly extend the opportunities for application of conditionally replication-competent adenoviruses as vectors for potent expression of therapeutic genes.

![Adenovirus constructs used for investigation of \textit{hsp70B'} activity in OAds](image)

\textbf{Fig.11: Adenovirus constructs used for investigation of \textit{hsp70B'} activity in OAds:} Schematic outline of replication-deficient adenovirus vector (Ad5Luc1) that was used as a non-replicating control and conditionally replication-competent adenoviruses (Ad\(\Delta 24E3\), Ad2xTyr, Ad2xTyr.hsp70B'pL) used for comparison in a cytotoxicity assay. \(\Delta E1\), E1 region deleted; \(\Delta E3\), E3 region deleted; \(\Delta 24\), E1A mutant featuring a deletion of the pocket protein-binding conserved region two; E1A and E4, early adenoviral genes; E1Ap and E4p, promoters of E1A and E4 genes; LITR, left inverted terminal repeat; pA, polyadenylation signal; RITR, right inverted terminal repeat; Tyr2E/P, artificial tyrosinase promoter with two enhancers and the core promoter of the human or mouse tyrosinase genes; \(\Psi\), packaging signal.
To investigate this strategy, the polyA-hsp70B’ promoter-luciferase-polyA cassette of pGL3hsp70B’p was inserted into the genome of the oncolytic adenoviruses Ad2xTyr (Banerjee, Rivera et al. 2004). Ad2xTyr features expression of both E1AΔ24, an E1A mutant with a deletion in the pRb binding domain, and E4 genes from optimized tyrosinase promoters and in consequence replicates with high selectivity for melanoma cells. The hsp70B’ cassette was inserted between right terminal repeat and E4 region, thereby generating Ad2xTyr.hsp70B’pL (Fig.11). Again a transcription termination signal was incorporated upstream of the promoter to prevent read-through transcription from the ITR. Because of the size of the inserted DNA sequence, it was necessary to delete the E3 region from the Ad2xTyr.hsp70B’pL genome.

First, the lytic activity and specificity of Ad2xTyr.hsp70B’pL by infection of melanoma (SK-MEL-28) and non-melanoma cells (HeLa) was determined. Cells were infected with Ad2xTyr.hsp70B’pL, Ad2xTyr, Ad5wt, AdΔ24E3-, and replication-deficient Ad5Luc1 that served as a non-replicating control at virus titers of 0.01 to 1000 vp/cell in 10-fold dilutions. Ad2xTyr and Ad5wt are E3-positive; AdΔ24E3- has a deletion of the pRb-binding domain of E1A, which does not reduce its lytic activity in cancer cells (Rivera, Wang et al. 2004), and is E3-negative. The E3 genes mainly have immunomodulatory functions, except the E3.11.6kd gene, also referred to as adenovirus death protein (ADP). ADP is expressed at very late times during Ad infection and promotes efficient lysis of the infected cell, thus facilitating virus release. In this context AdΔ24E3- was used as a matching control to Ad2xTyr.hsp70B’pL as both are deleted for the E3 gene region. Cell killing was detected by staining of viable, adherent cells with crystal violet (Fig. 12). Cytolysis of melanoma cells was observed for Ad2xTyr.hsp70B’pL, which were about 1.5 orders of magnitude attenuated compared to Ad5wt (E3+) and Ad2xTyr (E3+) and Ad5Δ24E3-. Importantly, lytic activity of Ad2xTyr.hsp70B’pL was severely reduced in HeLa cells, where more than 5 or 4 orders of magnitude lower cytotoxicity was observed relative to Ad5wt or AdΔ24E3-, respectively. In summary, Ad2xTyr.hsp70B’pL retains the melanoma-selectivity of viral cell lysis; however, it possesses a reduced lytic activity. Therefore, Ad2xTyr.hsp70B’pLuc allows for the characterization of the hsp70B’ promoter activity and inducibility in both cells in which the virus replicates (melanoma cells) and cells into which the virus can enter, but not replicate its genome (other cell types).
Next the activity of the hsp70B’ promoter of Ad2xTyr.hsp70B’pL was investigated. Therefore melanoma cell lines SK-MEL-28 and Mel888 and immortalized keratinocytes HaCat were infected with Ad2xTyr.hsp70B’pL or AdTL at 500 vp/cell. In HaCat cells Ad2xTyr viruses do not replicate (Schierer, Hesse et al. 2008). The replication-deficient AdTL, carrying the matching luciferase gene, was used as a control virus. At 2 hours (before replication of the virus genome) or 24 hours (after virus genome replication) post-infection, cells were either heat-shocked or mock-treated and luciferase activity was determined 4.5 h later (Fig.13). Luciferase expression after AdTL transduction was increased by heat shock 31.8-, 9.3-, or 6.5-fold (SK-MEL-28, Mel888, or HaCat, respectively, at 2h) and 5.4-, 2.8, or 12.0-fold (SK-MEL-28, Mel888, or HaCat, respectively, at 24h). The molecular basis for heat-shock-induced CMV promoter activity remains to be determined. In this regard, subsequent experiments showed that not all constitutive promoters were modified by heat-shock, neither after plasmid transfection (data not shown) nor after virus transduction (data not shown), arguing against a general promoter-independent increase in transcriptional activity. Importantly, for Ad2xTyr.hsp70B’pL strong heat-inducible luciferase expression was detected on the day of infection for all cell lines (SK-MEL-28, 2038.3-fold; Mel888, 1126.0-fold; HaCat, 136.2-fold induction) that was considerably stronger than for AdTL after heat-induction. In HaCat cells, in which Ad2xTyr.hsp70B’pL should not replicate, heat-induction of luciferase expression was
increased 24 hours post-infection (463.9-fold). Compared to 2 hours post-infection, luciferase activities for Ad2xTyr.hsp70B’pL (both with and without heat shock) were less increased than for AdTL, indicating the absence of replication of this virus in HaCat cells.

**Fig.13: Activity of the hsp70B’ promoter in a conditionally replication-competent, oncolytic adenovirus.** To investigate heat-inducibility of the hsp70B’ promoter of Ad2xTyr.hsp70B’Tyr-pL, melanoma cells (SK-MEL-28, Mel888) and non-melanoma cells (HaCat) were infected with Ad2xTyr.hsp70B’Tyr-pL or with replication-deficient AdTL (luciferase expressed from constitutive CMV promoter). Two or 24 hours post-infection, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5 h later. Infections were performed in triplicates. Columns show mean RLU values; error bars show standard deviations. RLU, relative luminescence units.

Notably, the increase in luciferase activity from 2h to 24h at normal temperatures was only 3.3-fold for Ad2xTyr.hsp70B’Tyr-pL, but 20.3-fold for AdTL indicating increase in CMV-promoter mediated transcription. In melanoma cells, heat-induction of luciferase expression for Ad2xTyr.hsp70B’Tyr-pL was drastically diminished (SK-MEL-28, 26.7-fold; Mel888, 5.0-fold) at 24 hours. Note, that residual heat induction was at least in part independent of the hsp70B’ promoter, as indicated by the results for
AdTL. These results imply that regulation of the *hsp70B* promoter in the context of an oncolytic adenovirus is lost after viral DNA replication. Importantly, promoter activity is not lost by insertion into the Ad2xTyr genome per se, as it is functional for Ad2xTyr.hsp70B’pL at early virus infection of melanoma cells, i.e. before replication of virus genomes, or after infection of cells that do not support virus replication.

A potential reason for the loss of promoter regulation in Ad2xTyr.hsp70B’pL could be the interference of adenoviral proteins or of cellular factors induced by progressive adenovirus infection with *hsp70B*’ promoter activity. However, the *hsp70B*’ promoter was only modestly deregulated after reporter plasmid transfection and super-infection with Adwt (Fig.8), arguing that other mechanisms must be involved in *hsp70B*’ promoter deregulation. The loss of inducibility of luciferase expression after infection of Ad2xTyr.hsp70B’pL might result from deregulation of the *hsp70B*’ promoter itself at high copy numbers, a mechanism which is not mimicked by the pGL3hsp70B’p transfection / Ad5wt super-infection experiment. Reason for such promoter deregulation might be the titration of activating transcription factors thereby preventing full activity of all promoters at high copy numbers. The transcription factor HSF-1 is responsible for heat-induced transcription initiation from the *hsp70* promoters (Baler, Dahl et al. 1993). To investigate if titration of HSF-1 is the reason for loss of *hsp70B*’ promoter regulation after virus replication, the effect of overexpression of a constitutively active mutant of human HSF-1, mHSF-1 was investigated (Fig.14).

![Graph](image_url)

**Fig.14:** Investigation whether overexpression of constitutively active HSF-1 mutant (mHSF-1) can rescue inducibility of the *hsp70B*’ promoter of Ad2xTyr.hsp70B’pL. Cells were co-infected with Ad2xTyr.hsp70B’pL and either AdmHSF-1 (mHSF-1 expressed from the CMV promoter) or AdGFP (CMV-GFP cassette), for the latter with or without heat shock 24 h post-infection. Further controls were AdTL, AdD fiberIL (luciferase expressed from a bicistronic viral mRNA) and Adhsp70B’pL each co-infected with either AdmHSF-1 or AdGFP (with or without heat shock). Cells were heat-shocked 24 h post-infection or remained at 37°C and luciferase activity was determined 4.5 h later. Infections were performed in triplicates. Columns show mean RLU values; error bars show standard deviations. RLU, relative luminescence units.
To this end, the replication-deficient AdmHSF-1, an adenoviral vector expressing mHSF-1 from the strong CMV promoter that had been generated earlier in the laboratory, was exploited. SK-MEL-28 cells were co-infected with Ad2xTyr.hsp70B’pL and either the non-replicating AdmHSF-1 or, as a control, AdGFP (the latter with or without heat shock 24 hours post-infection) that is a matching virus encoding GFP. Cells were first infected with Ad2xTyr.hsp70B’pL. After two hours the media was removed and cells were infected with the second adenovirus (AdmHSF-1 or AdGFP). Infections were performed at virus titers that result in approximately 100% infection/transduction efficacy to ensure double-infection (500 vp/cell). Co-infection of replicating adenoviruses with replication-deficient AdmHSF-1 results in strong over-expression of mHSF-1 (data not shown).

The results show minimal induction of luciferase expression by heat shock from Ad2xTyr.hsp70B’pL 24 hours post-infection. However, luciferase expression after co-infection with either AdGFP or AdmHSF-1 was only minimally stronger compared to heat-shocked cells. As further control, cells were infected with AdTL or AdΔfiberL, a replication-competent virus with the luciferase cDNA fused via an IRES to the late viral fiber gene. The results show that heat shock or co-infection with AdmHSF-1 had no major impact on luciferase expression by these viruses. In contrast, for non-replication competent Adhsp70B’pL strong induction of luciferase expression was observed by either heat-shock or AdmHSF-1 co-infection, thus demonstrating activity of the adenovirus-encoded mHSF-1.

In conclusion, these results argue against the hypothesis that limited amounts of HSF-1 are responsible for reduced heat-induction of luciferase expression by Ad2xTyr.hsp70B’pL after replication of the virus genome.

Furthermore, it was explored whether a block of viral DNA replication in permissive melanoma cells could restore regulation of the hsp70B’ promoter in Ad2xTyr.hsp70B’pL at 24 h post-infection. SK-MEL-28, Mel888 or HaCaT cells were infected with AdTL or Ad2xTyr.hsp70B’pL as described before and genome replication was either blocked by adding AraC at a concentration of 2µM or cells were mock-treated. At 24 h post-infection, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5 h later. AraC completely blocked virus genome replication (data not shown). As depicted in Figure 15, AraC treatment did not affect heat inducibility of the hsp70B’ promoter after infection of HaCaT cells and resulted in a modest increase in heat-induction of the hsp70B’ promoter for Ad2xTyr.hsp70B’pL, but not for AdTL, in both melanoma cell lines. This increase was
Results

from 3.8-fold to 29.8-fold for Mel888 and from 2-fold to 7.8-fold for SK-MEL-28 cells and was based on reduced promoter activity in the absence of heat shock. Nevertheless, heat-inducibility of the hsp70B' promoter was not completely restored by AraC treatment in melanoma cells and remained much reduced compared to melanoma cells 2 h post-infection or compared to HaCaT cells. Thus, hsp70B' promoter regulation of Ad2xTyr.hsp70B'pL was retained when virus replication was blocked in non-permissive cells by transcriptional control of early gene expression, but was partially lost in melanoma cells 24 h post-infection when viral genome replication was artificially blocked. Of note, early gene expression by Ad2xTyr.hsp70B'pL should be ‘off’ in HaCaT cells due to lack of tyrosinase promoter activity, but ‘on’ in AraC-treated melanoma cells, which might contribute to the observed discrepancy. In summary, these results show that regulation of the hsp70B' promoter is mostly lost in the genome of oncolytic adenoviruses specifically during virus replication in permissive cells.

Fig. 15: Analysis how a block of virus genome replication affects hsp70B' promoter regulation in Ad2xTyr.hsp70B'pL. SK-MEL-28, Mel888 or HaCat cells were infected with AdTL or with Ad2xTyr.hsp70B'pL. Cells were treated with 2 µM of AraC which was added at 1 h and 13 h post-infection or were mock-treated. At 24 h post-infection, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5 h later. Infections were performed in triplicates. Columns show mean RLU values; error bars show standard deviations; RLU, relative luminescence unit.
5.1.5 **Insulator elements as tools for improved regulation of the hsp70B’ promoter in replication-deficient but not replicating adenoviral vectors**

High baseline activities of inducible promoters (i.e. in the absence of the activating stimulus) are of major concern for potential clinical applications. In an effort to improve the induction rate of the hsp70B’ promoter in adenoviral vectors, specifically by reduction of basal promoter activity, the utility of insulator elements was investigated. It has been shown by several groups that viral DNA sequences can interfere with the activity of heterologous promoters. For example, transcription initiation in the left ITR or packaging sequence has been shown to mediate non-specific read-through transcription of transgenes inserted – with or without promoter – into the E1 region of the adenovirus genome. To avoid such interference with heterologous promoter activity, both viruses, Adhsp70B’pL and Ad2xTyr.hsp70B’pL, contained a transcription termination signal upstream of the hsp70B’ promoter. In addition to cryptic transcription initiation, viral sequences, such as the ITRs and packaging signal, show enhancer activity that might also disrupt the specificity of neighbouring cellular promoters. To investigate if such enhancer function might increase basal activity of the hsp70B’ promoter, insulator elements were inserted between the ITR/packaging signal and the polyA/hsp70B’ promoter of Adhsp70B’pL (Fig.16).

![Fig.16: Adenovirus constructs with insulated hsp70B’ promoter. Schematic outline of replication-deficient adenovirus vectors (Adl2hsp70B’pL, AdlC2hsp70B’pL) with the hsp70B’ promoter-reporter cassette and either a 2.4 kb tandem repeat of the chicken β-globin locus insulator (I2) or a 500 bp tandem repeat of the chicken β-globin insulator core (IC2). bp, base pairs; ΔE1, E1 region deleted; ΔE3, E3 region deleted; E4, early adenoviral gene; E4p, promoter of E4 gene; kb, kilobases; LITR, left inverted terminal repeat; pA, polyadenylation signal; RITR, right inverted terminal repeat; Ψ, packaging signal.](image_url)

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**Fig.16: Adenovirus constructs with insulated hsp70B’ promoter.** Schematic outline of replication-deficient adenovirus vectors (Adl2hsp70B’pL, AdlC2hsp70B’pL) with the hsp70B’ promoter-reporter cassette and either a 2.4 kb tandem repeat of the chicken β-globin locus insulator (I2) or a 500 bp tandem repeat of the chicken β-globin insulator core (IC2). bp, base pairs; ΔE1, E1 region deleted; ΔE3, E3 region deleted; E4, early adenoviral gene; E4p, promoter of E4 gene; kb, kilobases; LITR, left inverted terminal repeat; pA, polyadenylation signal; RITR, right inverted terminal repeat; Ψ, packaging signal.
Insulators are defined as regulatory DNA elements of the eukaryotic genome that prevent the activation of promoters by enhancers, but only if placed between them (Chung, Whiteley et al. 1993; Zhong and Krangel 1997; Filippova, Thienes et al. 2001). A well-studied insulator element is the 1.2 kb chicken β-globin locus insulator (Chung, Whiteley et al. 1993).

Initially, a 2.4 kb tandem repeat of this insulator (I₂) was analyzed in AdI₂hsp70B’pL (Fig. 17). As shown in Fig.17A, this insulator dramatically increased heat-inducibility of the hsp70B’ promoter in a replication-deficient adenovirus vector.

Fig.17: Activity of insulated hsp70B’ promoters in replication-deficient and replicating adenovirus vectors. (A) HeLa or SK-MEL-28 cells were transduced with replication-deficient adenovirus vectors Adhsp70B’pL, AdI₂hsp70B’pL, AdIC₂hsp70B’pL, or AdTL at 10 TCID50 per cell in triplicates. Two days post-transduction, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5 h later. Columns show mean RLU values after standardization with readings for AdTL; error bars show standard deviations; numbers indicate fold induction by heat shock. RLU, relative luminescence units. (B) 293 cells, which complement E1 proteins and thus allow for replication of ΔE1/ΔE3 adenovirus vectors, were infected with Adhsp70B’pL, AdI₂hsp70B’pL, AdIC₂hsp70B’pL, or AdTL at 0.1 TCID50 per cells in triplicates. Two or 24 hours post-infection, cells were heat-shocked or remained at 37°C and luciferase activity was determined 4.5 h later. Data are presented as described in (A).

Due to a marked reduction of basal promoter activity in the absence of heat stimulus, heat-induction for luciferase expression by AdI₂hsp70B’pL was improved from 38- to 1597-fold in HeLa cells and from 429- to 8749-fold in SK-MEL-28 cells relative to
Adhsp70B’pL. However, the considerable size of the I$_2$ element might be a drawback for specific applications of this element in adenoviral vectors. Therefore, a shorter 500 bp tandem repeat of the chicken β-globin insulator core (IC$_2$; Bell AC, 1999) was inserted into the Adhsp70B’pL genome, deriving AdIC$_2$hsp70B’pL (Fig.16). This should reveal whether the IC$_2$ element shows insulating activity similar to I$_2$.

Transduction experiments demonstrated that the IC$_2$ element indeed has insulator activity (Fig.17A). However, the increase in heat induction of the hsp70B’ promoter by IC$_2$ was approx. 3-fold (HeLa) or 2-fold (SK-MEL-28) reduced compared to the I$_2$ element. Of note, induction of transgene expression by heat shock for AdIC$_2$hsp70B’pL was still approx. 10-fold improved compared to Adhsp70B’pL.

The next step was to investigate if the insulator elements could also restore heat induction of the hsp70B’ promoter in an adenovirus genome after virus DNA replication. Therefore 293 cells, which support replication of E1-deleted adenovirus vectors, were infected with Adhsp70B’pL, AdI$_2$hsp70B’pL, or AdIC$_2$hsp70B’pL. When the cells were heat-shocked 2h post-infection, heat induction of luciferase expression from Adhsp70B’pL (Fig.17B) was observed. Because of marked basal luciferase expression at normal temperatures, heat induction was, however, only 26-fold. As observed for HeLa and SK-MEL-28 cells, heat induction of luciferase expression was increased for AdI$_2$hsp70B’pL (169-fold) and to a lesser extend for AdIC$_2$hsp70B’pL (81-fold) after the heat shock 2 hours post-infection.

These results show that both regulation of the hsp70B’ promoter and activity of the insulator elements are functional in the E1-complementing cell line 293. Notably, heat induction of luciferase expression from Adhsp70B’pL was reduced after replication of the virus genome (heat shock at 24 h) and only minimally (I$_2$) or not (IC$_2$) improved by insertion of insulator elements. Heat induction of luciferase expression from AdIC$_2$hsp70B’pL was not further improved by insertion of a second IC$_2$ element in sense or antisense orientation downstream of the luciferase gene (data not shown).

In summary, upstream insulator elements facilitate strongly increased heat-induction of the hsp70B’ promoter in first-generation adenoviral vectors due to reduced promoter activity at normal temperatures. Of note, the 2.4 kb I$_2$ element showed superior insulating activity compared to the 0.5 kb IC$_2$ element. However, regulation of the hsp70B’ promoter after adenovirus genome replication could not be rescued by the insulator elements.
5.2 Combining Rational Mutagenesis and Transgene Expression for Improving Efficacy of Oncolytic Adenoviruses

5.2.1 Spread of E1B19K-deleted, transgene-encoding oncolytic adenovirus in tumor cell cultures

The genomes of recombinant adenoviruses used in this second project are depicted in Fig.18. To study the combination of early host cell lysis and viral release with transgene expression the transgene-encoding oncolytic adenovirus Ad5/3.19K-.IL was generated (Fig.18).

Fig.18: The relevant genomic regions of the adenoviruses used in this project are shown. CMV, cytomegalovirus promoter; ΔE1/ΔE1B19K/ΔE3, deleted early genes; E1A/E1B19K/E3/E4, early viral genes; E1AΔ24, E1A mutant featuring a deletion of the pocket protein-binding conserved region two; EMCV IRES, internal ribosome entry site; LITR/RITR, left/right inverted terminal repeat; Luc, luciferase gene; pA, polyadenylation signal for transcription termination; Ψ, packaging signal; red boxes highlight the key difference between the two analyzed oncolytic adenoviruses that are either E1B19K-positive or negative.

This virus has the following features (i) deletion of E1B19K aiming at accelerated virus release and spread (see chapter 2.4.3.5), (ii) insertion of a transgene, the reporter gene luciferase into the late fiber transcription unit using an IRES (Rivera, Wang et al. 2004), (iii) Δ24 mutation of E1A for restriction of virus replication to tumor cells (see chapter 2.4.3.1), (iv) a chimeric fiber with Ad5 tail and shaft and Ad3 knob.
domains facilitating viral entry into CAR-negative cells (Krasnykh, Mikheeva et al. 1996; Rivera, Davydova et al. 2004) (Krasnykh, Mikheeva et al. 1996; Rivera, Davydova et al. 2004) and (v) deletion of the E3 genes due to size limitations. Ad5/3.19K+.IL was generated as the matching control virus with wild-type E1B genomic locus.

Initially, a virus spread and cell-killing assay was performed to analyze whether the enhanced spread phenotype of the $E1B19K$ mutation (Sauthoff, Heitner et al. 2000) is retained in the oncolytic adenovirus Ad5/3.19K-.IL in the context of E3-deletion, $\Delta 24$ mutation of E1A, transgene expression and chimeric fiber (Fig.19).

![Image of cell cultures and virus titration plots](image)

**Fig.19:** Spread-dependent cell killing of $E1B19K+$ or $E1B19K$- transgene-encoding oncolytic adenoviruses in different tumor cell cultures. A549, lung adenocarcinoma; C8161, SK-MEL-28, melanoma cell lines; PMelL, PMelA, low passage melanoma cultures; NCH82, NCH468; NCH89, low passage glioblastoma cultures; Cell cultures were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL, replication-deficient Ad5/3.Luc1 or wild-type Ad5 fiber containing Ad5$\Delta 24$E3- at indicated titers or were mock-infected (framed wells). Virus spread-dependent cytotoxicity was visualized by crystal violet staining of surviving cells. Therefore, stainings were performed when cytotoxicity was observed for the most potent virus at 0.01 or 0.1 vp/cell for each cell type individually. vp, viral particles.

To this end, A549 cells were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL, Ad5$\Delta 24$E3- and Ad5/3.Luc1 at virus titers of 0.01 to 1000 vp/cell in 10-fold dilutions. Ad5$\Delta 24$E3- is also $E1B19K$-positive and has the same $\Delta 24$ mutation of E1A and $\Delta E3$ mutation.
like the two oncolytic adenoviruses. Therefore, this virus served as a matching virus, although it has an Ad5 knob domain. However, at that time no matching virus with a chimeric fiber containing an Ad3 knob domain was available in the laboratory. The Ad5/3.Luc1 was used as a non-replicating virus control. When cell killing was observed for the most potent virus at the lowest titer, surviving cells were fixed and stained with crystal violet. Indeed, Ad5/3.19K-.IL caused a 1000-fold higher cytotoxicity in A549 cells compared to Ad5/3.19K+.IL, resembling the previously reported phenotype of the E1B19K-deletion (Subramanian, Kuppuswamy et al. 1984).

Next, a panel of melanoma and glioblastoma cell lines and low passage cultures were infected as described before to investigate virus spread and cell killing in a wider panel of cell cultures. The results show that the phenotype of the E1B19K-deletion depended on the individual cell line or culture (Fig.19): In two melanoma cell lines and in two of three low passage glioblastoma cultures virus spread of Ad5/3.19K-.IL was enhanced 10- to 100-fold compared to Ad5/3.19K+.IL. However, in two low passage melanoma cultures and one low passage glioblastoma culture cell spread and lysis by Ad5/3.19K-.IL was attenuated up to 100-fold. Thus, the deletion of E1B19K can result in either an enhanced or reduced spread phenotype of (transgene-encoding) oncolytic adenoviruses dependent on the infected cell type.

5.2.2 Apoptosis induction and cell type-dependent modulation of the adenovirus replication cycle by the E1B19K-deletion

To further investigate the cause for the cell type-dependent differences in virus spread of Ad5/3.19K-.IL, A549 and PMelL cells, representing the respectively enhanced or reduced spread phenotype, were studied in more detail. First, it was analyzed how the cells are affected by virus infection. Considering the anti-apoptotic activity of E1B19K, it was hypothesized that the opposing phenotypes of the deletion mutant result from differences in apoptosis induction between the cell types. To investigate apoptosis induction, A549 or PMelL cells were either infected with Ad5/3.19K-.IL or Ad5/3.19K+.IL at 10,000 vp/cell or were mock-infected. For a positive control A549 and PMelL cells were irradiated with UVB or UVC, respectively, to induce apoptosis. After 12, 24 and 48 hours post-infection, cells were harvested. Protein lysates were then separated in a polyacrylamide gel, followed by immunoblotting and detection of PARP and Caspase 3 cleavage, both characteristic for caspase activation, a hallmark of apoptosis induction. After infection with
Ad5/3.19K+.IL, cleavage of PARP and Caspase 3 was weak for A549 cells but emerged at 24 h for PMelL and was quite strong for these cells at 48 h (Fig. 20).

These results indicate that the melanoma cells showed a distinct apoptotic response to infection with E1B19K wild-type adenovirus. Importantly, for both cell types the deletion of E1B19K resulted in strongly increased/accelerated cleavage of PARP and Caspase 3 with marked cleavage observed starting at 24 h post-infection. These results reveal a similarly pro-apoptotic effect of E1B19K deletion mutant in both cell types arguing that differences in apoptosis induction are not responsible for the different oncolysis phenotypes.

In contrast to the first experiment analyzing spread-dependent cell killing, the next experiment tended to investigate direct, spread-independent virus cytotoxicity, i.e. before virus spread and re-infection could occur. Therefore, high titer infections (10,000 vp/cell) of A549 and PMelL cells with Ad5/3.19K-.IL, Ad5/3.19K+.IL and the non-replicating Ad5/3.Luc1 as a control were performed. Virus cytotoxicity was determined 48 h post-infection by crystal violet (Fig.:21A) and Annexin/PI (Fig.:21B) staining.
Results

Fig. 21: Spread-independent cytotoxicity of E1B19K+ or E1B19K- transgene-encoding oncolytic adenoviruses in A549 and PMelL cells. (A) A549 or PMelL cells were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL, or Ad5/3.Luc1 at indicated titers. Forty-eight hours post-infection cytotoxicity was visualized by crystal violet staining of surviving cells. Vp, viral particles. (B) A549 or PMelL cells were infected with Ad5/3.19K-.IL or Ad5/3.19K+.IL at 10,000 vp/cell and were harvested 48 h post-infection. Control cultures were mock infected or were treated by UV irradiation (UVB for A549, UVC for PMelL) for apoptosis induction. Cells were analyzed by Annexin/PI staining and flow cytometry. Data shown are the mean percentages of Annexin negative, PI positive (PI+), Annexin positive, PI negative (A+), double positive (A+/PI+) or double negative (A-/PI-) cells of three independent experiments. For mock-infected cells, mean percentage of double negative cells was 95.0% for A549 and 88.8% for PMelL. For UV irradiated cells mean percentage of Annexin positive, PI negative cells was 40.19% for A549 and 58.1% for PMelL.

In this setting Ad5/3.19K-.IL showed higher cytotoxicity than Ad5/3.19K+.IL for both cell types. In the cytotoxicity assay, this difference was more pronounced for A549 cells. In the Annexin/PI staining increased numbers of total dead cells were observed for both A549 and PMelL cells (for A549 from 20% to 60%; for PMelL from 30% to 70%). Increased numbers of Annexin-positive/PI-negative cell populations in both cell types are indicative for enhanced apoptosis induction confirming the results of PARP and Caspase 3 cleavage. Overall, these results indicate increased cytotoxicity of Ad5/3.19K-.IL in both A549 and PMelL cells. Thus it can be hypothesized that the reduced cell killing in low passage melanoma cells 14 days post-infection (Fig. 19) was due to reduced or delayed virus production, release and/or spread.

To test this hypothesis, virus replication in these cells was investigated more closely. Specifically, the major goal was to delineate what step of the adenovirus replication
cycle was affected by the $E1B19K$-deletion. To this end, virus burst assays were performed, which over a single round of virus replication measure the infectious virus particles in a cell population, i.e. virus particles in the cell and supernatants, and therefore allows determining the efficiency with which infectious particles are produced and released. For that purpose, A549 and PMelL cells were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL and the non-replicating Ad5/3.Luc1 at 10 vp/cell. Two hours post-infection, the medium was removed and cells were washed twice with PBS to remove all unbound viruses. Two and four days post-infection supernatants and cells were harvested separately and 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 (Fig.22).

![Graph showing viral replication and release](image)

Fig.22: Viral replication and release of $E1B19K^+$ versus $E1B19K^-$ transgene-encoding oncolytic adenoviruses in A549 and PMelL cells. A549 or PMelL cells were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL or replication-deficient Ad5/3.Luc1. Two or 4 days post-infection, supernatants and cells were harvested separately and titers of infectious viral particles were determined by TCID$_{50}$ assay. Left panels show total infectious viral particles (cells + supernatants). Right panels show the ratio of infectious viral particles in supernatants to infectious viral particles in cells to indicate virus release. Infections were performed in triplicates. Columns show mean titers, arrow bars show standard deviations. Numbers show fold titer differences and asterisks indicate statistical significant differences ($p < 0.05$).

For A549 cells similar amounts of total infectious virus particles (supernatant + cells) were produced for Ad5/3.19K-.IL or Ad5/3.19K+.IL and no or a minimal increase in virus titers was observed from 2 to 4 days. However, virus release into the supernatant was markedly increased for the $E1B19K$ mutant at two and even more so at 4 days post-infection, when release was more than 2 orders of magnitude.
higher than for the wild-type $E1B19K$ virus. For PMelL cells a different picture was observed: total infectious virus particle production was significantly lower for Ad5/3.19K-.IL compared with Ad5/3.19K+.IL at 4 days post-infection. For Ad5/3.19K+.IL an increase in total infectious virus particles from 2 to 4 days post-infection was observed. The amount of infectious viral particles in the supernatant was also lower for Ad5/3.19K-.IL than for Ad5/3.19K+.IL (not shown), even though virus release (ratio supernatant/cells) was slightly higher. Thus it was concluded, that deletion of $E1B19K$ interferes with adenovirus replication resulting in reduced production of infectious virus particles in PMelL cells, but not in A549 cells where the deletion rather strongly enhances virus release.

Next, it was investigated what step of the adenovirus replication cycle was influenced by the $E1B19K$ deletion in PMelL cells. Therefore, the efficiency of messenger RNA (mRNA) expression from the viral late genes fiber and hexon was analyzed.

![Fig.23: Kinetics and efficiency of messenger RNA expression from viral late genes fiber and hexon and quantification of virus genome copy numbers of $E1B19K^+$ and $E1B19K^-$ transgene-encoding oncolytic adenoviruses in A549 and PMelL cells.](image)

(A) For quantification of messenger RNA copy numbers of late fiber (left panels) and hexon (right panels) genes A549 and PMelL cells were infected with Ad5/3.19K-.IL and Ad5/3.19K+.IL at 10 vp/cell (A549) or 1000 vp/cell (PMelL). At 8, 16, 24, 32, and 40 h after infection RNA copy numbers of late viral genes were determined by Real Time PCR and are shown after normalization with mass of cellular DNAs determined by GAPDH messenger RNA copy numbers. (B) To analyze adenovirus genome replication PMelL cells were infected with Ad5/3.19K-.IL and Ad5/3.19K+.IL or replication-deficient Ad5/3.Luc1 at 10 vp/cell in triplicates. Forty-eight hours after infection DNA was purified and the ratio of adenovirus genome copy numbers to nanograms cellular genomic DNA was determined by Real Time PCR. Columns show mean values and error bars show standard deviations of one representative experiment out of three. Numbers show fold genome copy number differences and the asterisk indicates a statistical significant difference ($p<0.05$). n.d., not determined.
Results

To this end, A549 and PMelL cells were infected with Ad5/3.19K-.IL and Ad5/3.19K+.IL at 10 vp/cell (A549) or 1000 vp/cell (PMelL). At 8, 16, 24, 32 and 40 h post-infection mRNA copy numbers in cell lysates were measured by Real Time PCR (Fig.23A). It turned out, that late viral fiber and hexon expression for the $E1B19K$-deleted virus were 3.1 to 7.7-fold lower than for the $E1B19K$+ virus in PMelL cells, but not in A549. This attenuation became apparent 26 h post-infection indicating that replication of the $E1B19K$- virus in PMelL cells was inhibited during late infection. 

This was followed by the quantification of the adenoviral genome copy numbers in PMelL cells that were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL or the replication-deficient Ad5/3.Luc1 at 10 vp/cell. Forty-eight hours post-infection DNA was purified from cell lysates and adenovirus genome copy numbers were determined by Real Time PCR (Fig.23B). Here, the $E1B19K$- virus resulted in 2.1-fold lower genome copy numbers than its $E1B19K$+ counterpart. In summary, these results show, that the deletion of $E1B19K$ interferes with late adenovirus infection in PMelL cells leading to diminished adenoviral late gene expression and adenovirus genome copy numbers, which in turn results in less amount of total infectious viral particles and thus reduced viral release. In contrast, in A549 cells the $E1B19K$ deletion did not affect adenovirus replication but rather correlated with a strongly increased viral spread.

Next it was investigated whether these effects of the $E1B19K$-deletion on adenovirus replication and release in A549 and PMelL cells are dependent on apoptosis induction. To this end, burst assays were performed in the presence of the pan-caspase inhibitors Q-VD-OPH (for A549) and Z-VAD-FMK (for PMelL) (Fig.24). Initially, the appropriate concentration for blocking apoptosis by the caspase-family inhibitors had to be determined. Therefore, different concentrations of Q-VD-OPH or Z-VAD-FMK (both were replenished after two days) had been tested on PMelL and A549 cells that were either UV-irradiated (UVB for A549; UVC for PMelL) or infected with Ad5/3.19K-.IL at 10.000 vp/cell. Cells were harvested and lysed 16 h (PMelL) or 26 h (A549) post-irradiation or 72 h post-infection. Protein lysates were prepared, separated on a polyacrylamide gel, followed by immunoblotting and analyzed for PARP cleavage. It turned out, that in A549 cells UVB- and Ad5/3.19K-.IL-induced PARP cleavage was efficiently blocked with Q-VD-OPH at a concentration of 50µM, whereas Z-VAD-FMK resulted only in partial inhibition of PARP cleavage. However, in PMelL cells UVC- and Ad5/3.19K-.IL-induced PARP cleavage was completely inhibited by Z-VAD-FMK at a concentration of 50µM (data not shown).
Regarding the burst assay, A549 and PMelL cells were infected with Ad5/3.19K-.IL or Ad5/3.19K+.IL at 10 vp/cell. Two hours post-infection, the medium was removed and cells were washed twice with PBS to remove all unbound viruses. Afterwards growth medium with 10% foetal bovine serum was added in the presence or absence of the caspase-family inhibitors Q-VD-OPH (A549) or Z-VAD-FMK (PMelL) at a concentration of 50µM, which was replenished every two days. Three days post-infection supernatants and cells were harvested separately and lysates were prepared by three cycles of freezing and thawing. Serial dilutions of the lysates were then titered on 293 cells using the TCID50 assays.

Fig.24: Effect of caspase inhibition on viral replication and release of E1B19K+ or E1B19K- transgene-encoding oncolytic adenoviruses in A549 and PMelL cells. Cells were infected with Ad5/3.19K-.IL or Ad5/3.19K+.IL at 10 vp/cell in the presence or absence of the caspase inhibitors Q-VD-OPH (for A549) or Z-VAD-FMK (PMelL) at a concentration of 50µM, that were refreshed every two days. Three days post-infection, supernatants and cells were harvested separately and infectious viral particles were determined. Left panels show total infectious virus particles (cells + supernatants). Right panels show the ratio of infectious viral particles in supernatants to infectious viral particles in cells indicating virus release. Infections were performed in triplicates. Columns show mean titers, arrow bars show standard deviations. Numbers show fold titer differences and asterisks indicate statistical significant differences (p< 0.05).

By Q-VD-OPH treatment during infection of A549 cells, the enhanced release phenotype of Ad5/3.19K-.IL was mostly reverted. Total infectious particle production for Ad5/3.19K-.IL and Ad5/3.19K+.IL and viral release of Ad5/3.19K+.IL, however, were only marginally affected. In PMelL cells, inhibition of caspase activity during virus infection resulted in an approximately 10-fold increase in total virus particle production, but reduced virus release for both Ad5/3.19K-.IL and Ad5/3.19K+.IL.
Furthermore, attenuation of infectious virus particle production by $E1B19K$-deletion in these cells was partially reversed. These results argue that early apoptosis induction resulting from $E1B19K$-deletion is required for increased release of Ad5/3.19K-.IL in A549 cells and at least contributes to the reduction of replication efficacy of this virus in PMelL cells.

### 5.2.3 Modulation of the expression of transgenes inserted into oncolytic adenoviruses by $E1B19K$ deletion

Having demonstrated that the deletion of $E1B19K$ in transgene-encoding oncolytic adenoviruses results in early apoptosis of infected cells and modulation of virus spread, it was investigated how transgene expression is affected. For that reason, A549 and PMelL cells were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL or the replication-deficient Ad5/3.Luc1 at 500 vp/cell (A549) and 5000 vp/cell (PMelL) for 6, 10, 14, 18 and 22 hours (Fig.:X) or at 0.5 vp/cell (A549) and 5 vp/cell (Fig.:X) for 1, 2, 4, 6, 8, or 10 days. At the indicated time points cells were harvested and luciferase expression was measured. As expected from previous work (Rivera, Wang et al. 2004), expression of transgenes from the late transcription unit of oncolytic adenoviruses Ad5/3.19K-.IL and Ad5/3.19K+.IL was weaker than transgene expression directed from the CMV promoter of replication-deficient Ad5/3.Luc1 early after infection for both cell types (Fig.25). However, transgene expression for oncolytic adenoviruses increased dramatically over time surpassing transgene expression of Ad5/3.Luc1 at 14 hours (A549) or 2 days (PMelL) post-infection. Importantly, transgene expression after infection with Ad5/3.19K-.IL and Ad5/3.19K+.IL were quite similar during the first day post-infection for both cell types, excluding detrimental effects of early apoptosis induction due to deletion of $E1B19K$.

Transgene expression by Ad5/3.19K+.IL continuously increased until the last day of measurement in both cell types. In PMelL cells, transgene expression by Ad5/3.19K-.IL was lower than by Ad5/3.19K+.IL beginning at day 2 post-infection and from day 4 no further increase was observed, although no widespread cytopathic effect was observed by microscopy. This results correlates with reduced virus replication and spread of this virus in PMelL cells. In A549 cells, transgene expression by Ad5/3.19K-.IL clearly surpassed transgene expression by Ad5/3.19K+.IL at 4 days post infection, correlating with increased virus spread. Beginning at 6 days post-infection transgene expression by Ad5/3.19K-.IL dropped, parallel to increasing viral cell lysis, as observed microscopically, and thus loss of cells.
Results

Fig. 25: Transgene expression by E1B19K+ or E1B19K- transgene-encoding oncolytic adenoviruses in A549 and PMelL cells. Cells were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL or replication-deficient Ad5/3.Luc1 at 500 (A549) or 5000 (PMelL) vp/cell (left panels) or at 0.5 (A549) or 5 (PMelL) vp/cell (right panels). At indicated time points, cells were harvested and luciferase activity was determined. Infections were performed in triplicates. Shown are mean values. For clarity of presentation, standard deviations are not shown and were below 22% (A549, 6-22h), 20% (A549, 2-10 days), 23% (PMelL, 6-22h), or 33% (PMelL, 2-10 days).

To address this technical limitation of the experimental setting and allow for a better comparison of transgene expression between Ad5/3.19K-.IL and Ad5/3.19K+.IL, A549 cells were infected with increasing 10-fold virus dilutions and cytotoxicity and reporter gene assays were performed in parallel at 5 days and 10 days post-infection (Fig. 26). In general, increasing luciferase expression with both increasing virus titers and progressing infection time were detected (the latter only for the replication-competent oncolytic viruses but not for the replication-deficient virus). However, when cell lysis was evident by crystal violet staining, luciferase activities levelled or were undetectable due to complete loss of cells for both transgene-encoding oncolytic adenoviruses. The former was observed for Ad5/3.19K-.IL at 1 vp/cell on day 5 and at 0.001 vp/cell on day 10, but for Ad5/3.19K.IL only at the highest titer, 10 vp/cell, on day ten. Of note, transgene expression by Ad5/3.19K-.IL was superior to transgene expression by Ad5/3.19K+.IL by 2.5 orders of magnitude on day 5 (at
0.0001 to 0.1 vp/cell) and 4 orders of magnitude on day 10 (0.0001 vp/cell), i.e. at virus titers for which loss of cell substrate was not a limiting factor. Indeed, at 5 days post-infection transgene expression by Ad5/3.19K-.IL was already superior to transgene expression by the same titer of Ad5/3.19K+.IL at 10 days post-infection. This leads to the conclusion, that in A549 cells the deletion of E1B19K did not interfere with transgene expression during the first replication cycle (Fig. 6a) and resulted in a dramatically increased transgene expression beginning at 2 days post-infection due to accelerated virus spread.

![Fig.26: Comparison of transgene expression by E1B19K+ or E1B19K- transgene-encoding oncolytic adenoviruses in A549 cells by performing cytotoxicity and reporter gene assay in parallel. A549 cells were infected with Ad5/3.19K-.IL, Ad5/3.19K+.IL or Ad5CMVLuc at indicated titers for cytotoxicity assay and luciferase assay in parallel. At 5 days (left panels) and 10 days (right panels) cells were stained by crystal violet to visualize cytotoxicity and luciferase activities were determined for cells infected in parallel. Infection for luciferase assay were performed in triplicates. Columns show mean titers, arrow bars show standard deviations. # indicates that luciferase activity was below the detection limit. Asterisks indicate statistical significant differences for comparison of Ad5/3.19K-.IL to Ad5/3.19K+.IL with p < 0.05.](image)

Taken all together, this second project demonstrates that for oncolytic adenoviruses with luciferase in the late transcription unit, deletion of E1B19K caused early apoptosis of infected tumor cells. Oncolysis was affected in a cell type-dependent manner, resulting either in apoptosis-dependent early viral release and enhanced oncolysis (A549) or in reduced virus replication and oncolysis (PMelL). Early apoptosis and viral release by E1B19K deletion did not interfere with luciferase expression. Consequently, transgene expression by the E1B19K- virus was superior to the E1B19K+ virus over time due to better virus spread in A549 but reduced for PMelL. Thus, the E1B19K- virus showed >3 orders of magnitude increased oncolysis and transgene expression 10 days post-infection of A549 cells. Already at 5 days post-infection transgene expression by the E1B19K- virus was superior to transgene expression by the same titer of E1B19K+ virus at 10 days.
6 DISCUSSION

6.1 Insulated hsp70B' promoter: stringent heat-inducible activity in replication-deficient, but not replication-competent adenoviruses

6.1.1 Feasibility of the hsp70B' promoter fragment for heat-inducible transgene expression and its analysis in the context of a replication-deficient adenoviral vector

Therapeutic gene transfer represents an innovative strategy for targeted treatment of cancer and other diseases. To date, several therapeutic gene candidates for cancer gene therapy have been identified. These include tumor suppressor genes [(p53 (Bouvet, Bold et al. 1998; Hlavaty, Tyukosova et al. 2000; Ahn, Han et al. 2002; Shimada, Matsubara et al. 2002), retinoblastoma gene pRB (Fueyo, Gomez-Manzano et al. 1998), and p16^{INK4A} (Campbell, Magliocco et al. 2000)] and siRNAs [against ras (Rejiba, Wack et al. 2007), HER-2/neu (Yang, Cai et al. 2004), and VEGF (Gao, Mei et al. 2009)] for mutation compensation; suicide genes (cytosine deaminase/5-fluoro cytosine (Huang, Zhang et al. 2002; Ueda, Iwahashi et al. 2003), and herpes simplex thymidine kinase/ganciclovir (Sutton, Freund et al. 2000; Okabe, Arai et al. 2003) for tumor cell killing, cytokines [IL-2 (Gansbacher, Zier et al. 1992; Haddada, Ragot et al. 1993), IL-4 (Park, Kim et al. 2003), IL-12 ((Akiyama, Maruyama et al. 2002; Mazzolini, Prieto et al. 2003), interferon-γ (Zhang, Hu et al. 1996), GM-CSF (Guan, Ma et al. 2001)] for anti-tumor immune activation and it is expected that many more will be identified by genomic projects and large scale screening efforts. In contrast, the engineering of gene transfer vectors that specifically and efficiently deliver their therapeutic payload to the diseased tissue and provide high-level expression of the therapeutic transgene still represents a major challenge. Tumor-restricted transgene expression by adenoviral gene transfer vectors can be achieved by using tissue- or tumor-specific promoters, which have been tested in a variety of models with promising results. Among these are the α-fetoprotein promoter (AFP) for hepatocellular carcinoma (Hallenbeck, Chang et al. 1999), the tyrosinase promoter for melanoma (Nettelbeck, Rivera et al. 2002) and the prostate specific antigen (PSA) promoter for prostate cancer (Rodriguez, Schuur et al. 1997). Other groups implemented the use of exogenously inducible promoters. This is of special interest considering the delivery of cytotoxic transgenes that are not only expressed in situ but also secreted into the system and can have considerable tissue toxicity. For instance, although tumor-necrosis-factor alpha (TNF-α) exhibits cytotoxic effects on a wide range of transformed cells, high levels correlate with
increased risk of mortality due to liver toxicity and hypotension (Spriggs, Sherman et al. 1988; Rink and Kirchner 1996). In another phase I trial Motzer and co-workers administered IL-12 subcutaneously, which led to severe hepatic, hematopoietic and pulmonary toxicity (Motzer, Rakhit et al. 1998). Similarly, the intravenous administration of adenovirus with a CMV promoter driven FasL gene led to liver damage by systemic Fas activation (Rensing-Ehl, Frei et al. 1995; Nagata 1997). Thus, approaches to regulate the duration and the level of transgene expression exogenously would be beneficial. Therefore, several inducible gene expression systems in the context of adenoviral vectors have been developed, using radiation-inducible (Manome, Kunieda et al. 1998), drug-inducible (Yoshida and Hamada 1997; Rubinchik, Ding et al. 2000), hormone-inducible ((Senner, Sotoodeh et al. 2001) and heat-inducible promoters (Brade, Ngo et al. 2000; Borrelli, Schoenherr et al. 2001; Li, He et al. 2003).

This dissertation for the first time describes the development of novel adenoviral gene transfer vectors that feature improved and stringent regulation of transgene expression by using a 245bp fragment of the hsp70B' promoter together with insulator elements.

The feasibility of the hsp70B' promoter fragment for heat-directed transgene expression was initially confirmed by transient transfection assays. In the absence of heat the hsp70B' promoter exhibited low basal activities, whereas heat-treatment resulted in strong induction of the hsp70B' promoter and high levels of luciferase expression. Reporter gene studies by Leung and co-workers already demonstrated that the hsp70B' promoter in context of a plasmid features low basal activities and strong-induction upon heat-treatment (Leung, Rajendran et al. 1990), which could be confirmed by the results of the present study. Regarding the strength of heat-inducibility, Kato and coworkers found that heat-shock stress led to higher induction rates for hsp70B' mRNA than for other hsp70 genes (Kato, Akagi et al. 1997), suggesting that the hsp70B' promoter is extremely sensitive to cellular stresses. This high sensitivity of the hsp70B' promoter was also observed in the present study, as heat-treatment resulted in up to 3741-fold up-regulation of transgene expression. Furthermore, a 397bp fragment of the human hsp70B' promoter showed in another report sensitive induction by cadmium chloride (Wada, Taniguchi et al. 2005). This high sensitivity is presumably due to overlapping heat-shock-element (HSE) consensus sequences within the promoter region. Indeed, it was demonstrated that the hsp70B gene, which is the most closely related to the hsp70B' gene, but lacks
overlapping HSEs, had lower mRNA expression levels than the hsp70B' gene, when cells underwent stress stimuli (Parsian, Sheren et al. 2000). Therefore, the heat-inducible hsp70B' promoter system not only provides a high level of control over therapeutic transgene expression but also is characterized by its high efficiency with which the transgene is induced – both features are indispensable for applications of this system in clinical settings.

Nevertheless, this study also showed that the regulation of the hsp70B' promoter was slightly affected when transfection of pGL3hsp70B'pL was followed by early adenovirus infection (before genome replication), leading to higher basal activities and thus reducing the heat-induction rate. Even considerable higher basal levels for the hsp70B' promoter had been observed after transduction with Adhsp70B'pL. Notably, promoter deregulation was especially strong for HeLa cells, whereas in SK-MEL-28 cells lower levels of basal expression were observed. Such variability in basal and maximal levels of gene expression between different cell lines had also been noted for other cellular hsp70 promoters (Muramatsu, Kobayashi et al. 1995; Dressel, Johnson et al. 1998), suggesting that the inducibility of heat-shock promoters depends on the origin of each cell line. Activation of the hsp70B' promoter is, for example, highly dependent on the interaction of HSF-1 with HSEs (Baler, Dahl et al. 1993). The induction of HSF-1 in turn depends on a complex regulatory network involving several proteins. Thus there are many possible factors that might lead to variations in hsp70B' promoter activity. After stress stimuli inactive HSF-1 monomers convert to active homotrimers, allowing translocation in the nucleus and binding to HSEs, which lead to the transactivation of the heat shock genes (Zimarino, Tsai et al. 1990). This multistep process involves various chaperones and regulatory factors, like Hsp90, Hsp70, Hdj-1 and HSBP-1 (Satyal, Chen et al. 1998; Shi, Mosser et al. 1998; Zou, Guo et al. 1998) and alterations in certain oncogenes and cell signalling pathways could have an influence on HSF-1 and thus on the hsp70B' promoter. For instance, activation of the MAP kinase pathway has been demonstrated to repress HSF-1 and the heat shock response (Chu, Soncin et al. 1996; Kline and Morimoto 1997; Chu, Zhong et al. 1998), whereas mutated tumor-suppressor genes such as p53 and WT1 can induce gene expression in an HSE dependent manner (Tsutsumi-Ishii, Tadokoro et al. 1995; Maheswaran, Englert et al. 1998).

Interestingly, considerable differences in heat-induction of the hsp70B' promoter were observed in HeLa cells that either underwent transfection with pGL3hsp70B'pL and early adenovirus infection (before genome replication) or transduction with the
Discussion

replication-deficient adenoviral vector Adhsp70B’pL. Whereas the transfection/infection approach led to a slightly higher basal activity of the hsp70B’ promoter, transduction with Adhsp70B’pL resulted in considerable to strong basal activity. Deregulation of hsp70 promoters has been described to occur through transactivation by the adenoviral E1A protein (Williams, McClanahan et al. 1989). However in comparison to Ad5wt, Adhsp70B’pL is deleted for the E1 and E3 genes, arguing rather for non-viral proteins to be involved in the non-specific activation of the hsp70B’ promoter, which probably cannot be mimicked by the transfection/infection approach. This phenomenon, that some promoters, which show specificity in reporter plasmid transfections, partially or completely, loose their activity and/or specificity after insertion into the genome of adenoviral vectors had also been described by other groups (Buvoli, Langer et al. 2002; Hoffmann, Jogler et al. 2005; Hurtado Pico, Wang et al. 2005). In this regard, several groups have identified cryptic transcription initiation sites in adenoviral genomic sequences at the left end of the adenovirus genome upstream of the E1A gene, specifically in the inverted terminal repeat (ITR) and in the packaging sequence (Ψ) (Hatfield and Hearing 1991; Buvoli, Langer et al. 2002; Yamamoto, Davydova et al. 2003). Such cryptic transcription initiation is believed to mediate non-specific read-through transcription of whatever transgene or viral gene lies downstream, regardless if a promoter is located in-between or not. Indeed, considerable expression of promoterless transgenes inserted downstream of the left ITR and Ψ of adenoviral vectors has been reported (Buvoli, Langer et al. 2002; Yamamoto, Davydova et al. 2003). In order to prevent promoter interferences by read through transcription from cryptic initiation sites, poly A sites were inserted upstream and downstream of the hsp70B’ promoter luciferase cassette of Adhsp70B’pL. Nevertheless, it had also been described that enhancer activity of viral elements can affect the selectivity of inserted promoters. This effect has again been studied best for the ITR and Ψ, to which cellular transcription factors bind (Hearing and Shenk 1983; Hen, Borrelli et al. 1983; Miralles, Cortes et al. 1989; Buvoli, Langer et al. 2002) To overcome additional enhancer interferences, the two novel adenoviral vectors, Adl₂hsp70B’pL and AdIC₂hsp70B’pL, had been generated. These two replication-deficient adenoviral vectors contain either a tandem repeat of the 1.2kb chicken β-globin locus insulator (l₂) or the shorter 500bp tandem repeat of the chicken β-globin insulator core (IC₂), which aimed to separate the hsp70B’ promoter from the left ITR and the packaging signal. Both the l₂-or IC₂-containing adenoviral vector, showed in HeLa cells as well as in SK-MEL-28 cells a strong increased heat-
inducibility of the hsp70B' promoter compared to the parental virus Adhsp70B'pL. The reason for this strong heat-induction was a marked reduction in the basal activities of the promoter, a critical advantage for their application. Interestingly, transductions with Adl2hsp70B'pL resulted in superior induction ratios for the hsp70B' promoter compared to transductions with AdIC2hsp70B'pL that already showed higher basal activities. Nevertheless, induction of transgene expression by heat-shock for AdIC2hsp70B'pL was still approximately 10-fold increased compared to Adhsp70B'pL, indicating that the IC2 element is functional. Furthermore, the size of the IC2 elements might be beneficial when considering the limited cloning capacity of adenoviruses. Hence, the IC2 element would not only provide a tool to reduce the enhancer activity on inserted promoters but also would allow for the insertion of larger transgenes.

Of note, the results obtained for the I2 elements are in accord with a previous report that has demonstrated improved regulation of an artificial metal-inducible promoter in a replication-deficient adenoviral vector by the 1.2kb chicken β-globin insulator in vitro and in vivo (Steinwaerder and Lieber 2000). The induction ratios of the promoter after adenoviral gene transfer were improved by the insulator up to 40-fold by reduction of basal promoter activity. Notably, in the present study the 1.2kb (I2) insulator improved heat-induction up to approx. 15-fold compared to the induction rate of the parental virus Adhsp70B'pL. Steinwaerder´s report also showed that deletion of the 250bp insulator core resulted in loss of insulator activity, indicating the prominent role of this sequence. This is in agreement with the findings in this thesis that tandem copies of this element (IC2) show potent insulating activity, although somewhat less than the tandem repeat of the 1.2 kb element. A recent report shows that gene expression from the erbB2 promoter, that is mostly overactive in breast cancer cells, after adenoviral gene transfer to erbB2-negative cells was reduced by flanking of the expression cassette with the IC2 elements in antisense orientation (Martin-Duque, Jezzard et al. 2004). Of interest, this study shows that insulating activity was lost when high virus titers were used in transduction experiments. Thus, insulators might lose their activity at higher copy numbers, which might be of relevance for the application of replication-competent adenoviruses and therefore will be discussed in the next chapter.

Nevertheless, the chicken β-globin insulator has shown insulating activity in adenoviral vectors for different promoters and in various cell types. In addition, Alemany and coworkers demonstrated feasibility of the human insulator element of
the myotonic dystrophy locus to improve transcripational targeting of adenovirus replication (Majem, Cascallo et al. 2006). Like the chicken \( \beta \)-globin insulator, this element elicits its function via binding of the zinc-finger protein CTCF (Filippova, Thienes et al. 2001).

The remarkable potential of externally controlled adenovirus-mediated therapeutic gene expression is highlighted by previous reports on a different inducible system, radiation-induced expression of TNF\( \alpha \) via the egr1 promoter (Ad.EGR-TNF) (Weichselbaum, Kufe et al. 2002). This system resulted in effective concentrations of the potent anticancer drug TNF\( \alpha \) at the tumor site; however, avoiding the systemic toxicity of the drug that excludes its systemic application. Most importantly, intratumoral application of AD.EGR-TNF with concurrent radiotherapy was well tolerated and showed potent anticancer activity in early clinical trials. Interestingly, the egr-1 promoter mediates considerable gene expression after adenoviral gene transfer even without irradiation (Yamini, Yu et al. 2004; Anton, Gomaa et al. 2005), arguing for the incorporation of insulator elements to improve the therapeutic outcome. Furthermore, induction of the promoter has frequently resulted in consistent but less than 10-fold upregulation of transgene expression and in an activity that was weaker than for CMV promoter constructs. Thus, the Adhsp70B' viruses described in this study constitute an induction range and maximal activities that are superior to the AdEGR virus. In addition, AdI2hsp70B'pL and AdIC2hsp70B'pL represent alternatives to the AdEGR virus in clinical settings that exclude irradiation.

6.1.2 Analysis of heat-directed transgene expression in the context of a replication-competent/oncolytic adenoviral vector

The co-expression of transgenes by CRAds is a promising strategy that is expected to improve various aspects of viral oncolysis and can be harnessed to incorporate additional therapeutic strategies, such as genetic immunopotentiation, apoptosis induction or anti-angiogenesis. But since virus replication cannot be controlled, i.e. by virostatic drugs, and strong expression of the delivered transgene is expected, regulatable gene expression is highly desired. However, this has not been successfully achieved yet. Based on the results of Adhsp70B'pL in SK-MEL-28 cells that showed strong heat-inducible transgene expression, it was investigated whether the hsp70B' promoter retains its activity and regulation in an oncolytic adenovirus. In this regard, the novel melanoma-selective oncolytic adenovirus Ad2xTyr.hsp70B'pL was generated. Of particular note, in a cytotoxicity assay this novel replication-
compotent adenovirus retained its melanoma specificity, as no lytic activity was detectable in HeLa cells. In contrast, in SK-MEL-28 clear cytolysis was observed, although it was 1.5 orders of magnitude attenuated versus its parental virus Ad2xTyr and Ad5wt (Banerjee, Rivera et al. 2004). This stringent melanoma-selectivity of the tyrosinase promoter made it possible to further characterize the hsp70B' promoter activity and inducibility in the context of an adenoviral genome in both cells in which the virus replicates (melanoma cells) and cells into which the virus can enter, but not replicate its genome (other cell types). However, the reason for reduced lytic activity of Ad2xTyr.hsp70B'pL in melanoma cells remains to be determined. This might have been caused by the missing E3 region, which had to be deleted because of size limitations. The E3 region codes for seven different polypeptides and nearly all of them have immunomodulatory functions, except E3-11.6K, also known as adenoviral death protein (ADP). The ADP gene is required for efficient lysis and release of viral progeny from infected cells (Tollefson, Ryerse et al. 1996; Tollefson, Scaria et al. 1996) and therefore important for lateral viral spread. Thus, the deletion of the E3 region within the Ad2xTyr.hsp70B'pL could lead to diminished cytolysis activity compared to E3 containing oncolytic adenoviruses. Nevertheless, it is worth mentioning that the E3-deletion can only in part explain the observed effect, because attenuation of AdΔ24E3- compared to Ad5wt was only minimal. Lytic activity of Ad2xTyr.hsp70B'pL, however, might be increased by insertion of the transgene cassette into a different position in the oncolytic adenovirus genome or by choosing an appropriate candidate transgene, which further increases cytotoxicity (e.g. TNF-α) or enhances viral spread (see chapter 2.4.3.5).

In the two melanoma cell lines tested, heat-inducibility of the hsp70B' promoter in Ad2xTyr.hsp70B'pL was retained at early timepoints. However, at later timepoints regulation of the hsp70B' promoter was nearly lost leading to high basal activities. Similar results were observed after the transduction of the E1-complementing cell line 293 with AdI2hsp70B'pL and AdIC2hsp70B'pL, which are able to replicate in these cells. Two hours post-infection (before genome replication) both adenoviral vectors maintained heat-inducibility of the hsp70B' promoter. However, 24 h post-infection (after genome replication) heat induction was considerably reduced, indicating that not only promoter regulation but also insulating activity might get lost when viral replication takes place. Solely in HaCat cells, where Ad2xTyr.hsp70B'pL cannot replicate, heat-induction of luciferase expression was retained at early timepoints as well as at later timepoints.
The finding that heat-inducibility of the *hsp70B'* promoter can get lost when viral replication takes place is new, since this promoter had not been investigated before in the context of a replicating adenoviral genome. However, equal results were observed in a previous report that examined the activity of a 400bp fragment of the related and similarly regulated *hsp70B* promoter in an E1B55K-deleted, conditionally replication-competent adenovirus (Lee, Galoforo et al. 2001). The promoter was shown to be active without heat-shock in cells permissive for virus replication 1 or 2 days post-infection. Residual heat-induction of the promoter at these time-points was approximately 12-fold and thus in the range of the reported results for Ad2xTyr.hsp70B'pL in melanoma cells 1 day post-infection. However, this report did not further investigate the reason for promoter deregulation.

One reason for the loss of promoter regulation in Ad2xTyr.hsp70B'pL might be the insertion of *hsp70B'* promoter-luciferase cassette into the Ad2xTyr per se. As already mentioned in the previous chapter, the loss of cellular promoter selectivity in adenovirus genomes even in the absence of virus replication can be caused by cryptic transcription initiation or enhancer activity of viral genomic sequences or protein activity (Steinwaerder and Lieber 2000; Yamamoto, Davydova et al. 2003; Hurtado Pico, Wang et al. 2005). However, this was not the case for the *hsp70B'* promoter in Ad2xTyr.hsp70B'pL, as can be concluded from functional promoter regulation in HaCaT cells, that do not support virus replication, and during early infection in melanoma cells, i.e. before replication of virus genomes.

Therefore, it was speculated that the loss of inducibility of luciferase expression after infection of Ad2xTyr.hsp70B'pL might result from deregulation of the *hsp70B'* promoter itself at high copy numbers. However, when virus genome replication in melanoma cells was artificially blocked by AraC treatment the heat-inducibility of the *hsp70B'* promoter of Ad2xTyr.hsp70B'pL could only be improved to some extent, which was based on reduced basal promoter activity in the absence of heat-shock. Complete restoration could not be observed, thus indicating that copy numbers are not the major factor leading to deregulation of the *hsp70B'* promoter.

Next, it was hypothesized that interference of adenoviral proteins or of cellular factors induced by progressive adenovirus infection with *hsp70B'* promoter activity could cause a loss of promoter regulation in Ad2xTyr.hsp70B'pL. In this regard, it had been reported that the adenoviral E1A can transactivate the human cellular *hsp70(A)-1* promoter by interacting with the basal transcription complex (Williams, McClanahan et al. 1989). However, this cannot be the major factor leading to deregulation of the
Discussion

*hsp70B'* promoter as it was only modestly deregulated after reporter plasmid transfection and super-infection with Ad5wt. Further it was speculated, that limited amounts of HSF-1 are responsible for reduced heat-induction of luciferase expression from Ad2xTyr.hsp70B’pL after replication of the virus genome. Therefore, co-infection of SK-MEL-28 cells with Ad2xTyr.hsp70B’pL and either AdGFP (with or without heat-shock) or AdmHSF-1 had been performed. However, co-infection with AdGFP followed by heat-shock as well as co-infection with AdmHSF-1 resulted only in minimal induction of luciferase expression from Ad2xTyr.hsp70B’pL. Notably, co-infection of the non-replication-competent adenoviral vector Adhsp70B’pL with AdmHSF-1 or heat-shock led to increased luciferase activities. These data strongly argue against the hypothesis that most of the *hsp70B'* promoter copies are not activated due to a deficit of the transcription factor HSF-1. Nevertheless, other factors of the transcription initiation complex of the *hsp70B'* promoter might be limiting.

All together, these findings reveal that cellular promoter fidelity in replication-competent vectors can be lost in permissive cells, which should be considered in future strategies for transcriptional regulation and/or targeting of transgene expression by oncolytic adenoviruses. The results of this thesis therefore suggest that future studies need to elucidate whether vector replication results in loss of promoter regulation also for other inducible promoter systems that have been successfully used in replication-deficient vectors (Toniatti, Bujard et al. 2004).
6.2 Transgene Expression by Oncolytic Adenoviruses is Modulated by E1B19K-Deletion in a Cell Type-Dependent Manner

This study demonstrates that E1B19K deletion of transgene-encoding oncolytic adenoviruses in susceptible cell types not only mediates accelerated viral release and spread, but also dramatically increases transgene expression from late viral transcription units. Both accelerating viral spread and combining viral oncolysis with therapeutic gene expression have been reported to improve virotherapy in preclinical and clinical studies (Sauthoff, Heitner et al. 2000; Harrison, Sauthoff et al. 2001; Hermiston and Kuhn 2002; Yan, Kitzes et al. 2003; Subramanian, Vijayalingam et al. 2006; Freytag, Stricker et al. 2007; Gros, Martinez-Quintanilla et al. 2008). It has not been clear to date, however, as to whether a combination of both strategies is feasible. In this regard, three scenarios could have been envisioned: (i) attenuated viral replication resulting in an extended lifespan of infected cells allows for sufficient time for therapeutic protein synthesis whereas early lysis aborts production of therapeutic proteins, (ii) transgene expression is efficient before early host cell lysis and viral release and consequently accelerated virus spread causes enhanced therapeutic protein expression from an increased number of infected cells, or (iii) a combination of both, i.e. early lysis and viral release reduces therapeutic protein expression by each infected cell, which is however compensated by increased virus spread. This study shows that the second scenario holds true if early viral release and enhanced spread by E1B19K deletion is combined with transgene expression from the late adenoviral transcription unit.

The deletion of E1B19K expression has been the most widely investigated mutation featuring a quite strong increase in cell killing activity (Sauthoff, Heitner et al. 2000; Harrison, Sauthoff et al. 2001; Subramanian, Vijayalingam et al. 2006). This thesis clearly demonstrates that lytic activity of a transgene encoding oncolytic adenovirus can be increased by E1B19K deletion up to several orders of magnitude in different tumor cells. Further it could be demonstrated that the induction of apoptosis, i.e. caspase activation, is responsible for this outcome by causing accelerated release of infectious viral particles at least in A549 cells. This, however, was of concern to the “arming” approach, because in addition to the reduced time for transgene expression by early lysis, early apoptosis induction might further interfere with transgene expression. Nevertheless, our results reveal that deleting E1B19K does not affect transgene expression and activity in infected cells that show the accelerated spread phenotype. Even more, accelerated virus spread resulted in dramatically increased
cumulative transgene expression. Specifically, both viral spread/oncolysis and transgene expression were increased 1,000 to 10,000-fold at 10 days post-infection of A549 cells by the E1B19K mutation. Of note, transgene expression by the E1B19K mutant virus was already at 5 days post-infection superior to transgene expression observed at 10 days post-infection for the matching E1B19K wild-type virus.

These results were obtained even in the context of restricting transgene expression to late viral replication by inserting it into the late viral transcription unit. Transgene expression from late viral transcription units has been widely implemented using internal ribosome entry, as we did here, or alternative splicing (Fuerer and Iggo 2004; Jin, Kretschmer et al. 2005; Cascante, Abate-Daga et al. 2007) mechanisms (for an overview of gene expression strategies see also (Nettelbeck 2008). This strategy is considered advantageous, because of the high efficiency of the expression of transgenes from viral transcription units and because late transgene expression is dependent on replication of the viral genome and should in consequence be tumor-specific for oncolytic adenoviruses. Transgenes have also been inserted into early viral transcription units of oncolytic adenovirus genomes (Hawkins, Johnson et al. 2001; Rivera, Wang et al. 2004) or as full transcription unit including a promoter (Freytag, Rogulski et al. 1998; Wildner, Blaese et al. 1999). It remains to be determined how transgene expression in these formats is influenced by the E1B19K deletion. However, it can be speculated that earlier onset of transgene expression is even less sensitive than late transgene expression towards mutations that result in early apoptosis induction and/or viral release.

Ad5 mutants other than the E1B19K-deletion have been reported to cause an early viral release/enhanced spread phenotype in tumor cells, namely C-terminal truncations of the l-leader or of the E3/19k protein (Yan, Kitzes et al. 2003; Subramanian, Vijayalingam et al. 2006; Gros, Martinez-Quintanilla et al. 2008). For these mutants the molecular basis of early viral release was reported to be distinct from apoptosis induction. Rather, early onset of virus genome replication and thus entry into late phase of the viral life cycle (truncated l-leader) or membrane permeabilization (truncated E3/19k) were revealed. The results of this thesis demonstrate that early viral release resulting in enhanced viral spread per se does rather increase transgene expression, it could be speculated that also these mutations can improve transgene expression from “armed” oncolytic adenoviruses.

The results of this study imply that the interaction of adenovirus infection with host cell apoptosis pathways is more complex than previously thought. Several adenoviral
genes have pro- or anti-apoptotic activity when investigated individually and anti-apoptotic activity during early replication is thought to be required to prevent premature cell death and abortive virus replication in normal host cells. Recent studies, however, reported that adenoviral cell killing of cancer cells is not apoptotic, since (i) minimal or no caspase activation was detected after adenoviral infection and (ii) neither expression of proteins with anti-apoptotic activity nor treatment with caspase inhibitors blocked viral oncolysis (Abou El Hassan, van der Meulen-Muileman et al. 2004; Baird, Aerts et al. 2008). Similar results were obtained for A549 cells in this study. However, for low passage melanoma cells PARP and caspase 3 cleavage could be detected during late adenovirus infection. Furthermore, in these cells caspase inhibition during virus infection resulted in an approx. 10-fold increase in infectious virus particle production. Thus, adenovirus infection indeed activates apoptosis that counteracts productive virus replication. This corresponds with the observation that deletion of E1B19K, which showed anti-apoptotic activity, resulted in attenuated adenovirus replication as measured by infectious viral particle production (although it could only partially reverse this attenuation by caspase inhibition and thus caspase-independent effects might also be involved). In contrast, deleting E1B19K did not interfere with productive adenovirus replication in A549 cells but rather showed pro-release activity. As E1B19K deletion caused early apoptosis induction in both PMelL and A549 cells, the different consequences for adenovirus replication must be caused by molecular events downstream of apoptosis activation. Clearly, it will be of interest to further investigate the mechanism of virus release in cancer cells and how they are triggered by activation of the apoptosis program in A549 and other susceptible cancer cells.

An important result of this study is therefore, that the phenotype of the E1B19K mutant is cell-type dependent and indeed opposing effects on viral spread were observed in different cell types. Furthermore, cumulative transgene expression correlated with viral spread. In consequence, future applications of adenoviruses engineered for improved lytic activity should consider tumor type restrictions. For example, the results for low passage tumor cell cultures imply that the E1B19K mutant adenovirus is not advantageous for treatment of malignant melanoma, at least not for each patient, which is in accordance with previous observations (Schmitz, Graf et al. 2006). It remains to be shown whether the enhanced spread phenotypes of the I-leader and E3/19K mutants are cell type-dependent and more specifically, whether they mediate enhanced virus spread in those cell types in which
the E1B19K mutant was attenuated. In light of these results, the approach to identify viral mutants with increased cell killing potency by random mutagenesis and bioselection seems very attractive, because it allows for selection on the individual tumor type of interest (Yan, Kitzes et al. 2003; Subramanian, Vijayalingam et al. 2006; Gros, Martinez-Quintanilla et al. 2008). In this regard, the study suggests transgene expression as an attractive tool to monitor viral spread, as the results (Fig.26) show that it correlates with virus spread.
7 REFERENCES


References


References


Harrison, D., H. Sauthoff, et al. (2001). "Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved--


ionizing radiation or uptake of radioactive iododeoxyuridine." Hum Gene Ther 9(10): 1409-17.


References


References


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>aminoacid</td>
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<tr>
<td>Ad</td>
<td>adenovirus</td>
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<tr>
<td>Ad3</td>
<td>adenovirus serotype 3</td>
</tr>
<tr>
<td>Ad5</td>
<td>adenovirus serotype 5</td>
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<tr>
<td>amp</td>
<td>ampicillin</td>
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<td>approx</td>
<td>approximately</td>
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<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>AT</td>
<td>annealing temperature</td>
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<td>bp</td>
<td>basepair</td>
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<td>β-gal</td>
<td>β-galactosidase</td>
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<td>β-me</td>
<td>β-mercaptoethanol</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>C°</td>
<td>degree celcius</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>cytomegalovirus</td>
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<td>CsCl</td>
<td>caesium chloride</td>
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<td>CRAd</td>
<td>conditionally replicative Adenovirus</td>
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<td>Δ</td>
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<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleic acid triphosphate</td>
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<td>DTT</td>
<td>1,4 dithiothreitol</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>mA</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>PMSF</td>
<td>Phenyl-methyl-sulfonyl-fluorid</td>
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<td>rdAd</td>
<td>Replication-deficient adenovirus</td>
</tr>
<tr>
<td>rev</td>
<td>reverse</td>
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<td>RLU</td>
<td>relative light unit</td>
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<td>rpm</td>
<td>rounds per minute</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<td>Abbreviation</td>
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<tr>
<td>RT-PCR</td>
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<td>Simian virus 40</td>
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<td>TCID(_{50})</td>
<td>tissue culture infectious dose 50</td>
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<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
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<td>Tyr</td>
<td>tyrosinase</td>
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<td>U</td>
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<td>vp</td>
<td>virus particle</td>
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<td>WT</td>
<td>wild type</td>
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9 CURRICULUM VITAE

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11 PUBLICATIONS

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