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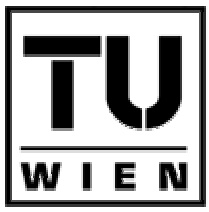

TECHNISCHE UNIVERSITÄT WIEN

VIENNA UNIVERSITY OF TECHNOLOGY

D I P L O M A R B E I T

Creating Universality for Cancer Gene Therapy in applying Adenovirus Vectors with Altered Tropism

Designing Adenovirus particles using K-Coil/E-Coil Technology to control receptor specificity

Ausgeführt am Institut für
Biotechnology Research
des CNRC-NRC in Montreal / Kanada

in Zusammenarbeit mit dem Institut für
Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften
der Technischen Universität Wien

unter der Anleitung von
Dr. Bernard Massie, **Dr. Rénald Gilbert** und **Dr. Yue Zeng**
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sowie

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Creating Universality for Cancer Gene Therapy in applying Adenovirus Vectors with Altered Tropism

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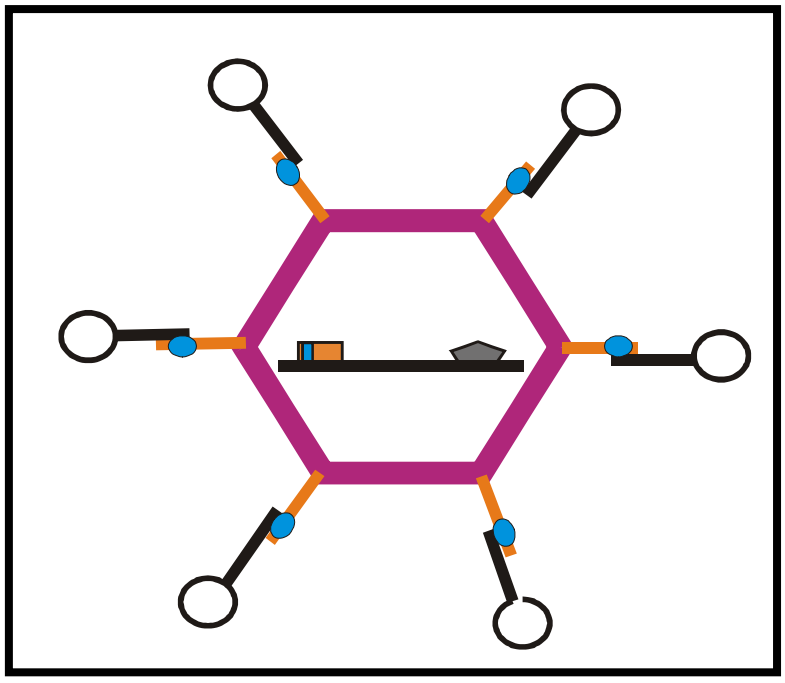

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1. Preface

1.1. Short outline of the project

For my Master Thesis project I was charged with the design of two recombinant types of adenoviruses. The first adenovirus should possess a K-coil sequence situated in the HI-loop region of the fibre's knob domain, the other one was supposed to have an ablated CAR tropism in addition to the identical K-coil insertion. The virus' transduction efficiency via EGF-receptors was monitored by fluorescents provoked by a GFP marker, that had to be inserted into the E1 region of the AdV genome for this reason.

1.2. The Research Institution – The Bioprocess Sector

The Biotechnology Research Institute (BRI) of the National Research Council Canada (NRC-BRI) is one of nineteen NRC institutes across Canada. It was established in 1987 and is Canada's largest laboratory dedicated to research and development in biotechnology. Upwards of 400 scientists and engineers are working in BRI's three research sectors health, environment and bioprocessing.

The Bioprocess Sector is also divided into three subgroups: Animal Cell Technology, Microbial and Enzymatic Technology plus the research group where I took part, Genomics and Gene Therapy vectors. BRI's Genomics and Gene Therapy Vectors Group is lead by Dr. Bernard Massie and designs and produces expression vectors by employing several organisms, including bacteria, yeast, insect and mammalian cells. These recombinant vectors like adenoviruses, adeno-associated viruses, retroviruses, baculoviruses or simlple plasmids are manipulated for vaccine applications or gene delivery in gene therapy. Additionally, the research group develops recombinant proteins and monoclonal antibodies for research and commercial applications.

2. Introduction

2.1. Reviewing Cancer and the Gene Therapy strategy

Cancer also referred as Neoplasm is a genetic disease and is characterized by escape of cells from the proper cell cycle control conferring them unlimited proliferartion potential and aberrant regulation of apoptosis resulting in increased survival of damaged or mutated cells. This fast cell replication is caused by changes in the cells' growth and repair genes. Subsequently the cancer cells may spread out to adjacent tissues. In the final stage of disease, cancerous cells affect other organs, by bloodstream or lymphatic system transport, building up so-called metastases. Patients suffering from cancer predominantly die by consequences of this fatal invading process, 13% of all deaths or 7.6 million people per year are ascribed to malignant tumours, cancer is thus the leading cause of death world-wide (WHO report [1], [2], [3]).

Genetic background of cancer Cells are normally controlled by proto-oncogenes that encode positive growth and division signals. However, a proto-oncogene can change its character by mutation or increased expression and become an oncogene that induces cancerous ("neoplastic") transformation [4]. On the other hand, there is a set of genes which cope with malign cell development if they are not mutated and causing cancer by themselves: tumour suppressor genes inhibit cancerous cell growth, and apoptosis-related genes further control cell growth by either promoting cell survival or cell death. In particular, this benefits the whole cell tissue e.g. by avoiding amplification of those cells which are already in fatal condition. Nevertheless, it requires a series of gene mutations to form a single cancer cell, a subversion of only one of these control genes is by far not enough [1], [3].

Gene therapy generally describes the insertion of transgenes in person's cells to fight disease by manipulation of intracellular DNA and chemistry. The principles for the control or destruction of cancer cells by Gene

therapy can be inactivation of oncogenes, replacement of damaged tumour suppressor genes or inhibition of "angiogenesis", a formation of blood vessels which is considered as a fundamental step in the transition of tumours from a dormant state to a malignant state. The specific transgene could have the capacity to enhance the production of toxic agents, it could stimulate the immune system to fight against the cancer cells, or it could sensitise tumour cells to radiation or drugs. For selective and efficient tumour cell administration the therapeutic transgene is either inserted into viral or non-viral targeting vectors [2], [3], [5], [6], [7], [39].

Although gene therapy came to the forefront in recent years as new option to cure cancer patients, there are several challenges needed to be overcome to bring it closer to the clinic. These obstacles include development of useful and safe vehicles for the delivery of foreign genes into target cells and minimising complications and undesired side-effects of the gene therapy. After development of viral vectors more than 25 years ago, retroviral vectors became very popular due to their ability to integrate into the host cell genome and to achieve persistent gene expression. However, this adverse event known as insertional mutagenesis can trigger malignant transformation by up-regulating and constitutive activating of cellular proto-oncogenes. Moreover, induction of immune response to viral vectors has become a serious hurdle in the field, thus vector-neutralizing antibodies reduce the ability of the viral vectors to transduce important therapeutic target cell types. With inflammatory responses the vector's effectiveness respectively the percentage of volunteers responding to the viral vector is blunted and gene expression gets short lived. Recombinant vectors based on modified adeno-associated virus (AAV) became the vehicles of choice for gene transfer because most humans display naturally acquired immunity to adeno-associated viruses, circumventing neutralizing antibodies. Very recently, a transient but strong toxicity was observed in clinical trials after application of AAV indicating that better understanding of vector biology and of the

interaction of viral vectors with the immune system is of fundamental importance. Remarkably, vectors developed on the basis of lentiviruses, members of the retroviral family, are considered to be much less genotoxic than the frequently used gamma-retroviral vectors.

Interestingly, lentiviruses possess the ability to infect cells in distinct cell cycle phases. It opens up the possibility to target both dividing and non-dividing cells. The same is true for adenoviruses, they are capable of infecting proliferating and quiescent cells but unlike the lentiviruses they do not integrate into the host genome. They therefore pose no risk of insertional mutagenesis. Even though Trentin et al. (1962) demonstrated that some adenovirus strains can cause cancer in laboratory animals, they have not been shown to cause human cancer and seem benign to the researcher. However, there is a risk that the viral DNA of adenoviruses, may disrupt host genes and possibly cause cancer in some patients after vaccination of a large population. The immunogenicity of adenovirus vectors is generally considered as second drawback in gene therapy application. Indeed, replication-competent adenoviral vectors have produced the most impressive cellular immune responses seen so far! On the one hand immune responses can impede safe and efficient use of adenoviral vectors for therapy approaches, so current studies focus on strategies to eliminate their immunogenicity by altering the adenoviral genome and its replication competence or replacing natural tropism of adenoviruses by a ligand mediated, CAR-independent binding mechanism. On the other hand moderate immunogenicity may be even advantageous in certain clinical applications like suicide gene therapy where inflammation responses may intensify the elimination of tumour cells. In summary replication-deficient adenoviral vectors have emerged as very popular and safe vectors: They have a broad host range, are stable and easy to manipulate, they can be easily produced at high titers and can accommodate larger inserts. In 2004 they had already been used in about one quarter of trials for gene therapy and it is expected that soon

replication-incompetent adenoviruses will be the most commonly used vehicle in clinical trials [64], [65], [66], [67], [68], [69], [70], [71], [72], [73], [74].

2.2. Vectors used in cancer gene therapy

Vectors have three strategic goals: they should enable gene delivery into tumour cells, protect the delivered transgenes from degradation and ensure stable transgene expression in the target cells. To be useful for medical application, the ideal vector needs to be easy and inexpensive to produce in large amounts as well. Besides, a vector should not cause side-effects such as infection, immunogenicity, host-cell mutagenesis or transmission. A broad spectrum of viral and non-viral vector systems has been developed with these intentions, and all types of vectors can score with some characteristics and their proper application fields but none of them are capable of unifying all the desired qualities yet [5], [8], [9].

2.2.1. Viral Vectors for gene delivery

Viruses are the most used gene transfer vehicles in gene therapy, thus they have the capability of introducing genetic material with high efficiency into target host cells. The viral vector techniques take advantage of the virus replication mechanism. They add a transgene into the virus RNA or DNA genome to enable a therapeutic gene transduction (gene delivery and expression).

For safety reasons, the viruses are modified for their use as vectors in their gene regions of replication, of assembling, or of infection to provide a replication deficiency to exclude pathogenicity. However, for gene therapy treatment, large amounts of virus vector particles are needed and therefore, the recombinant viruses that are designed as not-selfreproducing have to be replicated by engineered “packaging cell lines” (PLCs) which provide the deleted replication functions of the virus (see Figure I and II) [8], [9], [10], [11].

Viral transfer vectors can be divided into two classes: first, the non-lytic viruses such as retroviruses, and the more complex lentiviruses which

produce virions from the cellular membrane of an infected cell, leaving the host cell relatively intact but integrating their genome into the host DNA. Considering their advantages for the therapeutic gene delivery, retroviruses and lentiviruses offer nuclear targeting, long term expression, cell- and tissue-specific tropism as well as a large packaging capacity. Second, the lytic viruses including the human adenovirus and the *herpes simplex* virus destroy the infected cell after replication and virion production [12].

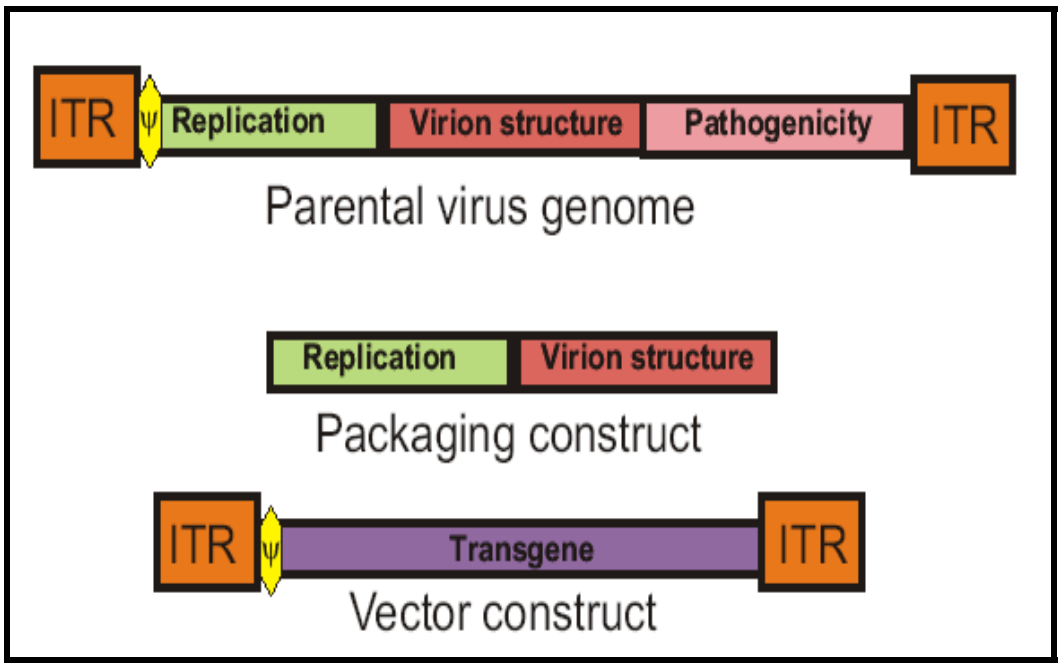

Figure I: Principle of generating a viral vector with packaging cell lines (PCL). ITR, inverted terminal repeats, origin of replication; ψ, packaging signal for encapsidation. For the production of recombinant viruses the components for replication and structural proteins in the parental virus genome are separated from the ones capable of causing disease. Therefore the packaging construct consists only of genes for replication and the protein structure of the virus, genes which cause the virus' pathogenicity are deleted. The vector construct contains the viral start of replication (ITR), the encapsidation signal ψ and the therapeutic transgene [10].

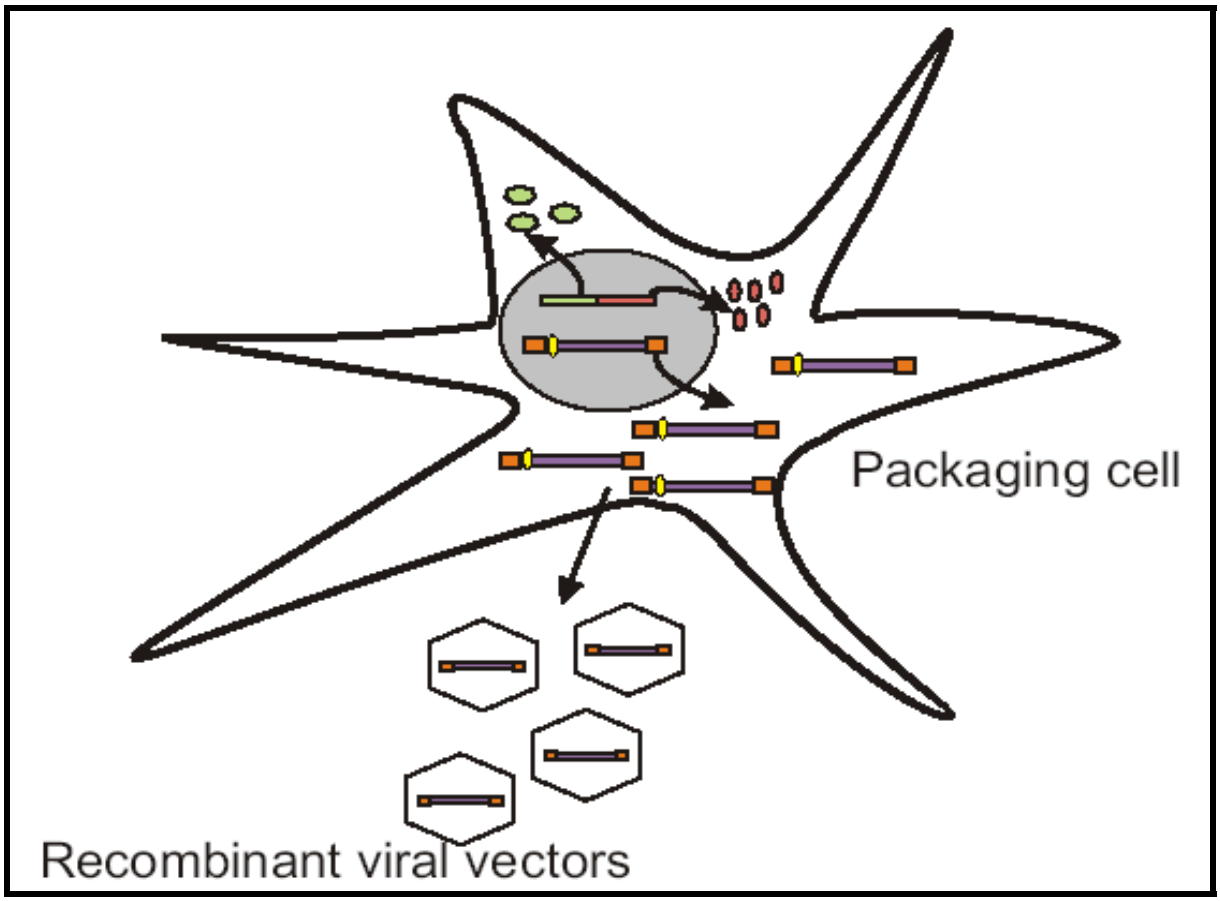

Figure II: Virus generation in PCL. The packaging constructs are located in the packaging cells (PCL) to express continuously the viral vector proteins for replication and encapsidation (green and red bubbles). For virus production the vector constructs are delivered into these PCL's, the vector genomes are replicated and encapsidated by the provided structural proteins to form finally a large amount of recombinant replication-deficient viral vectors [10].

Retroviruses are found in all vertebrates. They can be classified into three groups: (a) onco-retroviruses, (b) lentiviruses and (c) spumaviruses.

All retroviruses are small, enveloped, RNA viruses with two copies of the viral RNA genome in each particle (diploid). They enter by membrane fusion after binding to the host cell receptor. Subsequently they are converted into double-stranded DNA intermediates which are integrated into the host genome to form a provirus. All descendants of this infected host cell will now contain the new integrated viral genes. However sometimes the viral genome is not expressed immediately. Virus transcription is done by host cell transcription factors starting from the long terminal repeat region (LTR), a signal sequence for gene expression of the virus, to form new infective viral particles at the plasma membrane which are then released outside the cell.

There are three essential genes in the retroviral RNA: -*gag* which encodes for the capsid, -*pol* which is responsible for the expression of the viral enzymes and -*env* which encodes for the envelope glycoproteins, which mediate virus entry. Retroviral transfer vectors are made replication-deficient by replacing these structural genes *gag*, *pol* and *env* by a therapeutic gene with a possible size up to 10kb.

Retroviruses were among the first viral vectors to be developed mainly because of their easily manipulated genomes (see Figure III). Nevertheless, there are still some vital arguments against the in-vivo application of retroviral vectors [5], [8], [9], [10]: (a) the vector titer for *in vivo* therapies in humans will not exceed 10^7 infection particles/ml which is low in comparison to other vector delivery systems (see below); (b) the transcription of the transgene in host cells is not stable; (c) the randomness of integration of the viral vectors into the host genome causes the risk of mutagenesis; (d) the retroviruses are not able to transduce in non-dividing post-mitotic cells, therefore only proliferating

cells can become infected. Summarizing, retroviruses seem to be suitable at least for *ex vivo* gene therapy methods.

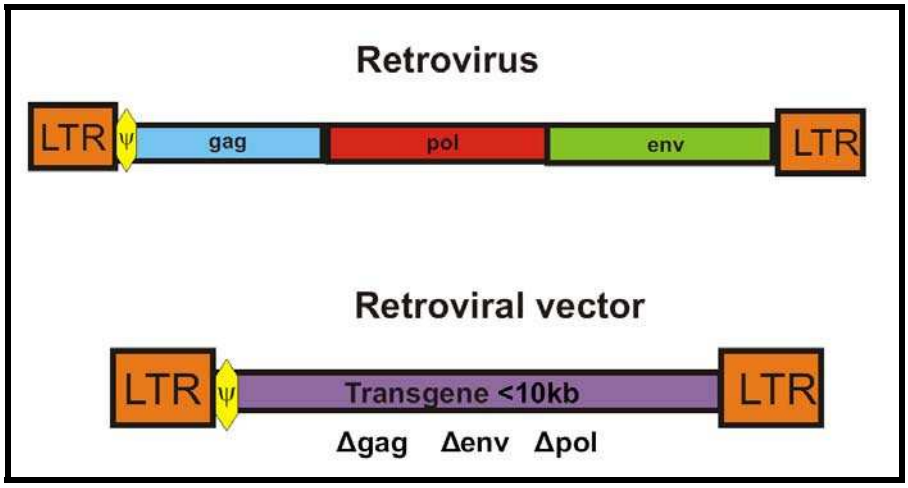

Figure III: Schematics of the wildtype retrovirus and the retroviral vector. The retroviral vectors are produced by removing the genes for virus replication (*gag*, *pol* or *env*) and by subsequent insertion of a therapeutic transgene. LTR: Long terminal repeats, that are the signal sequences for gene expression control; ψ: packaging signal sequence for encapsidation.

Lentiviruses (e.g. the human immunodeficiency virus type 1: HIV-1) are a special group of retroviruses with three to six additional viral proteins. They can infect proliferating but also quiescent cells, which is a desired tissue target for many therapies. Lentiviral vectors derived from lentiviruses have been engineered to overcome the limitations of retroviral vectors mentioned above. They confer persistent infection leading to stable transgene expression up to six months. Lentiviral vectors allow therefore the treatment of inborn pathological functions of metabolism and of chronic diseases in which a lasting gene expression is required [8], [9], [10], [13].

Adenoviruses are nonenveloped viruses encapsulated in an icosahedral protein capsid of 70-100nm in diameter. This capsid is made up of 252 capsomeres, among them a total of 12 pentons at the vertices each bearing a slender fibre. The 240 hexons construct the faces of the Adenovirus (see Figure IV). The adenovirus' genome is composed of linear, double-stranded DNA (32-36kb) associated with two major core proteins. At each 5' end of the linear double-stranded DNA a 55 kDa terminal protein is attached which ensures adequate replication of the virus' ends and serves as a primer.

The adenoviral DNA strand is composed by an identical inverted terminal repeat (ITR) at each end, one signal region (Ψ) required to package the viral DNA in viral particles and a region encoding for more than 40 proteins. This last area is divided into the early regions- E1 to E4- and the late regions- L1 to L5-, depending on the time course of their expression during the replication cycle. Therefore, the early genes E1-E4 are expressed before DNA replication, the late genes L1-L5 are expressed when the replication of viral DNA has being initiated (see Figure V).

The E1, E2 and E4 regions encode for regulatory proteins involved in replication, these regions are therefore essential for replication in cell culture. On the other hand, the genes in region E3 contribute to immune surveillance and suppression of the host's immune defences, but are nonessential for infection *in vitro*. The late genes encode structure proteins used for encapsidation of viral DNA.

In the 1950s the first adenovirus was isolated from the adenoids of patients with acute respiratory illnesses, and the respective virus was named after this origin of isolation. Until now there have been described 51 human adenovirus serotypes which can be classified as well by serological criteria into six subgroups A-F. Human adenoviruses are able to infect a wide range of organs such as the respiratory tract, the eye, the urinary bladder, the gastrointestinal tract, and the liver.

In gene therapy, the mostly applied adenovectors are derived from adenoviruses serotype 2 and 5 (Ad2, Ad5) of the subgroup C. Adenoviral

vectors have been created to overcome the limitations of retroviral vectors. The vectors can be produced at very high titers (10^{13} viral particles/ml), and they can accept large transgene inserts (up to 105% of their wild-type length). Moreover they are able to infect both dividing and post-mitotic cell types with high affinity.

For drug security reasons and to generate a place where to introduce the transgene in the viral vector, adenoviruses are made replication-defective by deleting the E1 region. To widen this region for the subsequent insertion of the transgene, the replication-dispensable E3 section is removed providing all in all a capacity of 8.5kb for the foreign DNA. The second generation of adenovectors has deletions in the regions E2 and E4, adenoviral vectors containing only the inverted terminal repeats (ITR) and the packaging signal have been created and named as "gutless vectors", they achieve an insertion capacity of 34kb for the transgene.

There are two main limitations of adenoviral vector :(a) they do not integrate their DNA into the host genome and therefore express transiently their foreign genes in dividing cells. The viral extra-genes are consequently not replicated in the course of cell division and are further absent in the progeny of transduced cells.
(b) Adenoviral vectors provoke inflammatory immune responses (the so called "immunogenicity") in the host. These effects might be harmful for the patient and besides they lower the transgene's expression period as well as they limit repeated administration of the vector [3], [8], [10], [11], [14], [18], [19].

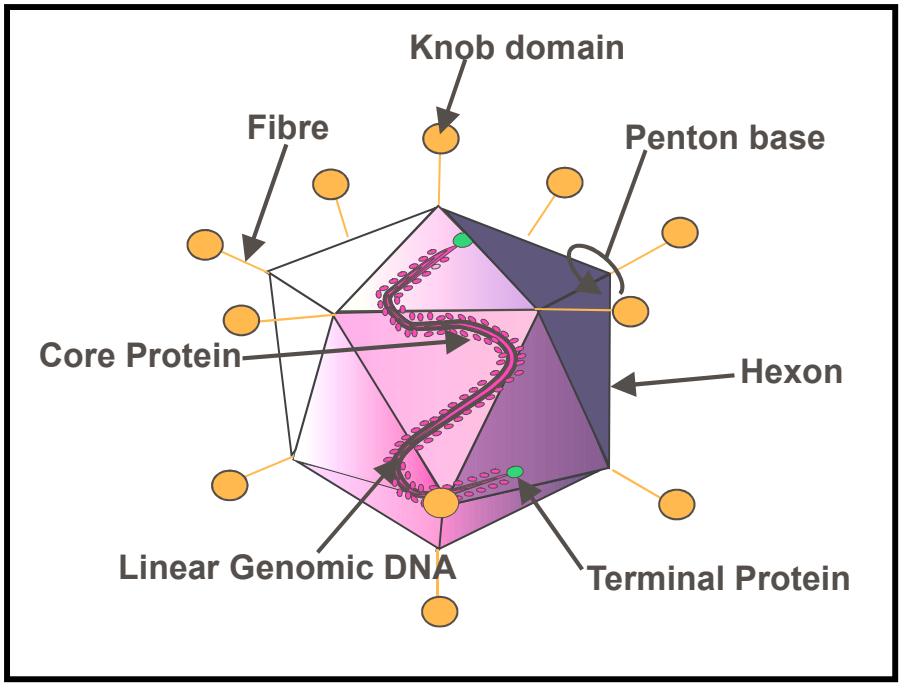

Figure IV: The three-dimensional adenovirus structure. The adenovirus icosahedron is made up of hexon and penton proteins. At each of the 12 vertices it pokes out a fibre composed of three domains: 1) the amino-terminal tail associated with the penton base protein, 2) the central shaft domain and 3) the carboxy terminal knob domain. This fibre-knob region generally interacts with the CAR-receptor on the cell surface. The adenovirus does not have a lipid envelope.

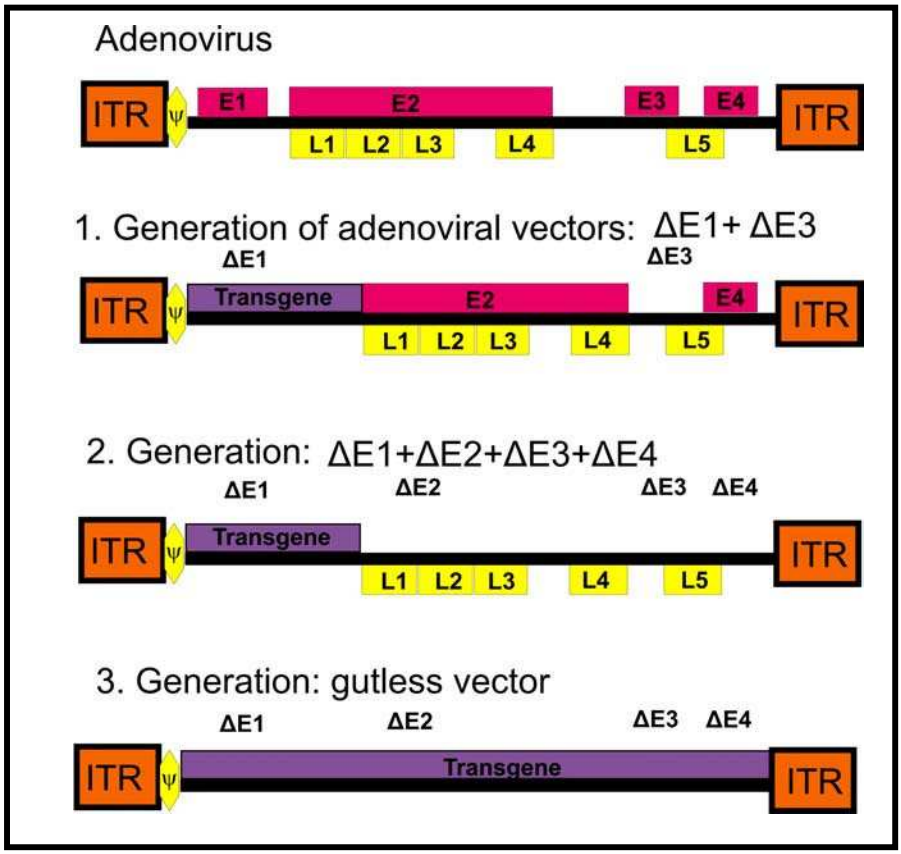

Figure V: Simplified diagrams of adenoviral genome and the three generations of adenoviral vectors. ITR, inverted terminal repeat; Ψ, packaging signal region; E1-E4, early regions which are protein encoding gene regions expressed before initiation of viral DNA replicaton; L1-L5, late regions expressed after initiation of viral DNA replication; Δ, deleted regions. First-generation adenovirus vector has a deletion in E1 genes, and optionally a deletion in the E3 region. This allows an insertion of a transgene with up to 7kb length. The E1 region ensures viral replication and DNA synthesis. Therefore its deletion should make the first generation of adenovectors replication defective. However immunogenicity and low-level replication are still notable in the ΔE1-adenovectors as the function of E1 region may be supplied by cytokines. Moreover the E1 deleted region can be replenished by co-infection of another DNA-virus. Second-generation adenovirus vectors, in addition to the deletion of E1 function, are characterized by the additional deletion of the E2 and/or E4 genes. In this case the transgene can have a maximum size of 10kb. Compared to the first generation the cytopathic effects and immune responses could be reduced significantly that way. The "gutless vectors" are the third generation developed by deleting all viral genes retaining only the noncoding inverted terminal repeats and packaging signal. The third generation is characterized by highly reduced immunogenicity and capacity up to 34kb.

The native adenoviral infection is designated by three different vector-receptor interactions (see also Figure VI and VII). Initially the adenoviral fibre knob region attaches onto the so called coxsackievirus-adenovirus receptor (CAR) of the host cell. This receptor is a 46-kDa transmembrane glycoprotein widely expressed in different tissues but still with unknown physiological function. Its two immunoglobulin-like domains in the extracellular region may however indicate a role in cell-cell adhesion [20].

Subsequently the motif RGD (Arg-Gly-Asp) located in the adenoviral penton base binds to the cellular α_v integrin receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) inducing virus internalization. The primal role of integrins is however cell adhension, migration, maintenance of cell polarity and regulation of the endo- and exocytic cycle [24].

Recently heparan sulphate glycosaminoglycan (HSG) was discovered to mediate adenovirus uptake and infection activity by interaction with the KKTK motif in the central shaft region of the adenovirus fibre. HSG originally modulates the Fibroblast growth factor (FGF) cell signalling by interacting with both FGF and FGF-receptor (FGFR); it is found ubiquitously on the cell surface and in the extracellular matrix [25].

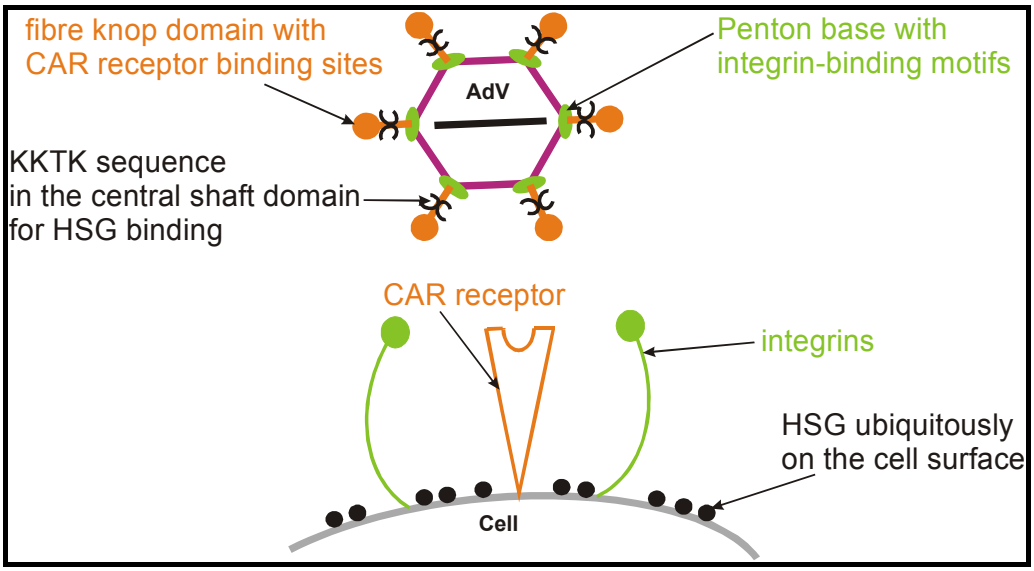

Figure VI: Cell receptors, mediation factors and the dedicated attachment regions of the Adenovirus. The AdV has three binding sites which regulate endocytosis: The carboxy terminal knob of the virus contains CAR related binding sites. The RGD motif of the adenoviral penton base interacts with the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors of the cell surface. The KKTK motif of the fibre's central shaft domain can bind HSG which enforces infectivity [3], [19], [21], [22], [26], [27].

HSG seem to be sufficient to mediate AdV5 and AdV2 infection. In recent studies inhibited HSG binding decreased virus delivery [21], whereas simultaneous disruption of AdV5 binding to CAR and alpha integrins did not change the infectivity at all [23].

The virus enters the cell within an endosome where it disassemblies stepwise until endosomal lysis takes place mediated by the penton base and low intraendosomal pH. The freed capsid is transported to the nucleus where the viral Ad DNA is imported through the nuclear pore for multiplication. The release of the duplicated multiple adenoviruses out of the host cell has no specific pathway. The infection may be productive, abortive, or latent. In productive multiplication, the viral genome is transcribed at discrete replication centres in the nucleus, mRNA is translated in the cytoplasm, and virions self-assemble in the nucleus, but the viral DNA generally does not integrate into the host genome. The

adenoviruses accumulated in the nucleus subsequently kill their host cell by lysis to be released for re-infection [3], [8], [10], [18], [19] , [21], [22], [23].

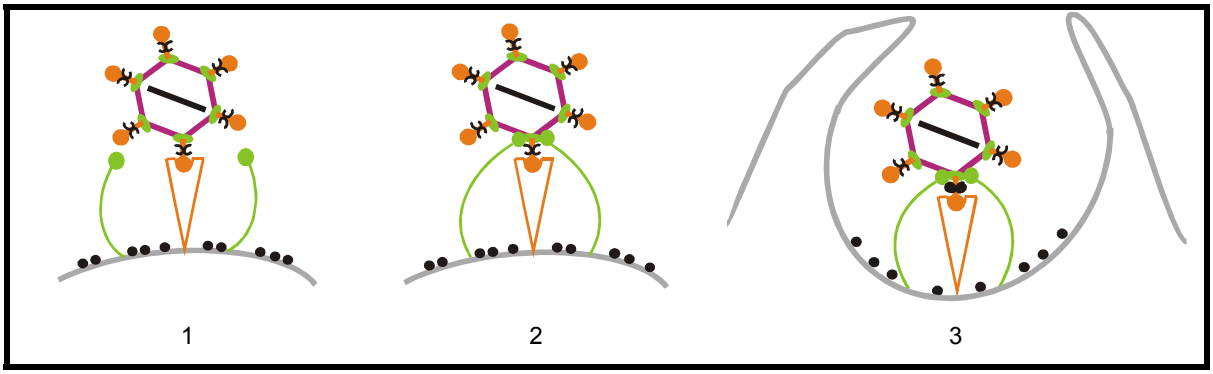

Figure VII: Mechanism of adenoviral attachment and endocytosis. 1: AdV-Attachment to cell surface by binding to the CAR-receptor (orange). 2+3: Virus internalization into an endosome by cellular integrin receptor (green) interaction with the adenoviral penton base (2), backed by HSG (black) which bind to the virus' central shaft domain (3).

The adeno-associated virus (AAV) is a non-pathogenic human parvovirus which causes a latent infection of human cells (e.g. fifth-disease by parvovirus B19). Parvoviruses are non enveloped DNA viruses with a diameter of about 25nm. Their genomes are linear, single stranded and contain about 4500 bases. AAV is classified as dependovirus as it requires co-infection with a helper virus (adenovirus, herpes simplex virus, vaccinia virus) for productive infection in cell culture.

The wild type of AAV is made up of a replication (*rep*) and a capsid (*cap*) gene sequence and is flanked by inverted terminal repeats (ITR). To constitute the AAV-based vectors the viral *rep* and *cap* genes are replaced by a transgene of maximal 5kb. Without their replication and capsid genes the AAV vectors are now replication-defective unless *cap* and *rep* is supplied in *trans* by co-transfection with a helper virus. That is, in AAV-vectors the ITRs are the only *cis*-acting elements needed, all viral coding regions are deleted and therefore there is no toxicity associated with gene expression (see Figure VIII).

AAV-vectors have already been used for muscle, retina, brain, liver and lung diseases. Their advantages are their high stability, efficiency, safety, heat-inactivation resistance and low risk of insertional mutagenesis. Moreover recombinant AAV-vector systems have a wide host range and broad tissue tropism, they can thus transduce even non-dividing cell types and have the ability to infect a wide spectrum of mammalian cells which allows the experimental use of vectors in a number of animal species.
On the other hand recombinant AAV vectors have a relatively small packaging capacity, vector production is difficult and the majority of human beings exhibits already neutralizing, transduction interfering antibodies against Adeno-associated viruses because of natural infections. Besides a single injection of AAV vectors elicits a strong humoral immune response against the viral capsid, this impairs continuative administration [8], [9], [10], [13].

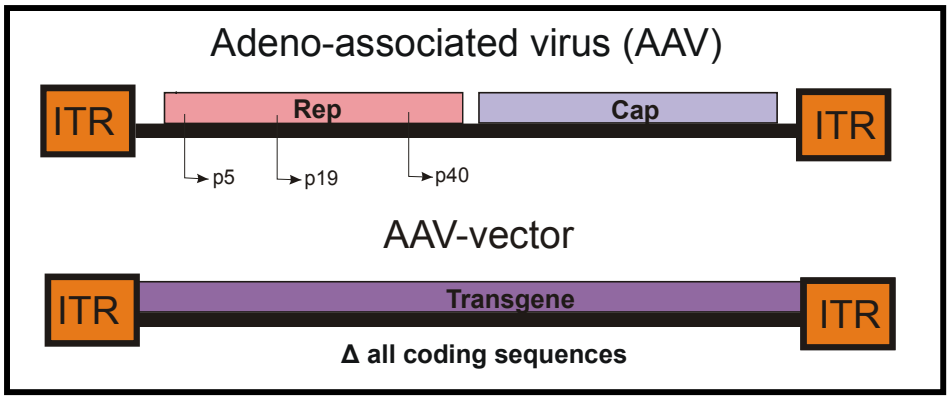

Figure VIII: Genomic structure of wild-type adeno-associated virus (AAV) and of its AAV-vectors. The genome of AAV contains the *rep* and *cap* open reading frames (ORFs) flanked by inverted terminal repeats (ITRs). The replication gene encodes for four overlapping regulatory Rep proteins: Rep78, Rep68, Rep52 and Rep40. The transcription of Rep78 and Rep68 proteins- necessary for DNA replication- is initiated by promoter p5. The DNA helicases Rep52 and Rep40 are transcribed from promoter p19 and facilitate the accumulation of progeny virus DNA. The three Cap proteins that form the capsid called VP1, VP2 and VP3 need promoter p40 for transcription. The AAV-vectors are *rep* and *cap* deleted, and refilled by a therapeutic transgene.

The pathogenic herpes simplex virus (HSV) is an enveloped, double stranded 150 kb DNA virus of about 20nm in diameter and encoding for about 90 proteins. It is naturally neurotropic in its latent state causing persisting neurologic sequelae, and replicating in epithelial cells after reactivation and entry into the lytic cycle. There are two serotypes of herpes simplex viruses: the HSV-1 which accounts for outbreaks of the face known as fever blisters, and HSV-2 that is associated with genital herpes. The HSV-1 type was rendered replication deficient to be used as gene transfer vector. These recombinant HSV-1 vectors are capable of delivering genes to non-dividing cells (neurons) and they can accommodate 30-150kb of foreign genes but both helper-virus-dependent and helper-virus-independent HSV-based vectors may recombine to a wild type phenotype and subsequently induct immune responses and cytopathic effects by viral expression.
Anyhow HSV vectors have already successfully been applied for therapy of neurological diseases, spinal nerve injuries, glioblastoma – a fast growing malignant brain tumour - and pain therapy [8], [10], [28], [29].

Poxviruses are the largest of all virions with 200nm of diameter. They are complex, brick-shaped viruses that contain a single molecule of double stranded DNA. Their way to replicate in the cytoplasm rather than in the nucleus without integration into the host genome stands out in the crowd of the DNA viruses. Poxviruses infect a wide range of mammalian cells but don't establish latency or persistence in the infected host.

The vaccina virus belongs to the poxvirus group and stems from a benign pox disease in cows. It was successfully administered as vaccine against the smallpox disease until official eradication of the infection in 1977 by the WHO. Nowadays vaccina serves as a gene transfer vector which is engineered by simple introduction of therapeutic transgenes in its genome without any inactivation or disabling. The major advantage to using the vaccina virus is that it can carry a large amount of transgenes

(up to seven to date). However vaccina's replication competence can cause immune responses as side effects. This problem may have been solved by the replication defective derivative of vaccina the so called MVA which lacks this toxicity [8], [18], [28], [30], [31].

The alphavirus is a single-, positive-stranded RNA virus which is up to 70nm in diameter, icosahedral and enveloped in appearance. The virus RNA has a length of 11-12kb, it can function intracellularly as mRNA and is already infectious by itself. Alphaviruses are distributed by a mosquito to vertebrate cycle, humans are among the defined hosts for this arthropod-borne virus. The different members of the alphavirus family cause either encephalitis or febrile illness.

The alphaviruses Semiliki Forest virus (SFV), Sindbis virus (SIN) and Venezuelan Equine Encephalitis virus (VEE) have similar properties and serve as gene delivery vectors. In infected humans all three virions produce acute febrile illnesses with malaise, rash, arthralgias and sometimes arthritis.

A recombinant alphavirus has great potential as gene delivery vehicle: Its insertion capacity is at least 7kb [32]. It provides an impressive transduction rate and a high transgene expression level. The RNA virion replicates in the cytoplasm and therefore does not integrate into the chromosomal DNA. This minimizes the risk of cell transformation. Besides, these recombinant viruses can be replicated rapidly and with high-titers. Alphaviruses transduce even neuronal cells; they show a wide host range, strong cytotoxic effects in cancerous cells and mediate tumour-specific immune responses paving the way for cancer therapy. However the aplhavirus' transient expression of heterologous genes represents a constriction for treatment [8], [18], [33], [34], [35], [36].

2.2.2. Targeted vectors in cancer gene therapy

On the one hand a vector system with a wide host range is useful as it is able to infect different cell types. This gives the opportunity to treat several different diseases with the same type of vector. On the other hand the wide host range of a vector unfortunately rises the number of toxicity reactions in normal and healthy cells which in fact also lowers the impact and the effectual dose of the therapeutic viruses.

Targeted delivery is meant to remove all these risks and could even minimise the vector doses. One way of targeted delivery is by local administration of a therapeutic gene. This is however only applicable with local, unresectable tumours like some head, neck, lung, brain, pancreatic, and liver cancers.

The other two strategies for targeted gene therapy are called targeted transduction and targeted transcription.

The targeted transduction is done by altering the host range of the vectors. There are three main methods in targeted transduction: a) pseudotyping - the replacement of the original envelop protein by a chimeric protein or by an envelop protein from another virus b) chemical attachment of ligands and c) direct genetic modification of the virus genome.

The targeted transcription strategy ensures that after a non specific transduction the transcription however starts only in the presence of tumor-specific promoters [3], [10], [13].

The E-coil/K-coil technology is an artificial patent-registered receptor-bispecific ligand system applied for adenoviruses (United States Patent 20070104732: Dr. Bernard Massie, Dr. Yué Zeng et. al.). The idea is to produce "universal viruses" that selectively localize gene expression to the tissue of interest (targeted transduction).

For production of the new modified virus including the E-coil/K-coil technology, firstly, the native CAR tropism is ablated in the Ad vector (AdV-ΔCAR). Then, the invented K-coil peptide (1 to 5 repeats of

KVSALKE, K5coil in this project) is incorporated into the fibre knob of the AdV (AdV-ΔCAR-K5-coil) to establish afterwards high affinity interaction with a bispecific ligand; in detail the so called E-coil peptide composed of 1 to 5 repeats of **E**VSALEK sequences (E5-coil in this project) fused to a ligand of choice (EGF in this project), depending on the desired tropism (compare Figure IX). Furthermore, a deletion of the E1 and E3 genome regions in the adenovector renders the virus replication incompetent in all cells except in 293 cells and their derivative such as the patented 293E packaging cells. These are HEK cells with an E-coil peptide as cell receptor backing the attachment and following endocytosis of the recombinant adenovirus expressing the K-coil [15], [16].

K5-coil shows very strong interactions with the E5-coil peptide. Therefore the K5/E5 system seems to be an ideal candidate for the construction of an efficient and potent *in vivo* Adenovirus retargeting system. Unfortunately the common fusion position for targeting motifs on the fibre's C-terminus impedes trimerization in the case of the chimeric K5c-fibre which can then not be incorporated in the AdV capsid.

To handle this trimerization problem the research group of Dr. Bernard Massie inserted the K5-coil peptide in the so called HI-loop site, a flexible, alternate region which contains mostly hydrophilic amino acids and which protrudes at the surface of the fibre knob. This region does not appear to be directly involved in trimerization while representing anyhow an alternative insertion site for targeting motifs [40].

Tests with the recombinant K5c-HI-loop fibre protein demonstrated that it is still expressed properly and that it keeps its trimer forming abilities.

During this Master Theses project the adenovirus carrying the K5c peptide in the HI-loop fibre region should be designed and tested *in vitro*.

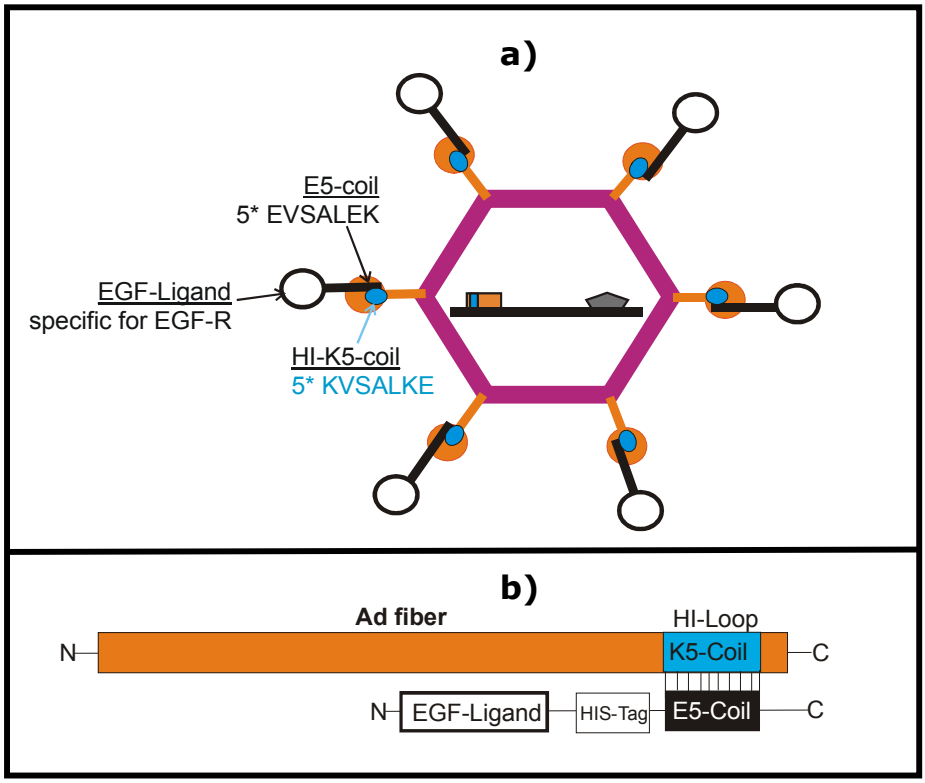

Figure IX: a) A scheme of an Adenovirus vector with a K-coil peptide sequence (K5-coil) in its genome and the K-coil peptide sequence incorporated in the virus' fibre knob (light blue). The E5-coil side of a bispecific ligand (black) is clued to the K5-coil of the Adenovector. The fused ligand is in this case an EGF-ligand, a frequently up regulated growth factor in breast cancer which binds to the receptor EGF-R. b) Interaction of E5-coil/EGF with the fibre containing K5-coil. The K5-coil peptide is inserted into the HI-loop region, a site which does not affect the trimerization capability of the fibre protein. His-Tag: six-histidine tag for purification

2.2.3. Non-viral vectors as delivery systems

Viral vectors are very efficient in gene transduction. However host immunogenicity is a frequent side effect that may be disadvantageous for certain cases of gene therapy. The patient's immune response is provoked by immune recognition of the viral vector's proteins. This leads to the destruction of infected cells and in consequence repeated administration for therapy is impeded. Moreover there is a difficulty to produce high quantities of viral vectors, viral vector systems are restricted in their intake capacity for transgenes, and several therapeutic virions tend to integrate into the host genome bearing the risk of mutagenesis.

Non-viral gene delivery systems represent an alternative to the viral gene delivery techniques. One of their features is their low toxicity and low immunogenicity. This enables repeated application. Besides they suspend the problem of large scale production which emerges with viral vectors. A drawback of non-viral gene delivery is their low transfection and expression level in the target cells [8], [28].

Naked plasmid DNA or naked PCR products can be injected into solid tumours with following transfection and transgene expression. However this methods secure a mere low-level and short-term expression. Genes could also be injected directly into nuclei of cells in particular used for gene delivery in embryonic cells and oocytes. That way DNA is not longer exposed to low pH levels in the endosomes [8], [28].

Similarly to direct injections, some chemical and physical methods enable the DNA penetration with high velocity. The method of calcium phosphate precipitation crystallizes the plasmid DNA. Crystals of DNA calcium phosphate are taken up by endocytosis and consigned to the nucleus. This method is nevertheless restricted to *in vitro* application. Electroporation introduces transient small pores in the membrane by short electric impulses. DNA gets into the cells by these pores. This cell penetration method does not only succeed *in vitro* but also *in vivo* when associated with naked DNA injection and electrodes located *in situ* in the target

tissues. This kind of gene transfection was already confirmed for muscle, brain, skin, liver and tumour cells. Another method of gene delivery by physical force is called the gene gun. DNA is coated on gold particles and shot into cells where it is released in the nucleus. This transfection method is however limited by its low efficiency and its unspecific tropism of DNA delivery [8], [28].

Hybrid vectors combine both viral and synthetic approaches by constructing one hybrid vector out of two or more vectors. This technique seeks to exploit the advantages of the combined vectors while overcoming their individual disadvantages. The virosome is a hybrid vector that is constructed by mixing lipoplexes (liposomes with DNA) with an inactivated HVJ virus (hemagglutinating virus of Japan). This hybrid vector enables efficient gene transfer into the epithelium of the respiratory tract. A second class of hybrid vectors improves the transduction efficiency in cells without viral receptors. They are produced by combination of liposomes or polymers with an adenoviral vector. The fusion of different viral vectors creates a third type of hybrid vectors [8].

Lipoplexes and polyplexes are completely synthetic gene delivery systems used as therapeutic nanoparticles in gene therapy. The Lipoplexes are DNA-liposome complexes providing attractive features for therapy applications including prolonged circulation in the bloodstream, protection from destruction and ability to deliver DNA into the nucleus for gene transcription with flexible tropisms. The liposomes can be divided into anionic, neutral and cationic particles. However attention is now turned to the cationic versions with their more convenient features: they don’t need DNA implementation like the neutral and anionic liposomes because they naturally complex with DNA. Construction is less demanding than for neutral and anionic liposomes. Besides, their positive charge ensures endocytosis by interaction with the negatively charged cell membrane, it ensures DNA release into the cytoplasm and the protection

against DNA degradation in the cell. The hydrophobic anchors of the different cationic liposomes may imply transfection specificity. Lipoplexes have been used in therapy against cancer, respiratory diseases and cystic fibrosis.

Complexes of polymers with DNA are termed polyplexes or "synthetic viruses". They were constructed to combine the efficiency of viral vectors and the advantages of liposomes. The polymer size influences the condensation level and thereby the protection of the DNA against digestion in the target cell. Specific targeting can be maintained by conjugating ligands for cell surface receptors to the complex [8], [28].

Synthetic oligodeoxynucleotides are alternative RNA or DNA molecules introduced into cells with the aim to inactivate or inhibit the expression of disease-related genes [8].

2.2.4. Bacterial gene therapy vectors

Some bacteria tend to replicate only in tumour areas. Some are even capable of delivering genes to mammalian cells. So the idea was to profit from these features by using strains of selective plus DNA transducing bacteria as vectors for targeted cancer gene therapy.

Bacterial vectors can be classified in auxotrophic and strictly anaerobic ones with regard to their exploitation methods of cancer microenvironments. On the one hand auxotrophic bacteria e.g. *Salmonella* are attracted by tumour specific nutrition factors. That means that they prohibit the growth of the tumour by depleting the cancer's proper nutrition factors for their own replication.

Anaerobic bacteria on the other hand like *Clostridium* and *Bifidobacterium* are renowned for their selectivity of colonization areas, thus they preferentially replicate in the hypoxic regions of tumours. Hypoxia occurs frequently in solid prolific tumours because these cancer regions can only develop a disorganised blood supply and thereby provide low oxygen proliferation levels. The well oxygenated normal tissues remain unaffected

by anaerobic bacteria. That way anaerobic bacteria finally affords a treatment for hypoxic cancer cells which were generally resistant to radiotherapy and chemotherapy!

To conclude pre-clinical studies have already proven that bacteria have a potential in tumour targeting. The attenuated or non-pathogenic strains of bacteria tested in humans showed an acceptable safety profile. However to achieve adequate efficacy by rising the dose of injection the tolerability still must be improved [8], [37], [38].

3. Material and methods

3.1. Transformation of E. coli strains

In transformation, naked, extracellular DNA is taken up by recipient bacteria. Bacteria that are naturally capable of transformation are called competent. Not usually competent bacteria can be made artificially competent however by laboratory manipulations such as calcium or heat shock methods, electroporation, gene gun and microinjection [18].

3.1.1. BJ5183 cells for homologous recombination

E coli. BJ5183 are not *recA* but posses the efficient recombination system *recBC sbcBC*. These electrocompetent Escherichia coli cells are K12 derivatives, with the genotype: *endA sbcBC recBC galK met thi-1 bioT hsdR* (Str^r). Briefly, BJ5183 provides high transformation efficiency and recombination capabilities and is therefore applied in the Adeasy system for homologous recombination [11].

Homologous recombination describes the alignment of equal or similar sequences of DNA to produce an exchange of material between the strands [48].

Electroporation This method induces a transmembrane potential with short electric pulses to form small pores in the membrane by which the foreign DNA can penetrate into the cell [8].

40 µl BJ5183 cells are thawed for 20 minutes on ice, then, the two DNA strands stipulated in co-transformation are pipetted into the BJ5183 cells. In comparison to the long vector DNA fragment, the short fibre- or deleted-fibre-region fragment should be 2 to 7 times in weight surplus. The mixture is incubated for 15 minutes on ice and electroporation is carried out at 2.2 Voltage. The transformed cells are cultured in 1ml LB at 37°C, 500µl are applied to LBA plates containing selective antibiotics for over-night incubation at 37°.

3.1.2. DH5α cells for amplification

DH5α is a regular *recA*, *endA* E coli. strain used for amplification of recombinant plasmid DNA in the Adeasy system, genotype: F^- φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-. Due to their *recA* status these cells cannot be used to produce recombinants of adenovirus plasmids by homologous recombination. *recA1* and *endA1* mutations increase the insert stability and improve the quality of amplified plasmid DNA.

Heat shock transformation 20-40μl DH5α cells are thawed 15min on ice, 3ml mini prep plasmid sample is added. The mix is incubated for 10minutes on ice, heat shocked for 20 seconds at 37° C water-bath temperature, 3 minutes put on ice, transferred in 1ml LB and incubated for 1h on the shaker at 37°C. 400μl are grown over night at 37° C on LBA plates supplemented with antibiotics for selection.

3.2. Bacteria cell cultures

Precultures are LB growth medium supplemented with an antibiotic, 100μg/ml ampicillin or 50μg/ml kanamycin. One plate is prepared with about 8ml LBA medium and 100μg/ml ampicillin respectively 50μg/ml kanamycin.

3.2.1. LB and LBA for cell growth

400ml autoclaved Luria-Bertani growth medium consists of: 400ml water, 4g Triptone, 2g yeast extract and 2g NaCl. For LB agar (LBA) plates 2.8g agar has to be added to 400ml Luria-Bertani medium before autoclaving.

3.2.2. Selection factor Ampicillin 40×

The growth medium should have the concentration 0.1mg/ml or 1× ampicillin. For the ampicillin concentration 40× or 4mg/ml of ampicillin 160mg ampicillin are dissolved in 40ml water.

3.2.3. Selection factor Kanamycin 40×

For a 40× concentration 100mg kanamycin are mixed with 50ml water (2mg/ml). The growth medium concentration has to be 0.05mg/ml or 1×.

3.3. Lipofectamine transfection of 293 HEK cells

Transfection describes the introduction of foreign genetic material or proteins into eukaryotic cells. The in vitro transfection for 5μg adenovector DNA samples into 1×10^6 293 mammalian cells was carried out with 15μl lipofectamine (Invitrogen) each.

Transfection protocol:

- tube 1 with 5μg *Pac I* linearized adenovector DNA and 600μl DMEM without serum (preheated to 37°C) is prepared and incubated for 5 minutes.
- tube 2 with 15μl lipofectamine plus 600μl DMEM without serum (preheated to 37°C) is prepared and incubated for 5 minutes.
- After 5 minutes the content of tube 1 is poured into tube 2 and incubated for 30 minutes at room temperature.
- In the meantime the 293A and 293E cells are washed two times with PBS.
- After 30 minutes incubation time the tube 2 content is applied evenly on the cells, 2μl DMEM added and the cells are incubated for 6 hours at 37° C.
- After 6 hours the DNA/lipo complexes are removed (no wash), and 10ml of 37°C-preheated DMEM 10% are added.
- Storage of the transfected cells at 37°C for two days before division.

Lipofectamine is a cationic lipid molecule formulated with a neutral helper lipid. Under normal physiological conditions cell membrane and nucleic acids would both have a negative charge. In order to allow nucleic acids to cross into the cytoplasm anyway the electrostatic repulsion of the cell membrane has to be overcome by using lipofectamine. The positive charge of the cationic liposome component provides an electrostatic

interaction with nucleic acids and enables contact with the negatively charged cell membrane. The neutral co-lipid component supports fusion of the liposome with the cell membrane enabling transfection. Lipofectamine is said to promote penetration of DNA through intact nuclear envelopes in the following [44].

293 cells are also known as Human Embryonic Kidney cells, HEK cells or HEK 293 and represent an epithelial cell line originally derived from embryonic human kidney. They are used as E1 complementing packaging cells for propagation of recombinant, replication defective E1 deleted adenoviruses. HEK 293 cells were generated by transfection of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA. Due to this imprecise cleavage method E1A- and E1B-Ad5 viral genes were also incorporated into the 293 cell genome [13]. The stock of 293 cells and its derivates used in this project was furnished by the research group.

293A cells The Adeasy system has selected a superior sub-clone referred to as 293A (A stands for attachment) for adenovirus production. Thus, the 293A cell line adheres strongly to plastic dishes which is an ideal feature for plaque assays and transfection experiments.

293E cells are 293 cells that express a pseudoreceptor, composed of E-coil fused with the transmembrane and cytoplastic domains of EGF-R. They are cultured under selective pressure with hygromycin.

3.4. Mammalian cell cultures

293 cells were washed with phosphate buffered saline (PBS), detached by Trypsin and cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with 5%, respectively 10% after transfection, in heat inactivated Fetal Bovine Serum and 2mmol/l L-Glutamine.

3.5. Adeasy system by Q·BIOgene

The Adeasy system is a technology which allows to construct in bacteria recombinant adenoviral DNA containing transgenes with up to 7.5kb. The desired expression cassette is first cloned into a transfer vector so-called pAdenoVator-CMV5-IRES-GFP, and subsequently transferred into the pAdeasy-1 vector, a plasmid including most of the adenoviral genome, by homologous recombination in the BJ5183 *E. coli* strain via electroporation. Once recombination is achieved and verified, the recombinant viral DNA is amplified by transformation into the DH5α strain. In the following viral particles can be produced by transfection into the 293A packaging cells.

3.5.1. Adeasy-1 plasmid - the adenovirus backbone

The pAdeasy-1 is a 33.4kb supercoiled plasmid containing most of the genome of adenovirus serotype 5 (Ad5), a copy of ampicillin resistance gene as well as the pBR322 origin of replication. The viral backbone vector is E1 (Ad5 nucleotides 1-3533) and E3 (Ad5 nucleotides 28130-30820) deleted, leading to replication defective adenoviruses. The replicative gene functions of E1 can be complemented in 293 packaging cells.

3.5.2. pAdenoVator-CMV5-IRES-GFP for gene transfer

This adenovirus transfer vector has 9456bp and includes the chimeric CMV5 promoter which coexpresses continuously the Green Fluorescent Protein (GFP) with the gene of interest (cloning capacity 4.6kb) by an IRES (Internal Ribosome Entry Segment) expression cassette.

The CMV5 promoter consists of the CMV (cytomegalovirus) promoter and enhancer with the adenovirus major late enhancer and splicing sequence and the major late protein tripartite leader sequence resulting in higher expression of recombinant AdV than the conventional CMV promoter in an equally broad range of cell types. The recombinant protein production in both complementing (293) and non-complementing cell lines approaches 15 to 30% of total cellular protein (TCP). This makes it possible to express

transgenes at significant levels *in vivo* at lower MOIs, thereby decreasing the toxic side effects associated with the load of adenovirus particles.
In the IRES expression cassette two cistrons are separated by the encephalomyocarditis virus Internal Ribosome Entry Site. In the dicistronic expression cassette the gene of interest can be cloned into the position of the first cistron and the GFP marker is encoded as second cistron.

3.6. Digestion and applied Enzymes

The restriction digest is a procedure of DNA analysis using restriction enzymes of type II for cleaving DNA strands selectively into shorter fragments. These restriction enzymes cut both strands of a DNA segment within their specific target sequence producing either blunt ends or overhanging sticky ends, a stretch of unpaired nucleotides in the end of a DNA molecule [41]. The main restriction enzymes of this project are discussed below.

3.6.1. *Srf* I enzyme

Srf I is a unique restriction site in the Adenovirus DNA located 814bp upstream the fibre gene. The palindromic recognition site 5'-GCCC/GGGC-3' with 8 nucleotides is cleaved by the restriction enzyme *Srf* I in the very middle, forming blunt ends (see Figure I). 700μg Adeasy-1 plasmid were digested over night in 50μl solution in a 37° C water bath, containing water, universal buffer and 4u *Srf* I.

5'-GCCC|GGGC-3'
3'-CGGG|CCCG-5'

Figure I: Target sequence and cleavage site (|) for the restriction enzyme *Srf* I.

3.6.2. *MUN* I enzyme

The *MUN* I digestion is a gene analysis step. 2μl of a mini prep sample are mixed with 5u *Mun* I, NEBuffer 4 and water to constitute a total volume of 12 μl. DNA fragmentation by *Mun* I is carried out for 90 minutes at 37°C producing sticky ends with an overhang of 5'-AATT (see Figure II). *MUN* I digestion was applied for analysis of recombinant Adeasy plasmids, Table I compares the fragments after *MUN* I digestion for Adeasy-dFbr and Adeasy-1.

		
#	**Adeasy-dFbr**	**Adeasy-1**
1	16 412 bp	16 412 bp
2	7227 bp	7227 bp
3	4091 bp	4126 bp
4	2368 bp	4091 bp
5	1615 bp	1615 bp

Table I: Fragments of *MUN* I digestion deployed for the verification of the recombination results. Both Adeasy plasmids Adeasy-dFbr and Adeasy-1 have 5 fragments after *MUN* I digestion. The two plasmids are however distinguishable in electrophoresis because only the Adeasy plasmid with the deleted fibre region (green triangle) has a 2368 bp DNA-fragment. Moreover Adeasy-1, which possesses a fibre region (orange rectangle), has two fragments of about 4000bp. They appear as one line in gel electrophoresis so Adeasy-1 seems to have only 4 fragments.

5'-C|AATTG-3'
3'-GTTAA|C-5'

Figure II: Target sequence and cleavage site for *MUN* I.

3.6.3. *Swa* I enzyme

The unique *Swa* I restriction site is located in the deleted fibre region. 20μl of mini prep sample are mixed with 20u *Swa* I, NEBuffer 3, BSA and water to constitute a total volume of 30 μl. The digestion lasts for 4 hours and is performed at the optimal temperature of 25°C. The recognition sequences of *Swa* I form blunt ends (see Figur III).

5'-ATTT|AAAT-3'
3'-TAAA|TTTA-5'

Figure III: Recognition sequences for *Swa* I. This digestion produces blunt ends.

3.6.4. *BSM* I enzyme

5μg pE4-HI-K5c plasmid are cut by 20u *BSM* I with NEBuffer 2 and water in 50μl for 4 hours at 65° C. The *BSM* I cut sites produce a CN-3' overhang (see Figure IV).

5'-GAATGCN|-3'
3'-CTTAC|GN-5'

Figure IV: The recognition sequence for *BSM* I is GAATGC.

After digestion the linearized plasmid is separated from the enzyme and buffer, by purification with the PCR Purification Kit 250, and solubilised in 30μl water.

3.6.5. *Kpn* I enzyme

30μl of the *BSM* I-linearized sample (see 3.6.4) are mixed with 20u of *Kpn* I, BSA and NEBuffer 1 for 40 μl volume in total. The restriction digest is carried out over night at 37° C. The recognition sequence of *Kpn* I forms sticky ends and is sketched in Figure V.

Figure V: DNA recognition sequence for *Kpn* I. This restriction enzyme produces a sticky end overhang of GTAC-3'.

3.6.6. *Pac* I enzyme

DNA fragmentation for gene analysis by *Pac* I (see Figure VI) is done with 2 μl of Mini prep sample, plus 4.6u *Pac* I adding BSA and water for 12 μl in total. The sample is exposed to 37°C for two hours.

Figure VI: *Pac* I produces a sticky end overhang of AT-3' in its target sequence.

3.7. Dephosphorylation by *CIP*

CIP is an alkaline phosphatase deriving from the calf's intestinal mucosa. The enzyme catalyzes the removal of 5´ phosphate groups from DNA, RNA, ribo- and deoxyribonucleoside triphosphates. This property is used to decrease the vector background in cloning strategies, since self-ligation of *CIP*-treated fragments is constrained by the lack of the required 5´ phosphoryl termini.

CIP is active in NEBuffers 2, 3 and 4, so in the majority of cases the phosphatase may be added directly into the digestion mix after the estimated fragmentation time has passed. For dephosphorylation 10u *CIP* are incubated at 37°C with about 10μg DNA sample for 60 minutes.

3.8. Electrophoresis

For gel purification or after restriction digest, DNA fragments with sizes between 200 and 50 000 kD can be separated and analysed using agrose

gel electrophoresis. In this method an electric field (120mA) draws the negative charged DNA, or RNA molecules to the positive pole separating them by size. Shorter molecules move faster and migrate farther than longer ones [42].

3.8.1. Agarose gel

The agarose is solved in 100ml water and heated for 2 minutes in the microwave. 12ml 5× TBE are added and the volume is adjusted with water to 120ml. The solution is cooled down under running water and poured into an electrophoresis ship to polymerise. 1% agarose concentration is used for <3kb- and 0.8% agarose for >3kb-fragments.

Tris-borate-EDTA (TBE) 5× is diluted and used as TBE 0.5× for gel polymerisation and electrophoresis buffer. The protocol for 1 litre TBE 5× is: Mix 54g Tris, 27.5g Boric Acid and 20ml EDTA (0.5M) and complete to 1 litre with distilled H_2O, adjust to pH8 by ≈30ml NaOH 10N and then autoclave the solution.

3.8.2. Marker: 1kb DNA ladder

The marker 1kb DNA ladder yields 10 bands from 0.5-10.0 kilobases: 10, 8, 6, 5, 4, 3, 2, 1.5, 1.0, 0.5 kb suitable for use as molecular weight standards for agarose gel electrophoresis. The 3.0 kb fragment shows increased intensity and is serving as reference.

3.8.3. Vistra Green Nucleic Acid Stain

Vistra Green is a sensitive in-gel stain for double-stranded DNA 10-fold more sensitive than Ethidium Bromide permitting detection of less than 20 pg/band in both agarose and acrylamide gels. This dye is used in association with fluorescence scanning instruments, but it is non-fluorescent until it binds non-covalently to the DNA backbone.

The Vistra Green dye is provided as a 10 000× stock solution in DMSO. 2μl of 1000:1 water diluted Vistra Green was applied in each sample of gel analyses.

3.8.4. Loading Buffer

Loading buffer visualizes the samples pipetted into the gel wells which allows to pursue and estimate the electrophoresis separation stage. For preparation of the loading buffer 1 w% (2,5ml) bromphenol blue, 4 w% (0,625ml) xylene cyanol and 100 w% (5ml) glycerol are mixed and completed with distilled water (1,875ml) to 10ml. 2μl of loading buffer per sample and well are used in the following gel analysis.

3.9. DNA Purification

3.9.1. Column Purification by QIAGEN

The QIAquick PCR purification Kit is used for DNA purification after PCR or enzymatic reactions. It is able to separate up to 10 μg of single- or double-stranded DNA fragments with sizes between 100bp and 10kb from primers with up to 40 nucleotides respectively from enzyme contaminations like alkaline phosphatase, restriction enzymes or polymerases.

In the presence of high salt DNA is adsorbed to a silica-gel membrane, situated in the middle of a spin-column, while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with 10-30μl water.

The purified DNA sample is suitable for any subsequent application, such as restriction, PCR, ligation and transformation or sequencing.

3.9.2. Gel Purification by QIAGEN

The QIAquick Gel Extraction Kit protocol is designed to extract and purify DNA of 70bp to 10kb from agarose gels in TAE (Tris-acetate/EDTA) or TBE (Tris-borate/EDTA) buffer. Up to 400mg agarose gel slices can be solubilised and up to 10μg DNA can be attached to the silica membrane in

the spin column. This kit may also be used for DNA cleanup from enzymatic reactions.

3.10. Enrichment of plasmids

The archetypes of plasmid DNA isolation from bacteria are the miniprep and the maxiprep/bulkprep method. The former can be used to quickly find out whether the plasmid is correct in any of several bacterial clones. The yield is a small amount of impure plasmid DNA, which is sufficient for analysis by restriction digest and for some cloning techniques. In the latter, much larger volumes of bacterial suspension are grown from which a maxi-prep can be performed. Essentially this is a scaled-up miniprep followed by additional purification.

3.10.1. QIAGEN's Minipreparation

Minipreparation of plasmid DNA with the QIAprep Spin Miniprep Kit is a rapid, small-scale extraction of plasmid DNA from bacteria. The extracted plasmid DNA resulting from performing a miniprep is itself often called a "miniprep". When bacteria are lysed under alkaline conditions both DNA and proteins are precipitated. After the addition of acetate-containing neutralization buffer the large and less supercoiled chromosomal DNA and proteins precipitate in an insoluble clot, but the small bacterial DNA plasmids can renature and stay in the supernatant [43].

The QIAprep miniprep method has a DNA binding capacity of 20μg dsDNA. The E. coli bacterial cells in 2ml of over night precultures (LB + 1× antibiotic) are lysed with alkaline conditions followed by adsorption of DNA onto a slica-gel membrane in the presence of high salt. Then the DNA is washed and eluted with 50μl water.

3.10.2. QIAGEN's Maxiprep preparation

The QIAGEN Plasmid Maxi kit was chosen for plasmid isolation. The bacteria DH5α including the designated plasmids are grown in 200 ml LB medium over night (12-16h) at 37° C to a cell density of approximately

3g/liter. The protocol uses a modified alkaline lysis procedure, followed by binding of plasmid DNA to an Anion-Exchange Resin under appropriate low-salt and pH conditions. All impurities of RNA, proteins, dyes, and low-molecular-weight fragments are removed by a medium-salt wash then. Finally up to 500μg plasmid DNA is eluted in a high salt buffer and concentrated plus desalted by isopropanol precipitation. The pellet was washed with 70% ethanol and redissolved in 300μl water.

3.11. PCR and applied Primers

The polymerase chain reaction (PCR) is used to amplify specific regions of a DNA strand using a polymerase enzyme (like *Taq*, *PHS* or *PfuTurbo*) plus buffer, primers and dNTP's. The PCR consists of a series of 30 repeated temperature changes called cycles; and each cycle consists of 3 discrete temperature steps.

The cycling is often preceded by an initial single denaturing step (called hold) at a high temperature (94°C), and followed by one hold at the end for final product extension (74°C). After the final extending step the temperature cycler remains at 4°C for DNA storage. The PCR standard protocol is outlined in Table II and the applied primers are listed in Table III.

94° C- 2' *Initial Denaturing step*			
94° C- 40'' *Denaturing step*	**T_A – 40''** *Annealing step*	**72° C - t_E** *Extending step*	*30 cycles*
72° C- 10' *Final extending step*			

Table II: PCR standard protocol. T_A, Annealing temperature, depends on the primers melting temperature (T_m); t_E: Extending time, depends on the length of the designated fragment, 2' for 1kb.

Name	T_m [°C]	Sequence (5'-3')
dFb 1s	56.3	5'-CGT AAC ATC CTG CAT TAC TAC C–3'
dFb 2as	59.8	5'-GTG TAT GCA CGT GTG CCT GTG-3'
33510R	62.0	5'-CGC TTG TGG TAT GAT GGC CAC-3'
PFR2	54.0	5'-CCT GCA AAC ATC AGG CC-3'
RFR4	54.2	5'-GCC AAA CTG CCT TTA ACA GCC-3'
FBM 408-409	69.6	5'-ACC ACA CCA GCT CCA GAG GCT AAC TGT AGA CTA AAT GC-3'
R-FBM-408-409	69.6	5'-GCA TTT AGT CTA CAG TTA GCC TCT GGA GCT GGT GTG GT-3'

Table III: The primers used in this project with their melting temperatures (T_m). FBM 408-409 and R-FBM-408-409 are the primers used for site directed mutagenesis of four nucleotide bases (orange, underlined) allowing ablation of CAR-tropism in adenovirus plasmids.

3.12. QuikChange™ Site-Directed Mutagenesis Kit

The Stratagene's mutagenesis kit is used to make point mutations, switch amino acids, and delete or insert multiple amino acids in double-stranded plasmids. It is a four-step procedure and uses either miniprep plasmid DNA or CsCl-purified DNA.

The basic procedure utilizes the supercoiled double-stranded pE4-HI-K5c plasmid (50.24ng, furnished by Maxime Pinard, a research group member) including an adenovirus fibre region with the CAR ligand region and the two synthetic primers FBM 408-409 and R-FBM-408-409 for PCR reaction (11pmol each, see Tale IV). These two oligonucleotide primers are each complementary to opposite strands of the vector and contain the desired four point mutations. During temperature cycling for denaturation of the plasmid, FBM 408-409 and R-FBM-408-409 are extended and incorporated by *PfuTurbo* DNA polymerase (1μl, 2.5u/μl). This is resulting in nicked circular strands. A nick is a point in a double stranded DNA molecule where there is no phosphodiester bond between adjacent nucleotides of one strand.

95° C- 30'' *Initial Denaturing step*			
95° C- 30'' *Denaturing step*	**55° C – 1'** *Annealing step*	**68° C – 25'** *Extending step*	*16 cycles*
72° C- 10' *Final extending step*			

Table IV: PCR parameters for the QuikChange Site-Directed Mutagenesis Method in a temperature cycler: furnished plasmid 50.24ng, Primer 11pmol, 1μl *PfuTurbo* (2.5u/μl), dNTP 200μM, Total volume 50μl.

Subsequently the methylated, nonmutated parental DNA template is digested with *Dpn* I endonuclease (target sequence 5'-Gm^{6}ATC-3'), specific for methylated and hemimethylated DNA, leaving the mutation-containing synthesized DNA strains. The nicked vector DNA containing the desired mutations is then transformed into DH5α cells. After transformation, the DH5α cells repair the nicks in the mutated plasmid.

3.13. Sequencing by University Laval

Sequencing is the determination of the precise sequence of nucleotides in a DNA sample. The biomolecular analysis platform uses an ABI Prism 3130XL and an ABI Prism 3100XL, two 16-capillary genetic analysers exciting fluorescent fragments with a laser beam. The fragments migrate by electrophoresis according to their size and show different fluorescence, specific to the specific fluorochromes in use. For sequencing 10μl (2ng/μl) of the DNA fragments (<1kb) of interest are prepared in a 0.5 ml Eppendorf tube. The starting primer should be furnished with a concentration of 1.5μM and a volume of 5μl per reaction.

3.14. Virus purification by CsCl-density gradient

The goals for adenovirus purification include the clearance of host-cell components like proteins and nucleic acids, the concentration of virus beyond conditions needed for formulation while separating empty capsids and unpacked viral DNA from mature virions.

Two steps of continuous CsCl gradient purification with an ultracentrifuge were performed. The first 24 hours-purification at 34 000 rpm and 4°C with a Type 60Ti rotor was mediated in saturated CsCl solution homogenously mixed with the adenovirus. Subsequently the virus band is collected carefully by a syringe and mingled in a smaller centrifuge tube with a diluted CsCl solution for a second round of continuous CsCl gradient purification. This ultracentrifugation is carried out for 24 hours at 4°C and 30 500 rpm using a SW41 Ti rotor. The virus band was extricated as before with a syringe (maximum 2.5ml) and subsequently the virus was desalted and purified with the NAP-25 column.

3.15. illustra NAP-25 Column

The illustra NAP-25 Columns allow DNA purification, desalting and buffer exchange by the process of gel filtration. Molecules like the adenovirus that are larger than the largest pores in the matrix elute straight away, small size molecules elute last. Enzymes are not denaturated with this method.

3.16. BioPhotometer

The BioPhotomeer calculates the concentration of DNA, RNA or oligonucleotides of an unknown sample via the measurement of extinction and dilution factor. This photometric quantitative analysis of nucleic acids is carried out at 260nm. Absorption results of 1 OD (E) correspond to about 50μg/ml dsDNA, 37 μg/ml ssDNA, 40 μg/ml RNA or 30 μg/ml oligonucleotides.

3.17. FACS or Flow Cytometry

The flow cytometry technology was used to measure green fluorescence values of the adenovirus-infected 293A and A549tta cells. The unique advantage of flow cytometry is that it measures fluorescence per cell rather than making a bulk measurement like for example spectrophotometry does.

For data acquisition the cells of interest are spaced singly at intervals in a fine stream of fluid by forcing them to pass with a large volume of saline through a nozzle also referred to as hydrodynamic focusing. For excitation, a laser light can now be directed onto an individual cell causing light scattering and emission of fluorescence in all directions (360°).

Forward-scattered light (FCS) results from diffraction and therefore yields information about cell size. The light scattered at 90° to the incident beam is called side scatter light (SSC) and is for example a parameter for surface irregularities. The fluorescent protein GFP in the cells is excited into emitting light at a higher wavelength than the light source. This combination of scattered and fluorescent light is picked up by detectors referred to as photomultiplier tubes (PMT) which convert the emissions into electrical signals. The electrical pulses are then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells. This type of amplification expands the scale for weak signals and compresses the scale for "strong" or specific fluorescence signals.

Initially FACS was an acronym for Fluorescence-Activated Cell Sorting. This stands for flow cytometers equipped to separate the identified cells during analysis. However in recent years "FACS" has become a synonym for flow cytometry in general using this term for all types of sorting and non-sorting applications [45], [46], [47].

The analyses of flow cytometry in this project were performed on a Coulter EPICS^{Tm} XL-MCL flow Cytometer equipped with a 15mW argon ion laser as excitation source and the following filters: a 550nm dichroic long pass plus a 525nm band pass filter set for green GFP-fluorescence

detection, and a 488 nm laser-blocking as well as a 488nm long pass dichroic filter.

3.18. Titre assessment by flow cytometry

A linear relationship between the multiplicity of infection (MOI), and the volume of diluted virus stock solution per 293A cell (V/C) can be drawn under the condition of a low multiplicity of infection (MOI<0.3 infectious viral particles/cell) and a virus harvest 24 hours post infection.

A dilution series of the purified virus stock have to be prepared with dilution factors between 10^{-2} and 10^{-7} in infection medium (DMEM 5%). 500µl of each virus dilution are incubated with 2.10×10^{5} 293A cells at 37° for 24h. Infected 293A cells loose their power for attachment when they have been infected with the adenoviruses, therefore the infected cells were tried to ablate by pipetting up and down with the cell's medium and collected in a tube per sample. 500µl PBS were used to wash the plate, subsequently this volume was added to the cell tube. In the end the remaining cells on the plate were supplemented and incubated with 500 µl Trypsin at 37°C for 3 minutes, the liquid was harvested and added to the cell's collection tube. After centrifugation at 1000rpm and 4°C the supernatant was removed from the cell pellet. Cell fixation of 30 minutes was performed with 200 µl Trypsin, 600 µl PBS and 200 µl 10% Paraformaldehyde added to the cell pellet. After filtration the cells were finally analysed by FACS.

The viral titre estimation was based on the number of GFP^{+} cells (P) for each viral dilution and was expressed as transduction units per millilitre of the virus stock solution [IVP/ml of virus]. The parameters for the titre calculation are the following: MOI, multiplicity of infection or number of infectious viral particles per cell [IVP/cell]; C, number of cells provided for infection [cell]; V, present volume of virus stock solution in dilution series [ml of virus]; P, probability of cell infection measured by counting the FACS [%] number of GFP^{+} cells and IVP/ml, transduction units per millilitre of the virus stock solution, infectious virus particles per millilitre.

The slope MOI×C/V of linear regression for MOI=-ln(1-P) determines the titre [IVP/ml of virus] of the virus stock.

3.19. AdV retargeting to EGF-R by K5c/E5c-EGF interaction

Retargeting in A549tta cells (furnished by the research group) evaluates the transduction efficiency of the designed Adeno-HI-K5c-CMV5-GFP virus. The A549tta cells are attaching cells with a tta promoter and expressing EGF receptors. A549tta cell culture requires fleomycin.

For the virus test 6×10^5 A549tta cells were seeded on 6-well plates and incubated for two days at 37°C with 13µl (MOI 5) or 2.6µl (MOI 1) of Adeno-HI-K5c-CMV5-GFP virus stock suspension with or without E5-coil-EGF ligands added (1µg E5c-EGF-ligand for MOI 5 and 215ng for MOI 1). The blank (T, Testimony) was prepared in the same way at MOI 1 and MOI 5 levels but Adeno-HI-K5c-CMV5-GFP was replaced by an adenovirus expressing the wild type fibre (furnished by Amelie Pilotte, a research group member) containing no EGF-ligand region but still a GFP-marker with a constantly working CMV5 promoter. The transduction efficiency was measured at two days post-infection via GFP expression with the flow cytometer.

4. Results

4.1. Design of Adeasy-dFbr

A deleted fibre region fragment (dFbr) was obtained by PCR with the primers 1s and 2as and a template DNA plasmid called Adeasy-dFbr-TR5CuO-GFP which was provided by Yue Zeng, a research group member (see Figure I). The amplified dFbr region fragment run on a 1% TBE 0,5x agarose gel and was gel-purified with the QIAquick Gel Extraction Kit to separate the designated strand from the template DNA and other possible amplified fragments.

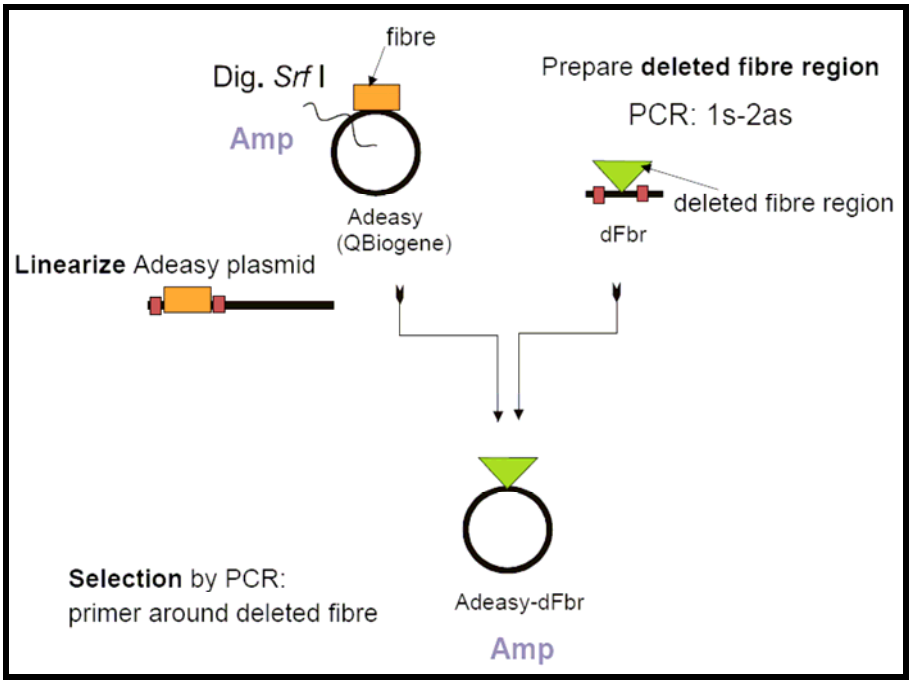

Figure I: Adeasy-dFbr design. The Adeasy-dFbr plasmid is ampicillin resistant, contains an adenovector with ablated fibre region and is generated by homologous recombination. The dFbr fragment and the Adeasy plasmid have similar sequences (red rectangles) which enables the recombination process. In theory there is only one recombination possibility for these two strands left, namely the designated one, if the sole dFbr fragment is provided by resection of its template DNA. This is Phase I of the Ad-HI-K5c-CMV5-GFP construction (see description above), an intermediate construction step which has to be done because otherwise it would be hard to replace a normal fibre region by a modified one (see also Phase II: introduction of an HI-K5c fibre region).

The ampicillin resistant Adeasy-1 plasmid was linearized by *Srf* I and dephosphorylated by *CIP* to avoid that the Adeasy vector itself grows on ampicillin containing LBA plates.

The linearized Adeasy plasmid and the dFbr region contain similar sequences around the fibre region. Consequently BJ5183 cells could be cotransformed with both strands via Electroporation trying to clone the Adeasy-dFbr plasmid by homologous recombination. As mentioned above ampicillin LBA plates where charged with these transformed BJ5183 cells and incubated over night at 37° Celsius.

The next day the plates were scanned for good colonies by PCR (PCR conditions in Table I, results in Figure II) using the primers 1s and 2as or 1s and 33510R which are located around the deleted fibre region.

At the end the promising colonies (#3, 4, 7, 10) where amplified in precultures containing ampicillin and LB, DNA was extracted by the QIAprep Spin Miniprep Kit and retransformed in DH5α bacteria cells by heat shock at 37° C for 20 seconds. Bacteria were streaked on ampicillin LBA plates and incubated over night at 37° Celsius. After this amplification step in every sample plate (#3, 4, 7, 10) 3 to 4 colonies of average size were chosen to be tested by digestion with *MUN* I (results in Figure III).

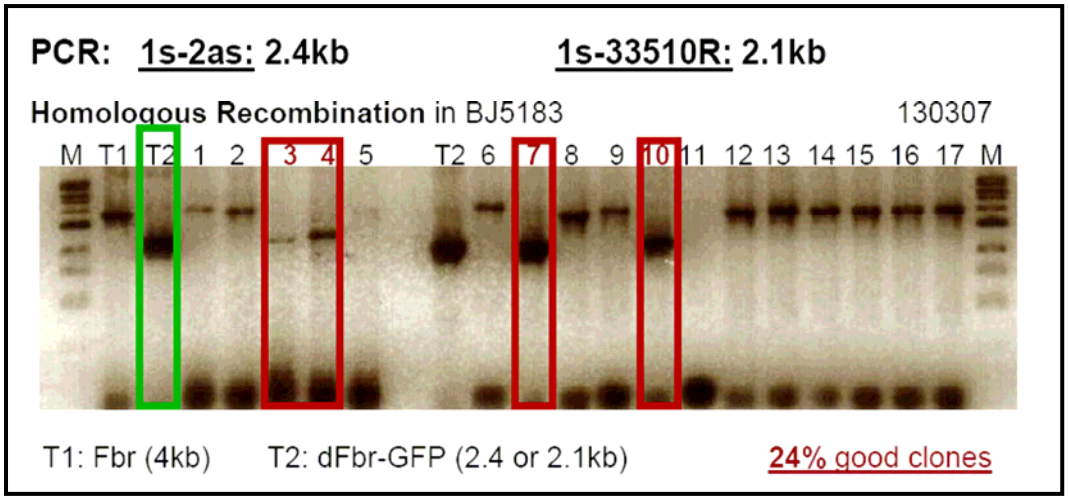

Figure II: Screening for clones with a deleted fibre region (Adeasy-dFbr) by PCR and primers situated around the fibre region. The pair of primers 1s with 2as should amplify a dFbr fragment of 2.4kb (samples: 1-5) the pair 1s with 33510R provides on the other hand a 2.1kb fragment (samples: 6-17). The negative testimony T1 represents a fibre region fragment with a length of 4kb (primers 1s, 2as). The positive testimony T2 is a deleted fibre region with 2.4 kilobases for 1s, 2as (green rectangle) and 2.1 kb for 1s, 33510. It was cut out of an Adeasy-plasmid with TR5CuO-GFP region. Screening results: 4 samples (red rectangles) of 17 have bands at 2.4kb respectively 2.1kb like the positive T2. That means the yield of good clones was 24%.

T_A	52°C
t_E	6’
Primer	20 pmol
Taq volume	0.4µl (5u/ml)
dNTP	200µM
Total volume	25µl

Table I: PCR conditions. The required fragment has 2.4kb or 2.1kb. Amplification is carried out with the primers 1s and 2as respectively 1s and 33510R. T_A, Annealing temperature; t_E, Extending time

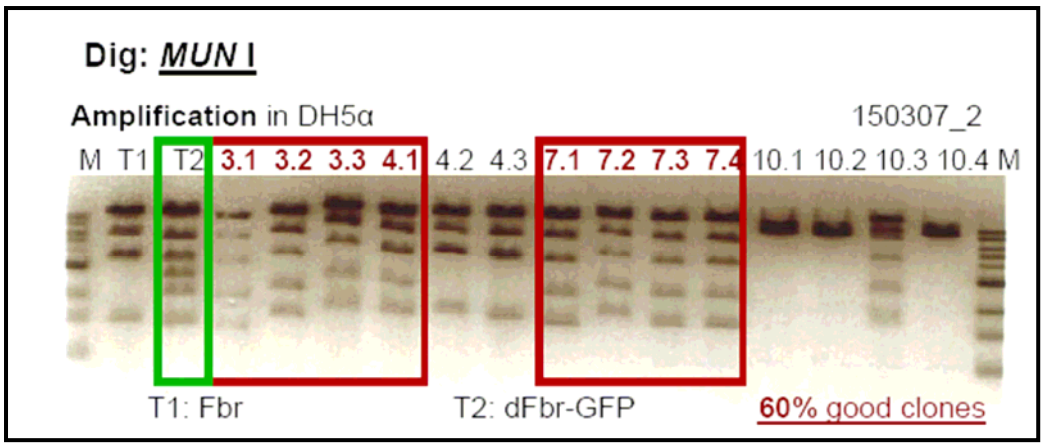

Figure III: Screening for the very best clones with a deleted fibre region (Adeasy-dFbr) by *MUN* I digestion after the amplification step in DH5α bacteria cells. The negative testimony T1 represents an Adeasy-plasmid with fibre region. The positive testimony T2 (green rectangle) contains an Adeasy-plasmid with a deleted fibre region plus a TR5CuO-GFP region which explains the difference between "good colony samples" having the predicted 5 bands and the T2 testimony sample with 6 bands. Screening results: T2 and 8 samples (red rectangles) out of 14 are very much alike. The yield of good clones was 60% in this step of amplification.

4.2. Construction of Adeasy-HI-K5c

The Adeasy-dFbr vector conveys ampicillin resistance and a subjoined *Swa* I-site in the dFbr region. So the plasmid had to be prepared by linearization via digestion with *Swa* I and dephosphorylated by *CIP* to avert survival on ampicillin containing culture media. Subsequently the linearized vector was separated from the digestion medium by the QIAquick PCR Purification Kit.

Secondly a fragment of the modified fibre region (fibre with inserted K5c in the Hi-loop region) was cut out of a template plasmid (pE4-HI-K5c - furnished by Maxime Pinard, a research group member) with two restriction steps by the enzymes *BSM* I and *Kpn* I and then separated from the other components by gel purification with a 1% TBE 0,5x agarose gel and the QIAGEN's QIAquick Gel Extraction Kit.

Homologous recombination in the BJ5183 cells was performed with the two prepared fragments by heat shock transformation. The whole design process of Adeasy-HI-K5c is depicted in Figure IV.

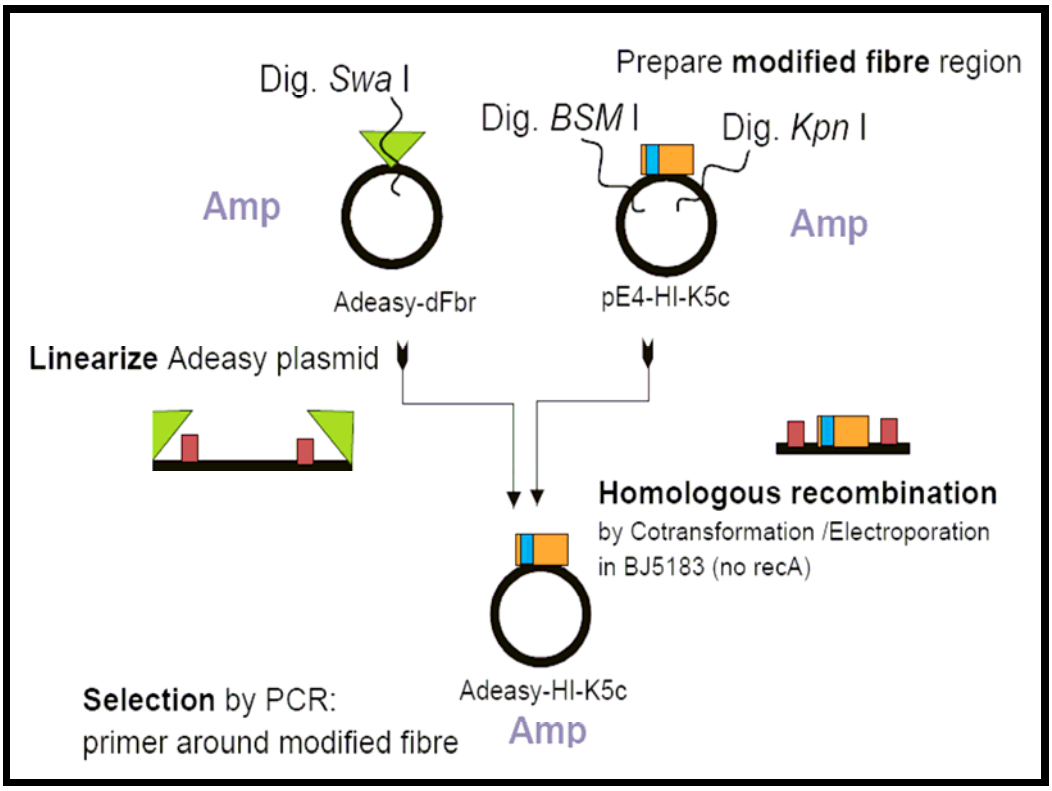

Figure IV: The Adeasy-HI-K5c plasmid construction (see text above). The fibre region (orange) is modified by K5-coil insertion (blue) in the HI-loop region of the fibre knob domain. The red rectangles represent the homologous regions in Adeasy-dFbr and in the modified fibre fragment. The latter fragment was obtained from the furnished pE4-HI-K5c plasmid by digestion with *BSM* I and *Kpn* I.

The transformed cells were put on LBA/ampicillin plates to grow over night at 37° Celsius. It was scanned for good colonies by PCR (conditions see Table II, results see gel electrophoresis Figure V) with the primers 1s and 2as situated at locations around the fibre region.

The chosen colonies (#1, 6, 7, 8) were again amplified via preculture, mini prep, and transformation into DH5α bacteria cells and 3 colonies of each sample were reselected for restriction enzyme analysis with *MUN* I (for results compare Figure VI).

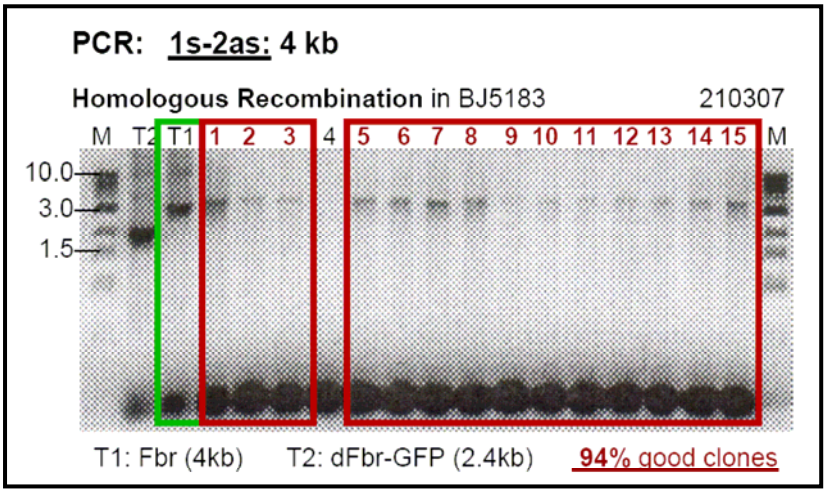

Figure V: Screening for clones with a modified fibre region (Adeasy-HI-K5c) by PCR and the primers 1s with 2as around the fibre region. This pair of primers should amplify a 4kb fragment from Adeasy-HI-K5c plasmids, like in the sample of Testimony T1 (green rectangle). The negative testimony T2 (2.4kb) represents a deleted fibre region fragment originally present in an Adeasy-plasmid with TR5CuO-GFP region. Screening results: 14 (red rectangles) out of 15 colonies showed up with bands around 4kb like the positive T1. This is a yield of 94%.

T_A	51°C
t_E	8′
Primer	20 pmol
Taq volume	0.4µl (5u/ml)
dNTP	200µM
Total volume	25µl

Table II: PCR conditions with primers 1s, 2as and a fragment to amplify with 4kb. T_A: Annealing temperature, t_E: Extending time

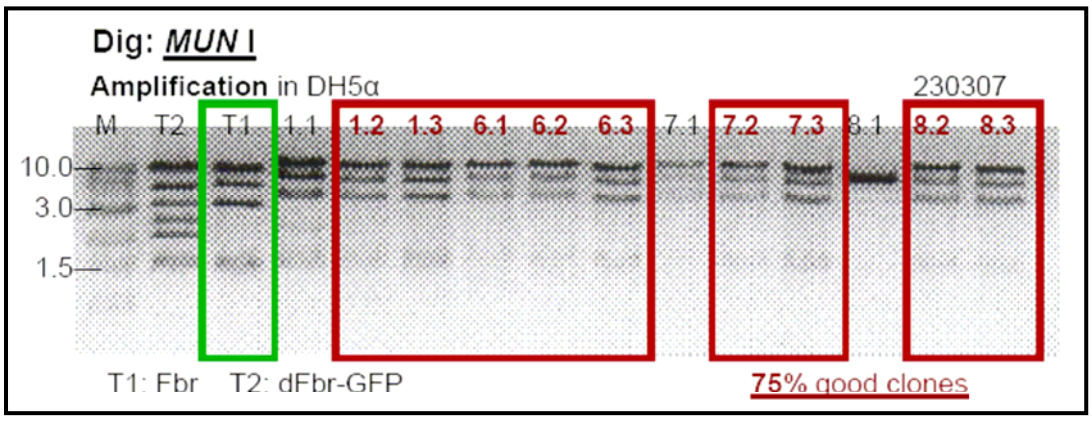

Figure VI: Screening for the very best clones holding a modified fibre region (Adeasy-HI-K5c) by *MUN* I digestion with 3 colonies of average size culled from each sample-plate (#1, 6, 7, 8). The positive testimony T1 (green rectangle) represents an Adeasy-plasmid with fibre region. The negative testimony T2 contains an Adeasy-plasmid with a deleted fibre region and a TR5CuO-GFP region. We expect to see 4 and not 5 bands for an Adeasy-HI-K5c plasmid digested by *MUN* I as two bands are at about 4kb (see Table 4.1.I). Screening results: 9 samples (red rectangles) of 12 have 4 bands, moreover these samples are lacking a 2368bp-fragment which would be typical only for plasmids lacking their fibre region. The yield of good clones was 75%.

4.3. Adeasy-HI-K5c-CMV5-GFP production

This third construction step inserts a kanamycin resistance and sequences for the Green Fluorescent Protein (GFP-marker) into the E1 region of the adenovirus genome Adeasy-HI-K5c. Furthermore the GFP-marker is accompanied by a CMV5 promoter providing a constitutive and high level expression of the green fluorescent protein. The adenovirus' transduction efficiency can then be measured by monitoring the intensity of fluorescence in the transduced cells.

Firstly the kanamycin resistant pAdenoVator-CMV5-IRES-GFP was linearized on its *PME* I site (target sequence 5'-GTTT|AAAC-3'), dephosphorylated with *CIP* to avoid its proper growth on kanamycin containing LBA plates and purified by the QIAquick PCR Purification Kit. The Adeasy-HI-K5c plasmid possesses two sites situated around its

sequences for ampicillin resistance which are homologous with two sites (homologous sites see red rectangles in Figure VII) at the pAdenoVator.
So, when homologous recombination (BJ5183 cells) of Adeasy-HI-K5c and the linearized pAdenoVator is executed, the ampicillin resistance sequence in the Adeasy-HI-K5c is replaced by the whole strand of the pAdenoVator. All construction steps for Adeasy-HI-K5c-CMV5-GFP design are sketched in Figure VII. The recombination products are streaked on kanamycin LBA plates and incubated at 37° over night. Good colonies can be selected by PCR analysis (see Table III for conditions, results see Figure VIII) using 1s and 2as primers that attach to sites around the fibre region. This is possible because firstly only recombinant Adeasy plasmids will have the ability to grow on kanamycin media and secondly a sole kanamycin resistant pAdenoVator does not have a fibre region. Consequently the existence of a fibre region in colony samples picked up from the kanamycin containing LBA plates confirms that the required recombination had taken place and that the sample represents a good colony.

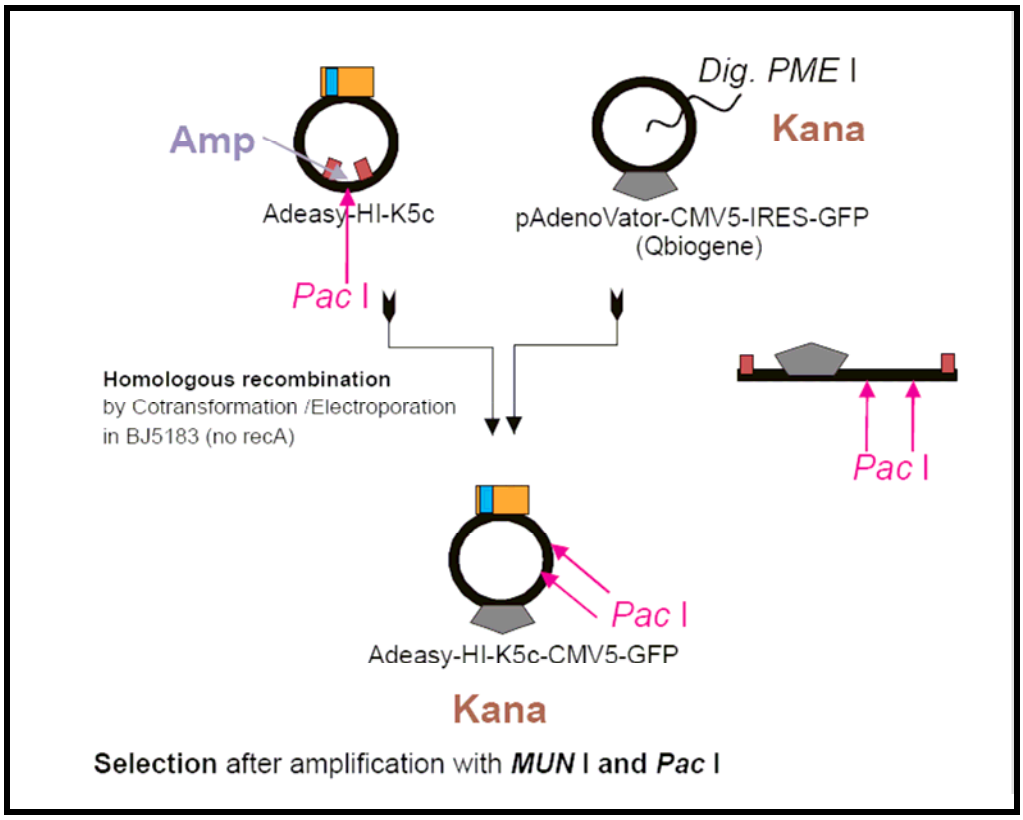

Figure VII: The Adeasy-HI-K5c-CMV5-GFP construction. The *Pac* I sites on the three different possible plasmids allow the differentiation by *Pac* I digestion: Adeasy-HI-K5c-CMV5-GFP and pAdenoVator-CMV5-IRES-GFP are cut in two fragments each while Adeasy-HI-K5c would be merely linearized by *Pac* I.

T_A	52°C
t_E	6’
Primer	12.5 pmol
Taq volume	0.5µl (5u/ml)
dNTP	100µM
Total volume	25µl

Table III: PCR conditions for primers 1s, 2as and an amplification fragment of 4kb. T_A, Annealing temperature; t_E, Extending time

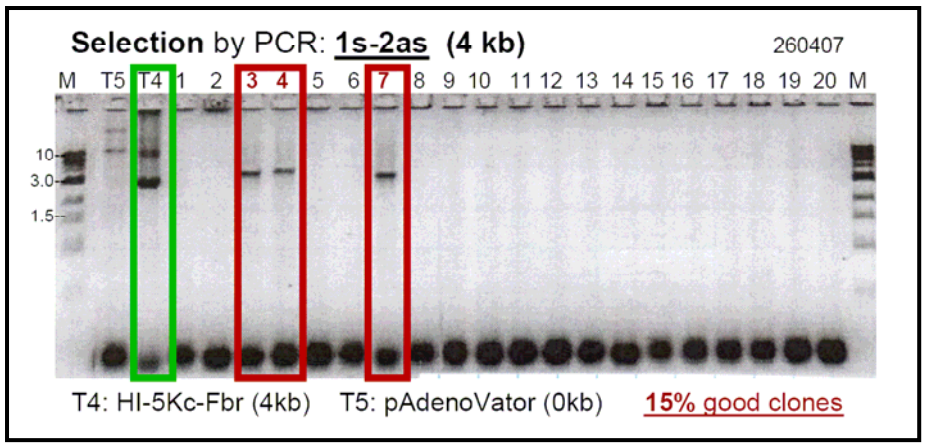

Figure VIII: We have to distinguish the colonies of pAdenoVator and the desired Adeasy-HI-K5c-CMV5-GFP plasmid on the kanamycin containing LBA plates. Screening for the fibre region via the primers around the fibre (1s, 2as) is a significant knock-out criterion against the pAdenoVator. 3 (#3, 4, 7) out of 20 samples contain a fibre region, consequently they are assumed to contain as well a GFP-marker region with CMV5 promoter. The yield is 15%.

The selected colonies (#3, 4, 7) are amplified by precultures with kanamycin, mini preps and by transformation into DH5α bacteria cells. The good colonies had to be selected this time by comparing the results of two digestion tests *MUN* I and *Pac* I (results see Figure IX).

Two good colonies (#2, 9) are amplified in big quantities for storage by the QIAGEN Plasmid Maxi Kit.

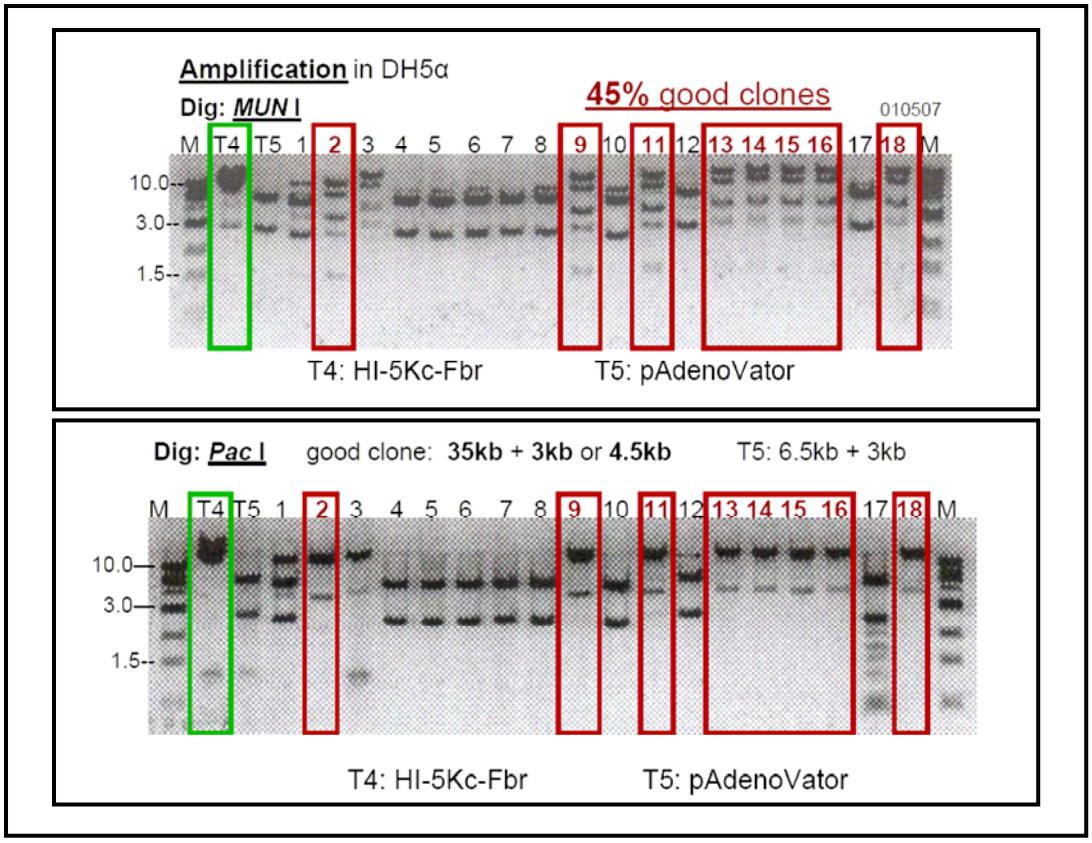

Figure IX: The *MUN* I digestion can testify that the obtained plasmid is no AdenoVator and that it contains a fibre region. The *Pac* I test delivers one fragment of the whole linearized Adeasy-HI-K5c, two small fragments for the pAdenoVator (6.5kb+3kb) and two fragments of 35kb plus 3kb respectively 4.5kb due to of two replication possibilities. Nevertheless both GFP integrations into the Adeasy-plasmid are good results. T5 is the negative testimony with a pAdeoVator plasmid, T4 should be a positive testimony with an Adeasy-plasmid and modified fibre region, but unfortunately it did not work right. Results of digestions: 8 out of 18 samples are good colonies, this is a yield of 45%. (1-6 stem from #3, 7-12 stem from #4, 13-18 stem from #7)

4.4. Designing Adeasy-dFbr-GFP

The construction of Adeasy-dFbr-GFP (sketch in Figure X) is done by homologous recombination in BJ5183 cells between the ampicillin resistant Adeasy-dFbr (see 4.3) and the kanamycin resistant, *PME* I-linearized and dephosphorylated AdenoVator-CMV5-IRES-GFP plasmid (compare procedure 4.3).

Good clones were selected by PCR (conditions Table IV, results Figure XI) with primers around the dFbr region (1s, 2as) as only plasmids with inserted GFP marker could have survived and grown on the kanamycin containing LBA plates during incubation over night at 37° C.

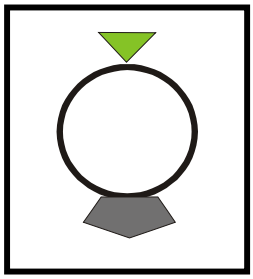

Figure X: The Adeasy-dFbr-GFP plasmid, an intermediate step in Adeasy-m-HI-K5c-GFP design.

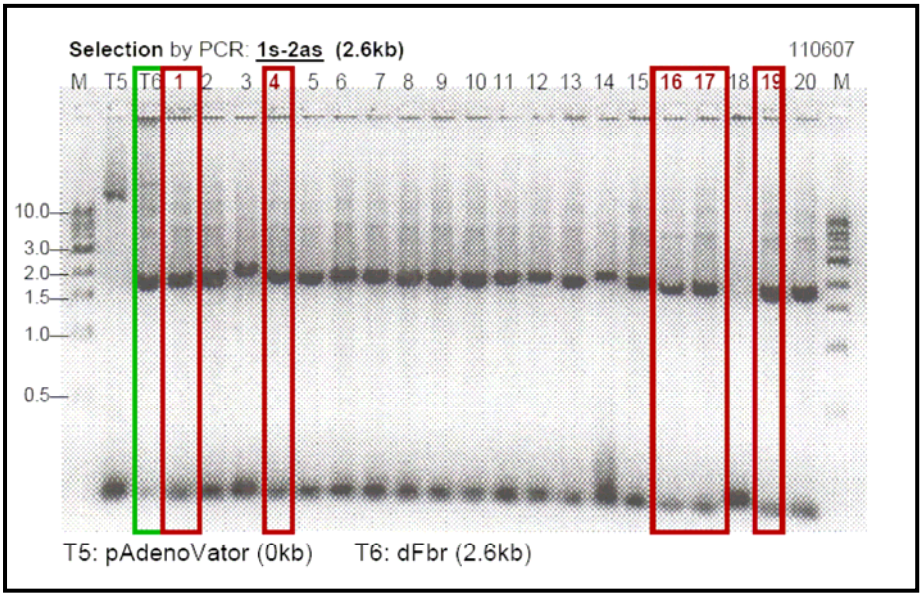

Figure XI: PCR is performed with the primers 1s, 2as around the fibre region. Results can differ pAdenoVator colonies from the desired Adeasy-CMV5-GFP plasmid. 5 of 20 samples (#1, 4, 16, 17, 19) resemble the positive testimony T6, thus a fragment of about 2kb with dissipated primers (primers appear as small fragments at the bottom of the electrophoresis picture). Admittedly we attended a 2.6kb fragment and T6 has not more than 2kb. We assume that the time of 6 minutes for the extending step was not sufficient for the amplification of the whole fragment. The yield of good colonies is 25%.

Four of these obtained Adeasy-dFbr-GFP samples (#1, 16, 17, 19) were amplified as before in DH5α bacteria cells and the very best colonies selected by *MUN* I and *Pac* I digestion (see Figure XII).

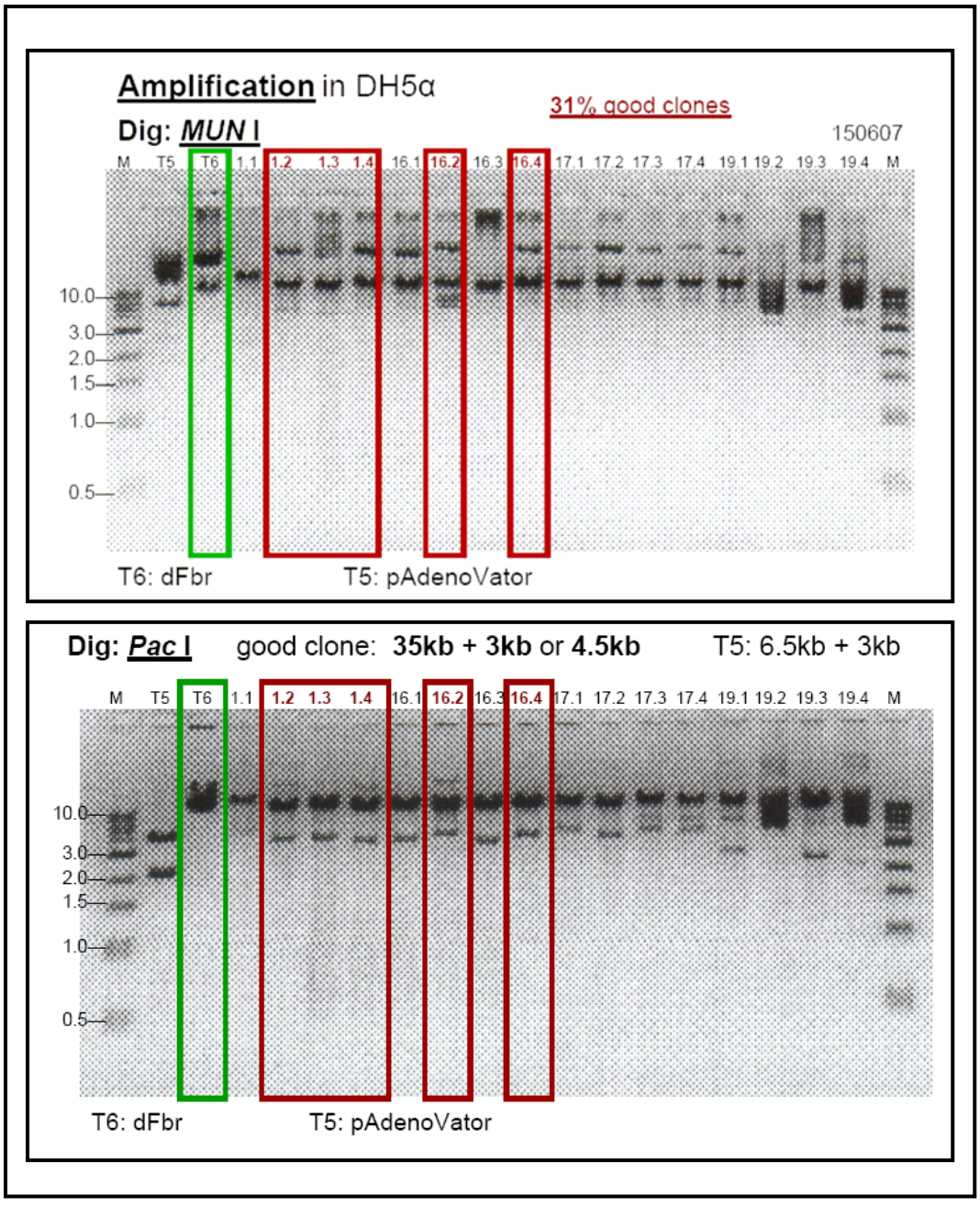

Figure XII: Unfortunately the *MUN* I digestion did not succeed. The digestion with *Pac* I delivers however 5 good samples which show two perfect bands at 35kb and 4.5kb, 31% yield. The negative testimony with pAdenoVator T5 bespeaks the difference to the good samples (#1.2, 1.3, 1.4, 16.2, 16.4). The positive testimony T6, a Adeasy-dFbr plasmid without fibre region (Maxi prep of sample # 3.1 picture 150307_2 in paragraph 4.1), was not successful neither in *Pac* I digestion.

T_A	52°C
t_E	6’
Primer	12.5 pmol
Taq volume	0.5µl (5u/ml)
dNTP	100µM
Total volume	25µl

Table IV: PCR conditions for primers 1s, 2as and an amplification fragment of 2.6kb. T_A, Annealing temperature; t_E, Extending time

4.5. Modifying CAR-region in a pE4-HI-K5c plasmid

The modification in the fibre region should ablate the tropism for natural CAR-receptors by replacing Serine[408] with a Glutamic acid and the Proline[409] with Alanine. This modification is realized by four point mutations in the CAR binding domain of the fibre using the Stratagene’s QuikChange™ Site-Directed Mutagenesis Kit, the pE4-HI-K5c plasmid, the primers FBM 408-409 plus R-FBM-408-409 and DH5α bacteria cells for plasmid amplification. The resulting pE4-m-HI-K5c plamid is displayed in Figure XIII. The modification was verified by PCR analysis (conditions Table V, results Figure XIV) with primers located in the fibre region (PFR2 and RFR4 around the modification site of the CAR region) and by sequencing (University Laval sequencing results Figure XV). The generation of the second recombinant adenovirus with CAR tropism ablation referred as Adeasy-m-HI-K5c-CMV5-GFP was not finished at the end of this Master thesis project. Thus, the last step of homologous recombination with Adeasy-dFbr-GFP and the fibre region of pE4-m-HI-K5c had still to be done.

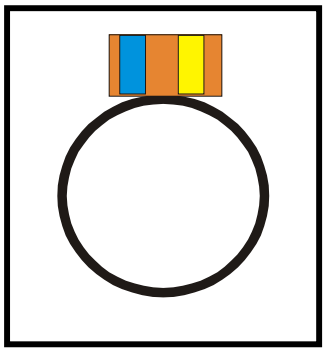

Figure XIII: The pE4-m-HI-K5c, with an additional modification in the fibre's (orange) CAR region (yellow) beside the insertion of the K5coil in the HI-loop region (blue).

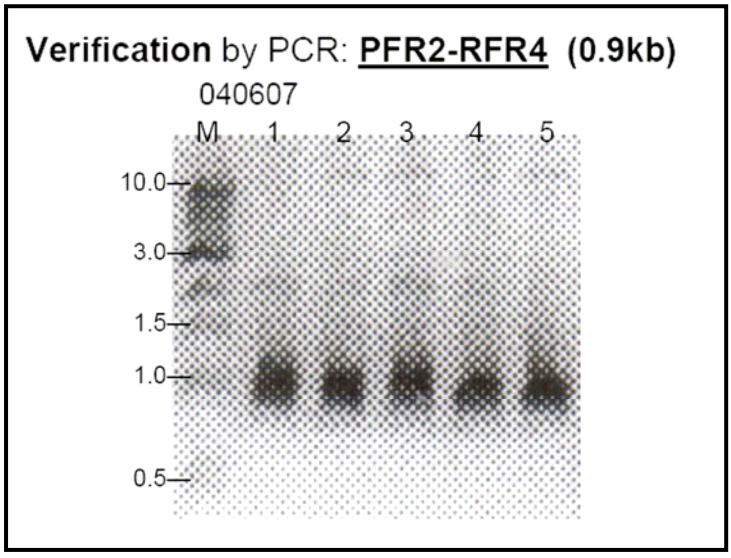

Figure XIV: PCR is merely able to detect a fibre region by a 900bp-fragment. This implies that after the modification step the sample still contains a pE4-HI-K5c plasmid with fibre region. The modification can only be proved by sequencing this fragment.

T_A	52°C
t_E	2’
Primer	40 pmol
PHS volume	1µl (2u/µl)
dNTP	400µM
Total volume	50µl

Table V: PCR conditions for primers PFR2, RFR4 and an amplification fragment of 900bp. T_A, Annealing temperature; t_E, Extending time

NNNNNNNNNNNNNNNNNNNNNNNNNNNNANNNNCNTNTNGTTANNCNANTGAGNTN
NNNNTCTTTCTCTGNNNNNNNNNNNNGTTAGCCTCTGGAGCTGGTGTGGTCNNNNNNNN
NNNNNTATCNTTATTTTTGNTTCCTACTGTAATGGCACCTGTGCTGNCAAAACTAAGGN
CAGTTCNTNNTTNANGAACCATANCCTTGTTTGAATCAAATTCTAGGNCATGGNCAATT
TTTGTTTTGAGGGGATTTGTGTTTGGNGCATTAGGTGAACCNAATTCNAGCCCATCTCC
TGCATTAATGGCTATGGCTGTAGCGTCAAACATCAACCCCTTGGNAGTGCTTANGTTNA
CCTCAANCTTTTTGGAATTGTTTGAANCTGTAAACAANTAAAGGCCTTTGTTGTTNTTAA
TANCCAANTTGTGGGNTGAGTTTATAAAAANAGGNCCCTGTCCTANTNTTAGATTTANT
TGGTTTTGANCATCAAACGGATAANTANCATCAAGTATAAGGNNTCTGTTTTGANAATC
AATCCTTNNCCTCCTGCTACATTAANTTGCATATTGCCCTTGNGAAANCAAAANCCAAG

Figure XV: The 900bp fragment obtained by PCR with PFR2 and RFR4 was analysed by sequencing with the starting primer R-FBM-408-409. The 5’-3’-sequence-result of sample #5 is listed above. It clearly accounts that the original GAGA sequence present in the CAR-ligand domain of the template plasmid was transformed as intended into CCTC (orange). The ablation of the CAR-tropism via DNA modification is confirmed that way. The pink nucleotide bases represent the circumjacent DNA bases present in both the primers of modification (FBM 408-409 and R-FBM-408-409) and the proper fibre region. The nucleotide base in blue is already part of the proper fibre region. N: not definable nucleotide bases.

4.6. Transfection of cleaved Adeasy-HI-K5c-CMV5-GFP in 293 cells

For the Adeno-HI-K5c-CMV5-GFP virus production the Adeasy-HI-K5c-CMV5-GFP vector (see 4.3) was cleaved by *Pac* I. This exposes the adenovector's ITR regions (Inverted Terminal repeats), the starting position for replication. After column purification the resulting linearized plasmid was transfected by lipofectamine (Invitrogen) into 293A and 293E cells and stored for 16 days at 37°C to enhance the virus particle production (compare Figure XVI). In 293E cells the recombinant virus can bind to CAR- and EGF-receptors and in 293A cells to the CAR-receptors, thus this virus is not CAR tropism ablated. Time till GFP expression in the two infected cell lines should be compared to choose the virus' future host cell type for a large scale infection.

The efficiency of transduction and production was monitored under the microscope by expression of the green fluorescence protein marker located in the adenovector's E1 region. The first slight GFP protein production could be observed on the ninth day after transfection. In the course of time the encapsulated adenoviruses went out of the transfected 293 cells and infected adjacent cells as well (self-infection).

The recombinant virus grew and amplified in both cell types of packaging cells, 293A and 293E, with equal velocity. That is why we decided to continue for the infection process with the 293A cells, which do not need antibiotics to be cultured and are consequently easier to handle.

Afterwards 50 plates (150mm diameter) of 293A cells should be infected with the Adeno-HI-K5c-CMV5-GFP adenoviruses to produce 10^9 infected cells. Therefore all viruses from the transfection plates were released from the cells by freezing the medium with the cells under –80°C and centrifuging it for 5 minutes at 1000rpm and 5°C and diluting in 5ml DMEM 5%.

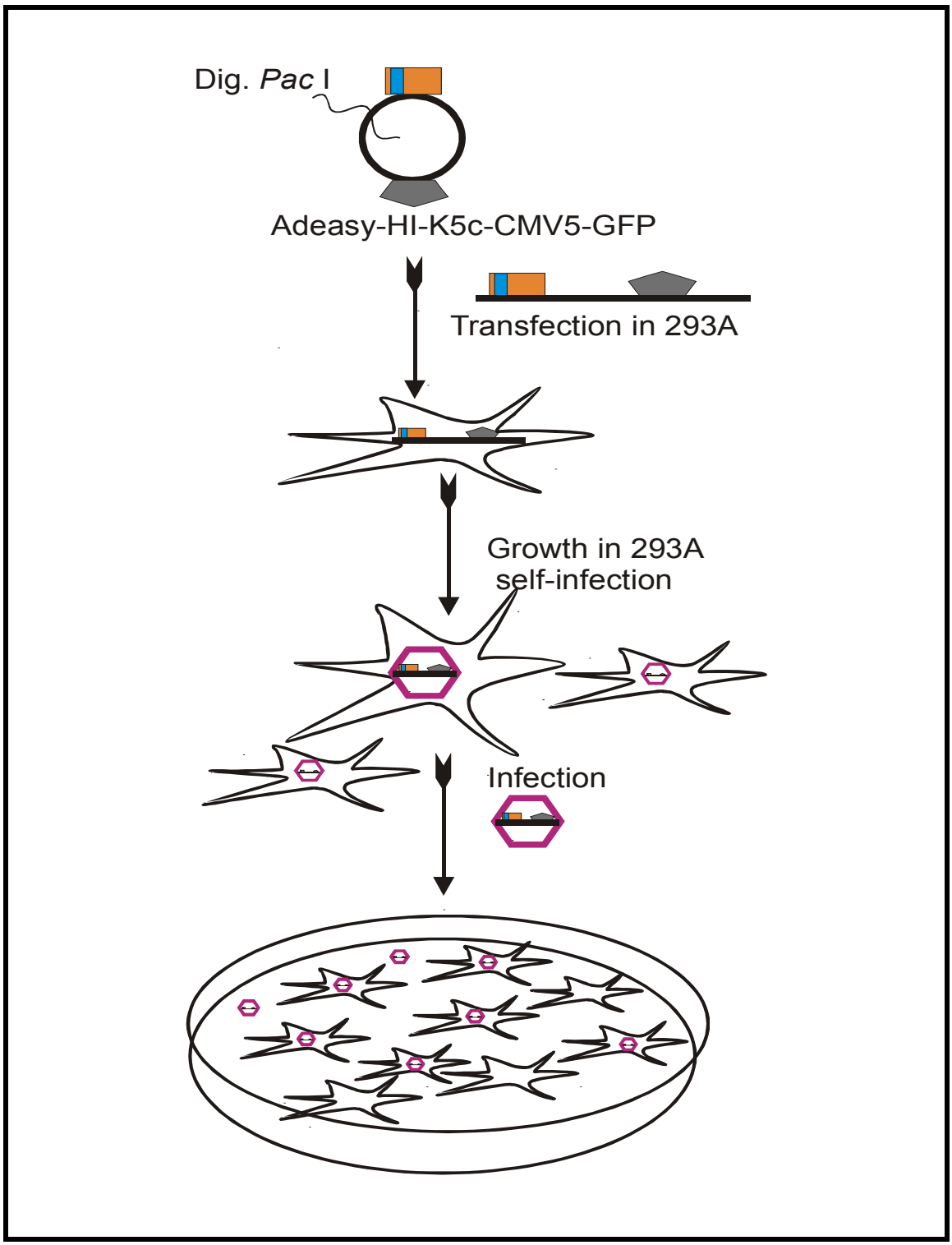

Figure XVI: Adeno-HI-K5c-CMV5-GFP virus production in 293A and 293E cells (procedure see description above).

4.7. Purification of Adeno-HI-K5c-CMV5-GFP adenoviruses

The 10^9 infected cells were harvested from the fifty 150mm-plates prior to autolysis (three days after infection) to maximize the cell-associated fraction. After that, cells were resuspended to roughly 2×10^8 cells/ml, lysed by 3 times freeze/thaw and clarified by centrifugation at 1000rpm, 5 minutes and 4° Celsius. The virus was subsequently purified through two rounds of a 24-hour ultracentrifugation by a continuous CsCl density gradient and the value of 7×10^{11} Particles per millilitre (P/ml) was detected with the BioPhotometer (Eppendorf) at 260nm after lysis with a virion lysis buffer (solution of Monobasic sodium Phosphate and Dibasic Sodium Phosphate). So the proper virus particles could be estimated roughly with 7×10^{10} Infectious Virus Particles per millilitre virus stock (IVP/ml of virus).

4.8. Virus Titration of Adeno-HI-K5c-CMV5-GFP

After the concentration and purification of the adenovirus particles they were quantified more precisely with a titre calculation in a flow cytometric assay with a dilution series of the virus stock solution in 2.10×10^5 293A cells (see Table VI). At the tested MOI levels ranging from 0.05 to 0.24 infectious virus particles per 293A-cell (see Table VII) it was calculated a titre of 2.54×10^8 infectious virus particles per ml virus stock solution (total stock volume: 5ml) in Table VIII respectively Diagram I and II.

Sample	Dilution factor	Volume virus stock in dilution	293A cell number	Probability of cell infection (FACS) (GFP^+ at MOI<0.3)
		V [ml of virus]	*C [cell]*	*P [%]*
4	3.7×10^{-6}	7,41E-06	2,10E+05	4,88%
3	1×10^{-5}	2,22E-05	2,10E+05	2,00%
2	3×10^{-5}	6,67E-05	2,10E+05	8,58%
1	1×10^{-4}	2,00E-04	2,10E+05	21,23%

Table VI: Dilution series of the virus stock solution with dilution factors between 1×10^{-4} and 3.7×10^{-6}, cell number (C) and percentage of infected cells per sample (P) measured with FACS via the green fluorescence signal of the GFP marker.

Sample	V/C [ml of virus/cell]	MOI [IVP/cell]
	X-value: V/C	*Y-value: -LN(1-P) = MOI*
4	3,53E-11	5,00E-02
3	1,06E-10	2,02E-02
2	3,17E-10	8,97E-02
1	9,52E-10	2,39E-01

Table VII: Calculation of the X- and Y-value for titre determination by slope calculation of linear regression

titre [IVP/ml of virus]	Range of samples
d=0, k (slope) = titre = Y/X	
2,55E+08	1-4
2,53E+08	1-3
mean titre [IVP/ml of virus]	
2,54E+08	

Table VIII: Slope determination by linear regression (see Diagram I and II) and calculation of the mean titre

Figure XVII shows the fluorescence effects in the Adeno-HI-K5c-CMV5-GFP infected 293A cells. The green fluorescing cells confirm that the former GFP-insertion into the virus vector succeeded.

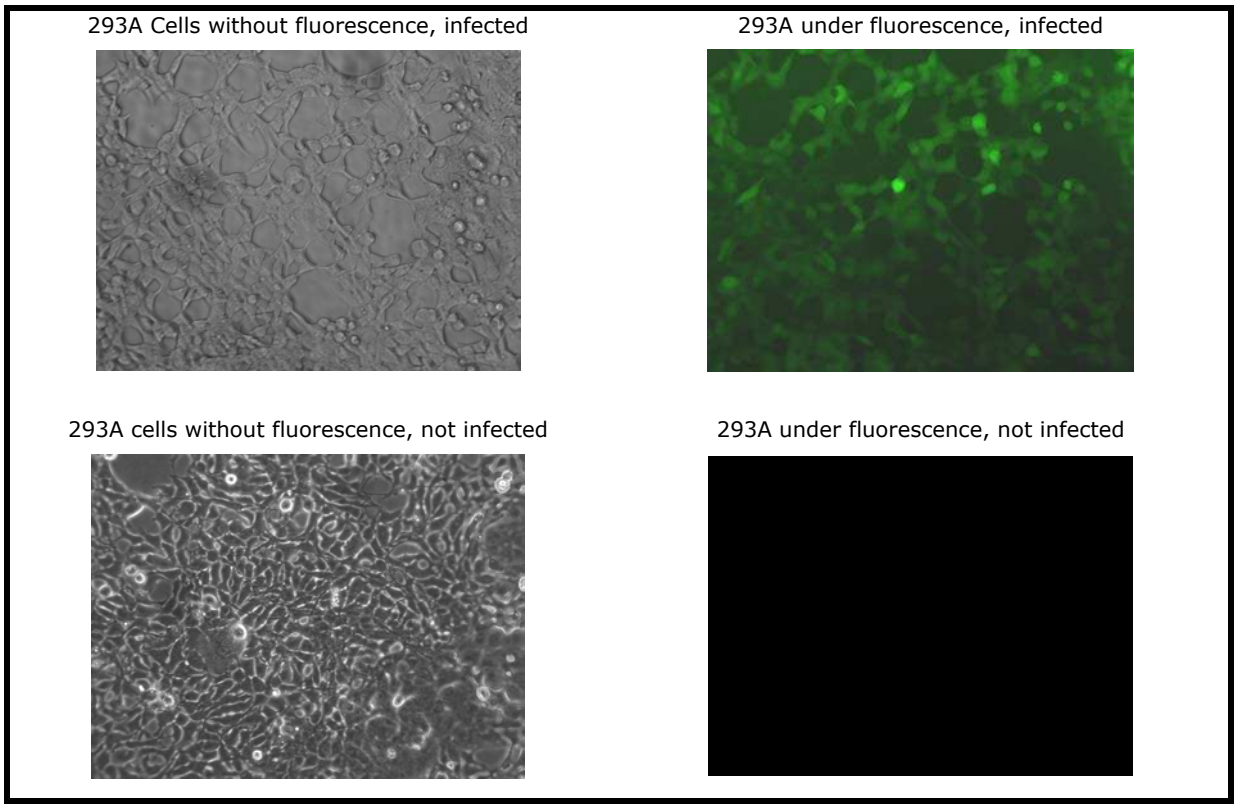

Figure XVII: Fluorescence effects to confirm GFP-insertion in the recombinant adenovirus Adeno-HI-K5c-CMV5-GFP. Protocol: 0.3×10^6 293A cells were infected with 10 MOI (20μl) purified adenovirus, incubation period at 37°C: 24 hours. Results: Only the infected cells showed green fluorescence, GFP marker insertion with CMV5 promoter is confirmed.

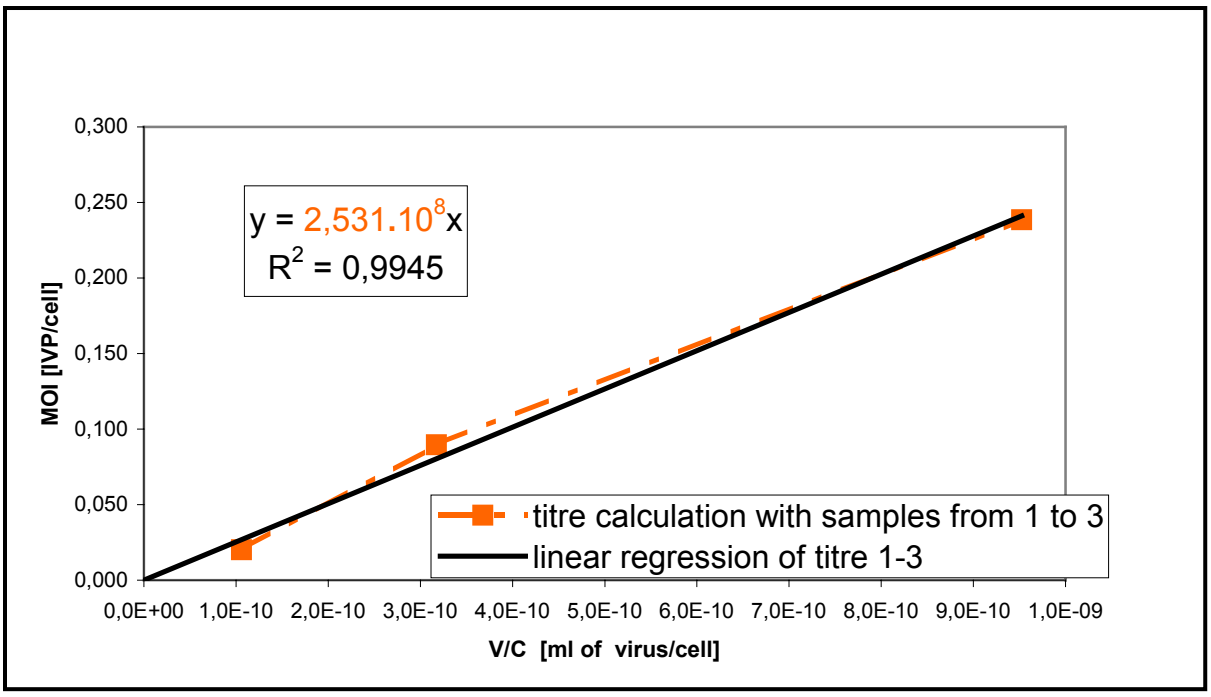

Diagram I: Titre calculation by linear regression in a flow cytometric assay with virus dilution samples from 1 to 3. The regression line is passing in the point of origin. Measured titre: $2{,}531 \times 10^8$ infectious virus particles per ml virus stock solution [IVP/ml virus].

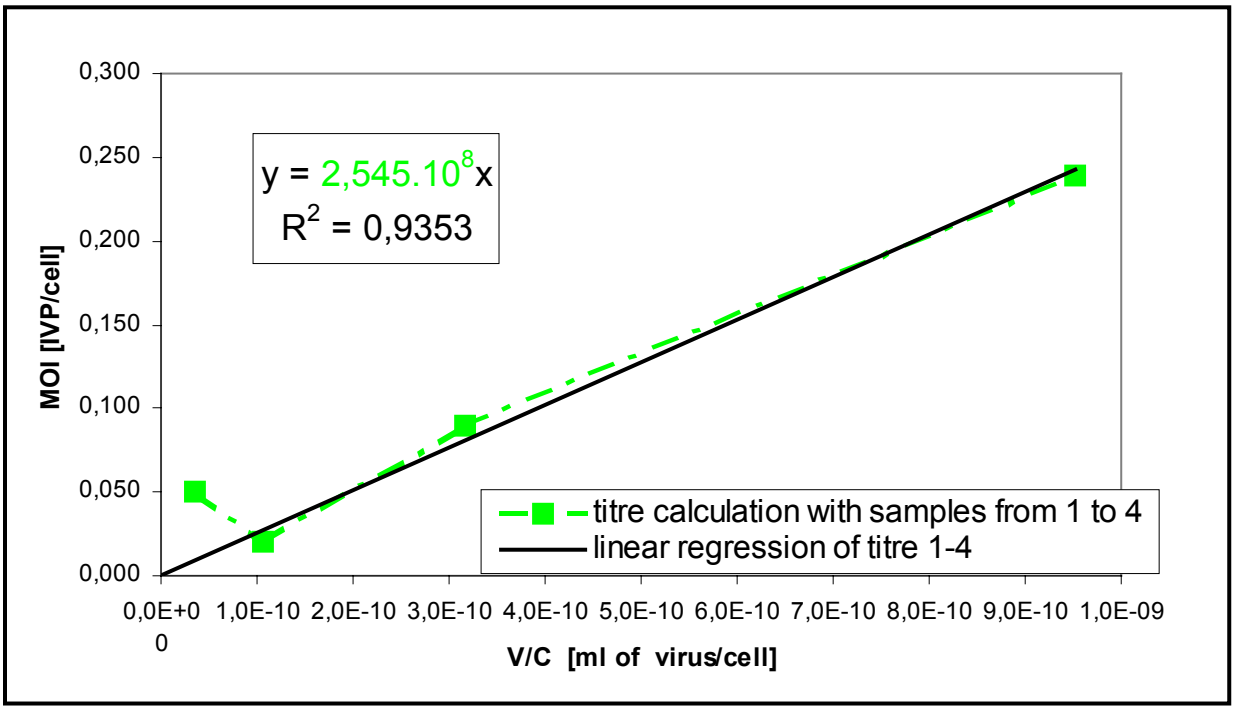

Diagram II: Titre calculation by linear regression in a flow cytometric assay with the dilutions 1 to 4. Titre: $2{,}545 \times 10^8$ IVP/ml virus.

4.9. Retargeting of Adeno-HI-K5c-CMV5-GFP in A549tta cells

The virus transduction assays of the recombinant adenovirus Adeno-HI-K5c-CMV5-GFP were accomplished via fusion to an E-coil-EGF ligand and infection of A549tta cells. These cells overexpress the EGF-receptors and express CAR as well.

Transduction efficiency was measured via flow cytometry (FACS) by monitoring the GFP expression and the X-mean for all virus concentration samples with and without ligands and blanks (vide procedure Figure XVIII and results Table IX).

The test results, see also Table X and Diagram III, draw out that the recombinant adenovirus, capitalising on the E5-coil/K5-coil ligand system, managed to infect 19% of the cells (effective GFP rise) at the MOI 5 level but did not infect the cells at level MOI 1 (negative rate of infection, -2%).

Sample	Multiplicity of infection	Data GFP$^+$%	Data x-mean
1	MOI 1 V	5,70	9,9
2	MOI 1 V	6,72	10,4
3	MOI 1 V+L	7,28	9,4
4	MOI 1 V+L	6,56	10,0
5	T(-) MOI 1 V	17,98	17,4
6	T(-) MOI 1 V+L	18,61	19,9

Sample	Multiplicity of infection	Data GFP$^+$%	Data x-mean
1	MOI 5 V	16,46	11,1
2	MOI 5 V	17,44	12,7
3	MOI 5 V+L	16,82	14,1
4	MOI 5 V+L	17,08	13,6
5	T(-) MOI 5 V	50,60	30,3
6	T(-) MOI 5 V+L	51,64	28,9

Table IX: Data for transduction efficiency tests measured via green fluorescence by FACS. The ratio in the assays between virus concentration and ligand concentration to A549tta cell quantity was 1:1 (MOI 1) and 5:1 (MOI 5). V, the engineered Adeno-HI-K5c-CMV5-GFP virus with CMV5 promoter; L, E-coil peptide with EGF-ligand; T(-), negative Testimony, adenovirus with natural CAR tropism and CMV5-GFP region in DNA but no EGF-ligands on surface; orange, blank results; blue, Adeno-HI-K5c-CMV5-GFP adenovirus results.

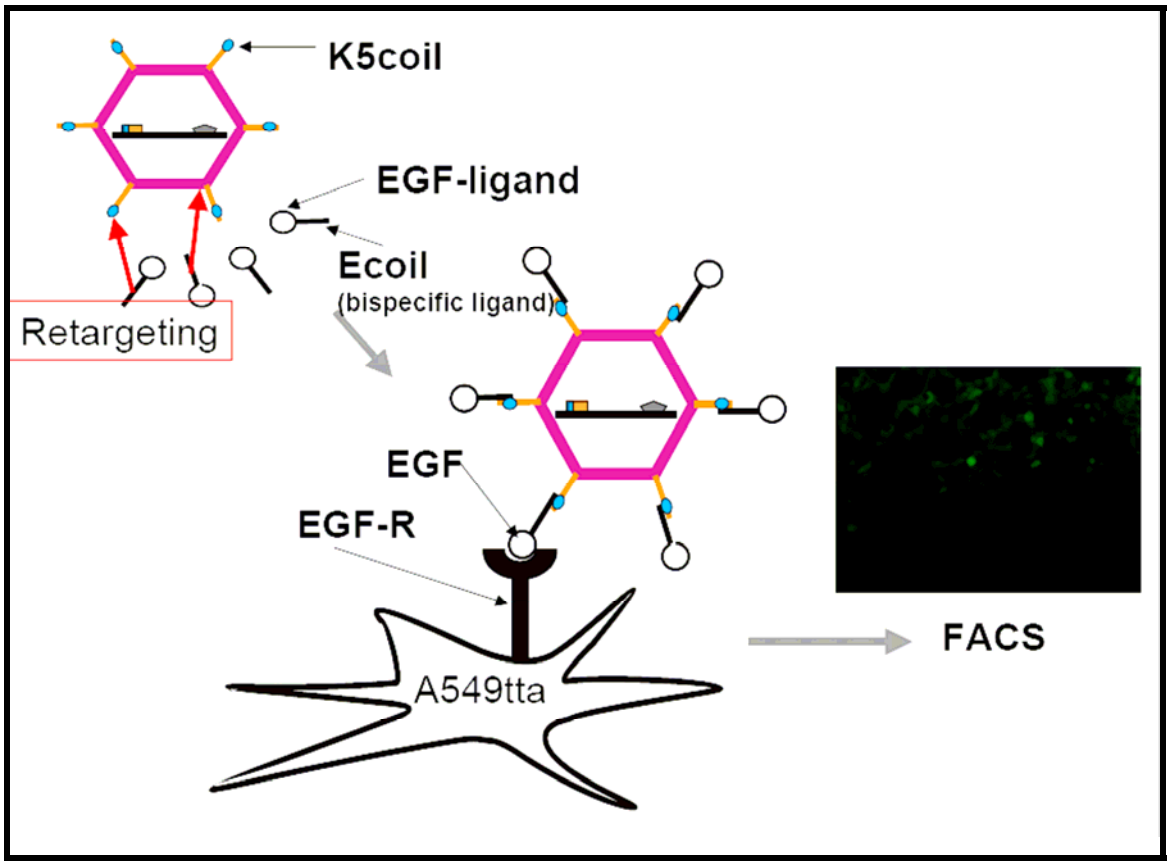

Figure XVIII: Retargeting procedure for the recombinant adenovirus Adeno-HI-K5c-CMV5-GFP in A549tta cells via Flow Cytometrie. An EGF-ligand on a E5coil-peptide is mustered with the adenovirus and the A549tta cells. The E5coil is a bispecific ligand and should clue to the K5coil fibre region of the adenovirus. Only this complex would now be able to dock at the EGF-R receptor on the A549tta cells for infection and protein expression. This supplemental second mechanism of attachment for the engineered Adeno-HI-K5c-CMV5-GFP virus assumingly should accelerate the internalization and lead to an augmentation of infected A549tta cells compared to the blank, hence the T(-) virus is internalizing into the A549tta cells only by CAR mediated infection. That way if in the sample of Adeno-HI-K5c-CMV5-GFP plus ligand (V+L) can be measured a significant augmentation of GFP expression via green fluorescence the insertion of the K5coil peptide into the HI-loop region of the adenovirus can be regarded as succeeded.

Sample	Multiplicity of infection	Total (GFP^+ * x-mean)	Mean of Total	GFP^+-rise in V+L [%]	Effective GFP^+-rise [%]
1	**MOI 1 V**	56,4			
2	**MOI 1 V**	69,9	63,2		
				6%	
3	**MOI 1 V+L**	68,4	67,0		
4	**MOI 1 V+L**	65,6			**-2%**
5	**T(-) MOI 1 V**	312,9	312,9		
				8%	
6	**T(-)MOI 1 V+L**	370,3	370,3		

Sample	Multiplicity of infection	Total (GFP^+ * x-mean)	Mean of Total	GFP^+-rise in V+L [%]	Effective GFP^+-rise [%]
1	**MOI 5 V**	182,7			
2	**MOI 5 V**	221,5	202,1		
				16%	
3	**MOI 5 V+L**	237,2	234,7		
4	**MOI 5 V+L**	232,3			
					19%
5	**T(-) MOI 5 V**	1533,2	1533,2		
				-3%	
6	**T(-) MOI 5 V+L**	1492,4	1492,4		

Table X: Calculations for the rise of GFP-expression when the Adeno-HI-K5c-GFP virus was completed by the bispecific E-coil peptide including an EGF-ligand. The resulting rise was for MOI 5 19%, the result for MOI 1 was unfortunately negative with -2%.

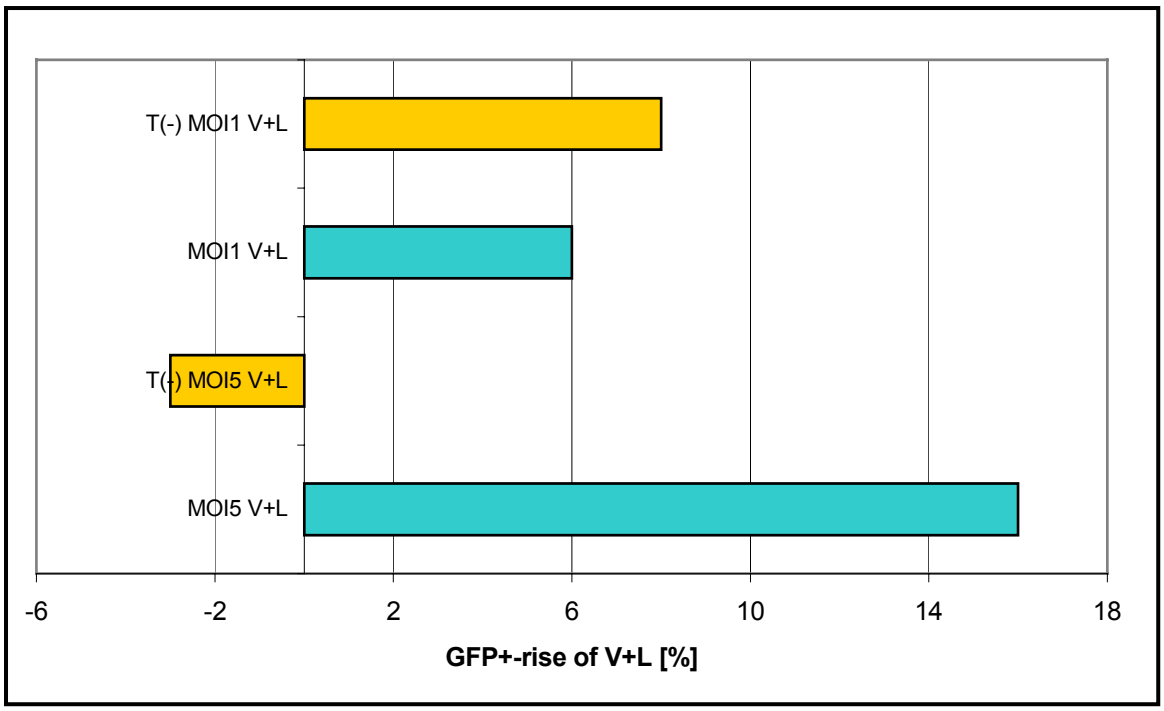

Diagram III: Testing the engineered adenovirus. Monitoring of GFP fluorescence for transduction efficiency tests of the Adeno-HI-K5c-CMV5-GFP adenovirus. Transduction takes place via the E5-l/K5-coil technology plus EGF-ligand/EGF-receptor docking system. Augmentation in percent between V+L and V for MOI 1 and MOI 5 in comparison with T(-) blank values, using viruses without neither EGF-ligands nor K-coil pseudo ligands. L, E-coil including EGF-ligand; T(-), blank, negative Testimony.

5. Discussion

Since 1989 hundreds of clinical trials for cancer gene therapy have been conducted or are underway, chiefly with terminal patients as subjects and most commonly using adenoviruses for gene delivery. Thus, adenovectors have many advantages like their high transduction efficiency, their high transgene capacity of about 6kb for adenoviral first generation vectors, moreover they can easily be produced with high titers and they are able to transduce both dividing and nondividing cells, this is an important property since only a small portion of the malignant tumour cell population may be mitotic at given time. In 2003, *Gendicine* was approved as first gene therapy product worldwide on the Chinese market. This drug is composed of a replication-incompetent adenovirus, containing a p53 tumour suppressor gene in place of the viral E1 region, and is applied in combination with radiotherapy against head and neck cancer. *Advexin*, a therapeutic virus similar to *Gendicine*, is pending approval in the USA and *Oncorine*, a conditionally replicative adenovirus, was already approved in China for head and neck cancer in 2006. Furthermore, an adenovirus with the prodrug activator Herpes simplex thymidine kinase transgene, the Anglo-Finish *Cerepro* against glioma, has reached Phase III trial [50].

Despite this first success with adenovirus vectors in cancer gene therapy, their field of application is limited by their natural CAR-tropism. Indeed, CAR receptors mostly do not represent an interesting and specific therapeutic target. One of the obstructions in employing CAR-tropism for therapeutic adenoviruses is for example caused by human liver parenchymal cells. Thus, these normal liver cells naturally highly express CAR on their cell surface which turns them into a competing target for adenoviruses with CAR-tropism. Subsequently this can lead to unintentional gene expression in liver cells instead of the intrinsically targeted tumour cells [3]. So cancer gene therapy which applies CAR-mediated infection mechanisms may compromise infection specificity or inhibit infection at all. In order to improve the adenovirus' targeted

transduction efficiency the vector's tropism obviously needs to be widened.

The three year research project of Dr. Bernard Massie tends to improve this therapeutic index for gene therapy against tumours which are overexpressing receptors other than CAR. The idea is to ablate CAR tropism and to retarget an AdV toward any desired target receptor by means of the universal and patented E-coil/K-coil technology. For the task of replacing the rather disadvantageous natural CAR entry either epithermal growth factor (EGF) or fibroblast growth factor (FGF) ligands are frequently chosen since their transmembrane receptors, EGF-R and FGF-R, are found to be closely linked to the malignant potential of cancer cells: So, in as many as 60% of head, neck, ovarian, cervical, bladder, oesophageal, gastric, breast, endometrial and colorectal cancer patients an increased EGF-R expression was associated with reduced survival rates [49]. Up regulation of IGF-R was documented as well for various cancer types like glioma[51], neuroblastoma[52], medulloblastoma[53], breast cancer[54], [55], endometrial carcinoma[56], colon carcinoma[57], and prostate cancer[58], [59]. Importantly, the IGF-R levels actually decline after puberty in normal cells but increase in malignant cells.
Dr. Massie and his research group decided to generate an adenovirus targeted toward the cancer specific IGF-R. In theory an adenovector "armed" with an IGF-R ligand will infect mainly the high IGF-R expressing cancerous cells and will spare the normal low-IGF-R-expressing epithelial cells. Although EGF- and IGF-ligands could be conjugated with bispecific antibodies to the adenovirus fibre, the consistent manufacturing of uniform antibody conjugates is very difficult. The simple and stable retargeting method with the E-coil-IGF-ligand fusion protein glued to an adenovector formulated with the fusion protein's binding partner called K-coil in its fibre knob, secures that way a big advantage when considering drug production and clinical application.

Cancerous cells should be infected selectively by this system and killed by molecular chemotherapy with the CD::UPRT suicide gene. This suicide gene encodes two enzymes able to convert in two steps the non-toxic pro-drug 5-fluorocytosine (5-FC) *in situ* into the cytotoxic product 5-fluorouracil-monophosphate (5-FUMP). The vector's preparation and clinical application for the recombinant AdV vector system of Dr. Massie would be the following: For the first part of "adenobody" construction the "universal" recombinant K-coil-adenovirus has to be clued to a bi-specific E-coil peptide already fused to IGF, the ligand of choice. After administration the recombinant vehicle system introduces selectively the therapeutic suicide gene CD::UPRT into tumour cells that are overexpressing the chosen ligand's adversary IGF-R. Subsequently the relatively non-toxic pro-drug 5-fluorocytosine (5-FC) can be administered systemically with high doses to the patient. The pro-drug-converting enzyme CD originates from E coli. It will be expressed only within the transfected tumour cells leading to local production of big amounts of the highly toxic therapeutic molecule 5-fluorouracil (5-FU). 5-FU inhibits the enzyme thymidylate synthetase and has already been applied in large scale for chemotherapy. In order to increase the potency of CD, the bacterial uracil phosphoribosyltransferase (UPRT) is added in the viral gene transfer constructs. UPRT phosphorylates FU-5 to its cytotoxic form 5-FUMP to overcome the FU-5 resistance of some tumours since blockade of FU-5 phosphorylation is one means of tumour resistance. In the medium term the transfected, suicide gene expressing cells, try to maintain survival by diffusion of the lethal chemical 5-FUMP through the gap junction into contiguous non-transfected cancer cells. In the end this "bystander effect" brings about the death of both, the transfected cells and the neighbouring tumour cells once the toxin reaches high enough concentrations. So, this suicide gene therapy should enable tumour regression even when only a part of the cancerous cells are infected [60].

For my six month participation in the whole project two recombinant replication incompetent adenoviruses should be generated for subsequent transduction studies. A K4-coil-fibre AdV had already been generated before. However, the research group of Dr. Bernard Massie found a 86-fold stronger interaction of the K5/E5 system than with the K4/E5 combination, so a K5-coil peptide instead of a K4-coil in the fibre knob region of the AdV promised improvement for infection potency *in vivo*. Unfortunately fusing the K5-coil to the common position on the fibre's C-terminus stopped the fibre's correct folding by trimerization. In consequence the chimeric fibre could not be incorporated in the AdV capsid. The HI-loop region in the fibre knob is known as insertion site for larger ligands. So a fibre protein was generated having the K5-coil inserted into the HI-loop to test its trimerization capabilities. The recombinant protein was expressed properly and could form trimers which was demonstrated by western blot analysis. Therefore, it was my task to construct an AdV expressing the K5-coil in the HI-loop region called Adeno-HI-K5c-CMV5-GFP, and furthermore a second recombinant AdV that should have in addition an ablated CAR tropism also referred as Adeno-m-HI-K5c-CMV5-GFP.

The Adeno-HI-K5c-CMV5-GFP virus with CMV5 promoter was generated by homologous recombination steps in bacteria and propagated in HEK 293 cells. The virus intermediate's transduction efficacy was tested roughly *in vitro* by retargeting with results of 19% infection rates at MOI 5 levels via the E5-coil/K5-coil mechanism using a pre-existing E-coil peptide fused to an EGF-ligand. The conditions for retargeting analysis need however to be improved before further manipulations of the adenovirus vector will be started like deletion of HSG and α_V-integrin binding sites, formation of disulfide bridges between the K-coil/E-coil peptides ensuring more stabilization of their interaction, and of course suicide gene insertion. Subsequently a synthetic E5-coil with IGF-ligand will be produced including a conjugating sulfide bridging system.

The second intermediate recombinant adenovirus with additional ablated CAR-tropism, called Adeasy-m-HI-K5c-CMV5-GFP, has not been completed yet lacking one homologous recombination step between modified fibre region and dFbr-GFP Adeasy plasmid. After propagation of Adeasy-m-HI-K5c-CMV5-GFP the infectivity of this virus shall be tested as well by retargeting.
Subsequently, the promising produced vectors will be tested for toxicity, specificity and tumouricidal effect in mouse and rat models for liver metastases and malignant glioma. Firstly the vectors will be administered by direct injection into subcutaneous tumours, in the second phase by injection via the intrasplenic route in mice with liver metastases and in the end, for the third assay, with intravenous injection followed by systemic treatment with the prodrug 5-FC.

Adenoviral infections are for the most part transient, that means the genome of the adenovirus is diminishing in a few weeks, which represents one of the disadvantages for certain gene therapy applications. However, for the goal of killing tumours by the suicide gene therapy approach sustained expression of the transgene is not required.

Regarding drug security wild type adenoviruses and replication incompetent adenovectors can be considered as extensively safe. The applied virus serotype AdV5 can cause respiratory tract infections. However in the majority of AdV infections in immunocompetent hosts no observable symptoms are present, so natural infections with adenoviruses are largely harmless in humans.

The most efficient administration method for the therapeutic adenovirus would be systemic injection. Unfortunately this injection method initiates on the other hand host immune responses caused by interactions of the AdV capsid with the host cells leading to shorter vector persistence and/or inadequate transgene expression. Despite all, for suicide gene therapy

these inflammatory responses may contribute to the treatment efficiency because immunogenicity might even intensify the elimination of tumour cells. Additionally, as already mentioned, systemic adenovector delivery is considered as the ultimate goal especially for metastasing cancer cells. However, for a long time it was not clear if the patient's immunogenicity reactions caused by repeated drug administration would reduce the vector's delivery efficiency. Li et al. could prove this theory wrong: In these studies multiple injections assays could yield much higher than single injection ones[61]; and others could affirm that multiple injections with small viral volumes result in a higher overall transduction efficiency than one single injection with a larger volume [3].

Another efficiency question raised about pre-existing antibodies. Thus, 97% of the human population already possess antibodies from previous exposure to wild type adenoviruses[50], the prevalence of special anti-AdV5 neutralizing antibodies is about 65% [3]. Nevertheless, the voiced concern that high presence of pre-existing antibodies may limit efficiency of AdV-based gene therapy is not borne out. In fact clinical trials substantiated that transgene expression is not precluded in patients with pre-existing immunity [62].

An other often mentioned problem associated with gene therapy is the lack of thorough understanding of biological mechanisms. The death of an 18-year-old volunteer in 1999, Jessie Gelsinger, in a human Phase I clinical trial designed as safety test of an E1/E4-deleted recombinant adenovirus vector for the treatment of patients with deficiencies in ornithrine transcarbamylase, raised much concern about adenovector-related toxicity. Little is known about the behaviour of the gene therapy delivery vehicles and the therapeutic transgenes in humans, so it is hard to anticipate unexpected pitfalls in experimental therapies. Jessie Gelsinger received the highest dose of the adenovector in the study and died four days later from an adenovirus vector-induced shock syndrome

resulting from a cytokine cascade and leading to multiorgan failure. Briefly a lethal response may occur in an individual treated with an "overdose" of recombinant adenoviruses administered [3], [10].
A further risk for E1-deleted replication-incompetent adenovirus production using 293 packaging cells, is that the virus genome is trans-complemented with the E1 region back to replication-competent, transgene eliminated wild-type viruses by homologous recombination with the frequency of 10^{-9} genome replications. The industry is trying to reduce this phenomenon by replacing the 293 cells with PER.C6 cells, human embryonic retinal-derived cells with precisely restricted DNA, which are said to avoid a significant overlap of cell and vector sequences [13].

The emerging question of ethicians concerns the fear if germs cells risk to be genetically altered unwittingly or will be even "enhanced" intentionally, changing forever the genetic makeup of the future generation. Actually, according to the "Weismann barrier", soma-to-germline feedback should be impossible, meaning that hereditary information moves only from genes to body cells but never in reverse. However, there are indications that the Weissman barrier could be breached which would enable infections of the germline without intending it by intrinsic somatic gene therapy [63]! All the more a selective gene delivery into tumour cells will be in demand in the near future.

Anyhow, gene delivery vectors still have to be improved for broader application fields. In early stage patients for instance, the cancer utilizes solely one or a few angiogenic factors for growth, but as the disease progresses the cancer cells attempt to recruit and secrete more angiogenic factors. Briefly, the more invasive the cancer is, the more likely it makes use of multiple oncogenic pathways. Therefore applying the present gene therapy drugs with selective vector targeting by one or limited pathways for late stage patients might fail. Moreover clinical studies have shown that a combinational therapy like conventional

chemotherapy or radiotherapy together with gene therapy greatly improves the potency of gene therapy vectors, indicating that this combinational therapy branch should be studied further [3].

6. Acknowledgments

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7. Appendix: Equipment and applied chemicals

7.1. Equipment

- PCR: Mastercycler; Epgradient
- DO-Photometer: BioPhotometer-Eppendorf
- Scanner: Molecular Dynamics, Fluor Imager 595
- Thermo cupboard: Scientific Johns
- FACS: Coulter EPICS^{Tm}XL-MCL flowcytometer, Beckman-coulter, Miami, FL
- Shaker: New Brunswick Scientific, Incubator Shaker
- Mini-Centrifuge: Biofuge pico, Heraeus (max 13.000rpm)
- Maxi-Centrifuge: Beckman J2-21-M, Induction Drive Centrifuge
- Ultracentrifuge: L*-70M Beckman, USA
- UV Light Microscope: Fotodyne Incorporated
- Escape: Canadien Cabinets
- Micropipettes (Eppendorf)

7.2. Solid chemicals

- Bromphenol blue (Sigma, St-Louis, Ca)
- Xylene cyanol (Biorad, Richmond, Ca)
- Triptone (BD, Le pont de claix, France)
- Yeast extract (Sigma, St-Louis, Ca)
- NaCl (Sigma, St-Louis, Ca)
- Agar (BD, Le pont de claix, France)
- Tris (MP biomedicals, Solon, OH)
- Boric acid (EMD, Gibbstown, NJ)
- EDTA (Sigma, St-Louis, Ca)
- Agarose (VWR international, West chester, PA)
- CsCl (J.T. Baker, Phillipsburg, NJ)

7.3. Liquid chemicals

- Ethanol (Commercial alcohols, Brampton, ON)
- Glycerol (EMD, Gibbstown, NJ)
- Isopropanol (Fisher scientific, Pittsburgh, PA)
- HCl (EM science, Darmstadt, Germany)
- DMEM: Dulbecco's Modified Eagle's Medium (Wisent, St-Bruno, QC)
- L-glutamine (Wisent, St-Bruno, QC)
- Fetal bovine serum (Hyclone, Logan, UT)
- PBS (Hyclone, Logan, UT)
- Trypsin (Hyclone, Logan, UT)
- Lipofectamine (Invitrogen, Carslbad, Ca)
- dNTP (GE Healthcare, little chalfont buckinghamshire, England)
- BSA (Sigma, St-Louis, Ca)
- Vista Green (Amersham Biosciences, Buckinghamshire, England)
- 1kb DNA Ladder (New England Biolabs)

- Paraformaldehyde (Sigma, St-Louis, Ca)

7.4. Antibiotics

- Hygromycin (Invitrogen, Carslbad, Ca)
- Phleomycin (Sigma, St-Louis, Ca)
- Ampicillin (Sigma, St-Louis, Ca)
- Kanamycin (Sigma, St-Louis, Ca)

7.5. Enzymes and Buffers

7.5.1. Polymerases and Enzyme of Dephosphorylation

- *Taq* (New England BioLabs)
- *PHS* (New England BioLabs)
- *PfuTurbo* (Stratagene)
- *CIP* (New England BioLabs)

7.5.2. Enzymes of digestion

- *Swa* I (New England BioLabs)
- *Srf* I (Stratagene)
- *Bsm* I (New England BioLabs)
- *Kpn* I (New England BioLabs)
- *Pac* I (New England BioLabs)
- *Eco* RI (New England BioLabs)
- *MUN* I (New England BioLabs)
- *PME* I (New England BioLabs)
- *Dpn* I (Stratagene)

7.5.3. Enzymes' Buffers

- Buffer 1 (New England BioLabs)
- Buffer 2 (New England BioLabs)
- Buffer 3 (New England BioLabs)
- Buffer 4 (New England BioLabs)
- *Taq* Buffer (New England BioLabs)
- *MUN* I Buffer (New England BioLabs)
- *PHS* Buffer (New England BioLabs)
- DMEM Buffer (New England BioLabs)

7.6. Kits

- Adeasy-Protocol (Qbiogene)
- PCR-purification (QIAGEN)
- Gel-extraction (QIAGEN)
- Mini-prep (QIAGEN)
- Maxi-prep (QIAGEN)
- DNA-mutation (QuickChange^{Tm}, Stratagene, Cedar Creek TX)
- illustra NAP-25 Column (GE Healthcare, Little Chalfont, UK)

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