Combinatorial engineering

of adeno associated virus vectors

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Die Natur kennt keine Probleme, nur Lösungen. Carl Amery

Für meine Familie

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1 Abstract

The use of viral vectors as delivery vehicles for therapeutic genes (gene therapy) is being investigated and improved since two decades. Despite exciting advances and promising results in animal studies and clinical trials, technical refinements are still needed to improve efficiency and safety of gene transfer. One of the most popular vector types derives from adeno associated virus of type 2 (AAV2). This virus has attracted the interest of gene therapists being safe, easy to produce at high titers and offering the possibility to target a plethora of cell types and to maintain detectable levels of transgene expression for long times. However, two major limitations hamper the use of this vector type. First, the broad tropism of AAV means that vectors are rather unspecific and therefore likely to infect neighboring tissues. Second, high seroprevalence in human populations of antibodies directed against wtAAV2 capsids reduces or abolishes transduction rates in individuals with pre-existing immunity.

The goal of my work was to address these two major issues and develop new methods to engineer AAV capsid variants with increased target selectivity and decreased antibody recognition.

Within a first project, I established for the first time a straight-forward *in vitro* protocol for the selection of viral mutants with increased selectivity for a melanoma cell line (BLM) out of a combinatorial viral library. The selection procedure consisted of a negative selection step on non-target cells and a positive selection step on BLM cells. Selected mutants showed almost wt levels of infectivity on BLM cells while transducing several tested non-target cells with a significantly lower efficiency, resulting in a up to 3.7-fold increase of target specificity.

Within the second project I generated a novel AAV2 capsid library by randomizing five amino acid residues that had been previously reported to play a role in antibody docking to the viral shell. Screening this library for particles that remained infectious despite pre-incubation with neutralizing antibodies allowed the isolation of several capsid variants that remained highly infectious even in the presence of antibody concentrations that completely neutralize wtAAV2.

2 Zusammenfassung

Die Verwendung viraler Vektoren als Genfähren zum Transfer therapeutische Gene in menschliche Zellen wird seit über zwei Jahrzehnten erforscht. Trotz aufregender Neuerungen und vielversprechender Ergebnisse aktueller Tierexperimente und klinischer Studien muss die Sicherheit und Effektivität dieser neuen Technologie noch weiter verfeinert werden. Das Adeno-assoziierte Virus vom Serotyp 2 (AAV2), ein sicheres, effektiv herstellbares Virus ist einer der beliebtesten Vektoren zum Einsatz in der Gentherapie, da es eine stabile Genexpression in einem breiten Spektrum verschiedener Zelltypen über lange Zeit ermöglicht.

Zwei Nachteile behindern den Einsatz dieses Virus jedoch in besonderem Maße: Auf der einen Seite zieht der breite Tropismus bei systemischer Gabe die unspezifische Transduktion von Geweben nach sich, die nicht infiziert werden sollen. Zum Zweiten ist ein Großteil der Menschheit schon unbemerkt mit dem Virus in Kontakt gekommen und verfügt über neutralisierende Antikörper, welche die Transduktion der Zielzellen durch die Genfähren verhindern.

Ziel der beiden Projekte meiner Arbeit war die Entwicklung neuer kombinatorischer Strategien um die oben genannten Einschränkungen zu beseitigen.

In einem Projekt wurde ein in-vitro Protokoll entwickelt, mit dem Virusmutanten mit gesteigerter Spezifität für eine Melanomzelllinie (BLM) aus einer kombinatorischen „Viruslibrary“ selektiert werden konnten. Das Protokoll besteht aus einem Negativselektionsschritt auf Fibroblasten und einem anschließenden Positivselektionsschritt auf BLM-Zellen. Die selektierten Mutanten zeigten mit Wildtyp vergleichbare Infektionslevel auf BLM-Zellen und signifikant herabgesetzte Transduktion der Fibroblasten und weiterer Nicht-Zielzellen.

Im zweiten Projekt habe ich eine neuartige „AAV2-Kapsidlibrary“ generiert, in der fünf zuvor als wichtig für Antikörperbindung charakterisierte Aminosäurepositionen vollständig randomisiert wurden. Nach vier Selektionsrunden mit neutralisierendem Serum konnte ich mehrere Mutanten isolieren, die trotz Anwesenheit von Serumkonzentrationen infektiös waren, welche den Wildtyp vollständig neutralisierten.

3 Introduction

3.1 Human gene therapy

Delivery of therapeutic genes to diseased tissues (gene therapy) holds the potential to heal a broad range of genetic and non-genetic pathologies by correcting genetic defects or by controlling the cellular metabolism by regulating gene expression. Different methods to bring foreign DNA into human cells ex vivo or *in vivo* are being developed. These techniques can be divided in two main groups. Non-viral methods exploit the possibility to deliver naked DNA/RNA or to couple this genetic information with different compounds (e.g. coating the nucleic acid with polyethylenglycole or lipofectamine). The other possibility is to use viruses as delivery vehicles. Until today, more than 1340 clinical trials have been conducted worldwide (Figure 1), aiming at delivering a plethora of different genes to address a wide variety of diseases.

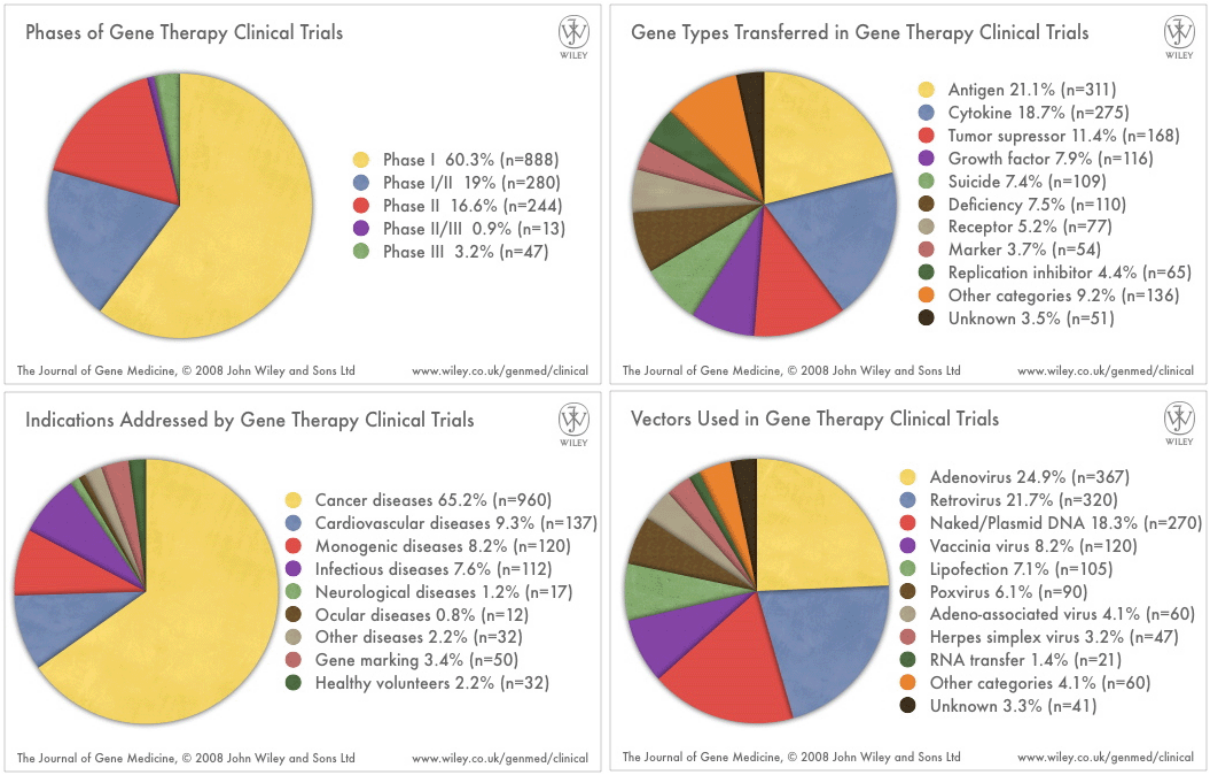

Figure 1: Overview over clinical trials with gene therapy vectors *(taken from http://www.wiley.co.uk/genetherapy/clinical/)*

Over 65% of clinical trials worldwide use viral vectors as vehicles. Natural viruses have evolved to deliver their genetic cargo into host cells and these characteristics can be exploited to deliver therapeutic genetic material in gene therapy approaches. However, before they can

be successfully employed, natural viruses need to be adapted to medical usage. In particular, two aspects are fundamental: safety and efficacy.

In 1999, Jesse Gelsinger, an 18 years old Ornithine Transcarbamylase (OTC) deficiency patient died in Pennsylvania, USA, after treatment with a high dosage of adenoviral vector containing a normal copy of the OTC gene. The following investigation allowed to conclude that the subministred vector triggered a severe immune reaction leading to multiple organ system failure (Somia and Verma 2000).

In 2002, a retroviral vector containing the common γ-chain gene for the cytokine receptors IL-2R, IL-4R, IL-7R, IL-9R and IL-15R was inoculated to ten X-linked severe combined immunodeficiency (X-SCID) patients. X-SCID normally leads to death within the first year of life. More than seven years later, 3 out of 11 patients have developed T-cell Leukemia as a consequence of the vector administration. One of them died of the cancer. The others live without SCID symptoms, two of them still fighting against leukemia (Cavazzana-Calvo et al. 2005; Cavazzana-Calvo and Fischer 2007). This example emphasizes the promises but also the challenges of this technology.

Advances in understanding diseases at molecular level and a better understanding of strengths and weaknesses of viral vectors is improving success rates of gene therapy protocols. Since the issues in this context are peculiar for each different vector type, the following paragraphs will focus on summarizing the relevant aspects with regard to the viral vector that has been the subject of my work: the adeno associated virus (AAV).

3.2 Adeno associated virus

3.2.1 Classification of adeno associated viruses

Adeno associated viruses (AAV) are members of the family of the *parvoviridae*. These non-enveloped viruses consist of a small icosahedral capsid with a diameter of 18-26 nm. The capsid contains a single-stranded DNA genome of approximately 4.7 kilobases. Parvoviruses can be divided into vertebrate- and insect-infecting viruses, classified as parvovirinae and densovirinae respectively. Human parvoviruses are not known to cause any disease except for erythrovirus B19 (Brown 2000) that was isolated by J. R. Pattison in the 1970s and can cause erythema infectiousum, hydrops fetalis and abortion. The first isolate of AAV was identified as contaminant in a simian adenovirus 15 preparation and therefore named adeno associated

virus. To date, thirteen other serotypes and more than 100 variants have been isolated in humans, other primates, rodents, or invertebrates. The serotypes have different levels of sequence homology causing differences in host and cell tropism and recognition by antibodies (Lukashov and Goudsmit 2001; Gao et al. 2002; Calcedo et al. 2009). In addition, AAV is classified in the genus of dependoviruses and in contrast to autonomous parvoviruses it requires for replication external helper functions that can be supplied by other viruses (e.g. adenovirus, herpes simplex virus, vaccine virus, human cytomegalovirus or papilloma virus) or by physical or chemical factors (e.g. UV- or γ-radiation, heat shock, cancerogenic compounds) (Atchison, Casto et al. 1965; (Richardson and Westphal 1981; Schlehofer et al. 1986; Yalkinoglu et al. 1991). AAV2, the best analyzed serotype, was found in an adenovirus 12 contamination (Hoggan et al. 1966). Its atomic structure is resolved to three angstrom (Xie et al. 2002) (see 3.2.3).

3.2.2 Genome organization of AAV2

In 1982, the complete nucleotide sequence of AAV2 was determined (Srivastava et al. 1983). The single stranded DNA genome consists of 4675 base pairs (bp) coding for two open reading frames (ORFs). The *cap* gene codes for three structural proteins that build the viral shell while the *rep* gene encodes four non-structural proteins needed for replication and encapsidation of the viral genome. The ORFs are flanked by 145 bp long inverted terminal repeats (ITRs) that build a T-shaped hairpin structure at both ends of the viral genome and are used as origin of replication, as packaging signals and for the integration or excision of the viral DNA into the host genome (McLaughlin et al. 1988; Kotin et al. 1990)(see Figure 2).

The four multifunctional proteins Rep78, Rep68, Rep52 and Rep40 (named according to their molecular weight in kilodalton) are expressed under control of promoters p5 (Rep78; Rep68) and p19 (Rep 52; Rep 40), named after their position in the genome, which was divided into 100 map units. *Rep* expression results in four proteins, *Rep68* and *Rep40* being splice variants of their larger counterparts. All Rep proteins share helicase and ATPase activities, while Rep78 and Rep 68 also have site specific DNA-binding, and endonuclease activities (Im and Muzyczka 1990; Berns and Giraud 1996; Smith and Kotin 1998; Collaco et al. 2003). Rep72 and Rep68 contain nuclear localization signals essential for replication, transcription and site-specific integration into the host genome (Cassell and Weitzman 2004) while Rep 52 and Rep40 are responsible for helicase-dependent packaging of the viral genome into the preformed capsid through pores (Becerra et al. 1985; Wistuba et al. 1997; Dubielzig et al.

1999; King et al. 2001; Bleker et al. 2005). Rep proteins also control the splicing of cap and their own expression in the absence of helper effects by repressing p5 and p19 transcription (Pereira et al. 1997; Kronenberg et al. 2001; Qiu and Pintel 2002).

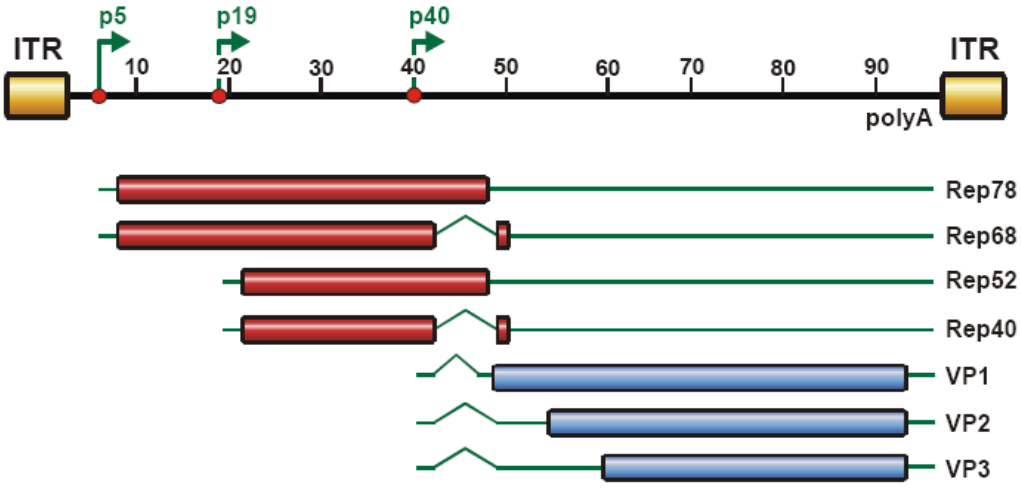

Figure 2: Organisation of the AAV2 genome and gene products. *Two ORFs rep and cap are expressed under the control of three promoters p5, p19 (rep) and p40 (cap) named after their map unit position in the 4675 bp genome that is divided into 100 map units. The ORFs are flanked by inverted terminal repeats . A shared poly adenylation signal at unit position 96 is shared by all transcripts, while use of alternative splicing, alternative promoters and alternative start codons results in four rep genes, responsible for replication and three cap genes that built up the viral shell. Open reading frames are shown in cylinders, untranslated regions as solid lines and introns as kinks.*

Three structural proteins VP1, VP2 and VP3 that share the same C-terminus are expressed from the *cap* ORF under the control of a promoter at map position 40 (p40). With respective molecular masses of 87, 72 and 62 kilodaltons (kDa), the three proteins are translated from two transcripts that arise from alternatively splicing of the cap gene. The longer one is translated into VP1, the largest of the three proteins that carries a phospholipase A2 domain at its N-terminus required for efficient infection (Zadori et al. 2001; Girod et al. 2002). VP2 and VP3 are translated from the shorter transcript from two separate start codons that supposedly have different translational activity. This gene architecture results in a VP1, 2 and 3 protein production at a 1:1:8 ratio respectively, which provides the appropriate proportion of capsid subunits (Becerra et al. 1988) to form a viral capsid of 60 subunits which is assembled with T = 1 icosahedral symmetry (Xie et al. 2002) by 5 VP1, 5 VP2 and 50 VP3 subunits. The numbering of amino acid positions in capsid proteins refers to the VP1 counting in the following.

3.2.3 Capsid structure

Recently the atomic structure of AAV-2 has been determined to 3 Å resolution by x-ray crystallography. Each viral capsid is composed of 60 subunits arranged with T=1 icosahedral symmetry of the three structural proteins VP1, VP2, and VP3 (Xie et al. 2002). They share overlapping sequences and differ only at their N-termini but these portions could not be resolved in the 3-D structures due to low electron density. Between the strands of a β-barrel core (Fig. 2) which is highly conserved among parvoviruses, large loop insertions are found that share only low similarity among the parvovirus family. These loops comprise two-thirds of the capsid structure and constitute the capsid surface features that interact with antibodies and cellular receptors.

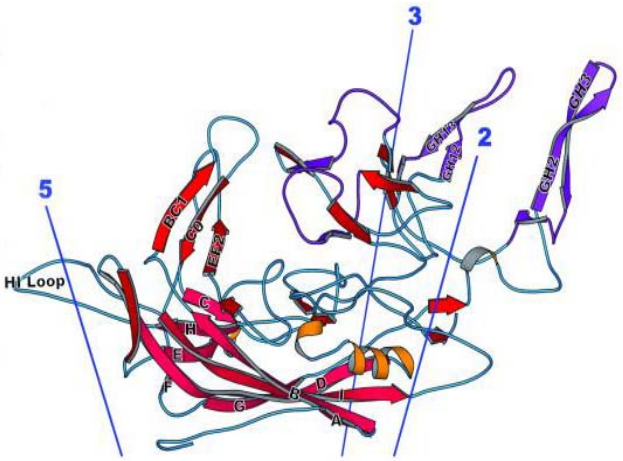

Fig. 2: 3D structure of the VP3 capsid protein (Xie et al. 2002). *See text for details. Blue numbers and lines indicate the symmetry axes.*

Three of these loops contribute to the formation of the 3-fold-proximal peaks, which cluster around the 3-fold symmetry axis. In the valleys separating the three peaks of one 3-fold axis, clusters of positive charges are located, which are implicated in receptor binding. Mutational analyses have identified these locations being involved in binding to the primary receptor of AAV-2 (Wu et al. 2000). The basic aa R487, R585, R588 and H509, which are at the side of the peak seem to play a crucial role (Wu et al. 2000; Grifman et al. 2001; Xie et al. 2002; Opie et al. 2003). Mapping of antibody binding epitopes (Wobus et al. 2000) (Huttner et al. 2003) have suggested the importance of the 3-fold proximal peaks not only in receptor binding, but also for the recognition of the viral particles by antibodies. Moreover, it is possible that other important viral functions are also located at this prominent feature.

Since the VP1 and VP2 regions of the capsid proteins are located within the lumen of the viral particle (Kronenberg et al. 2005), all virus:cell interactions that take place before entry and the interations between capsid and antibodies only depend on the surface of the VP3 protein.

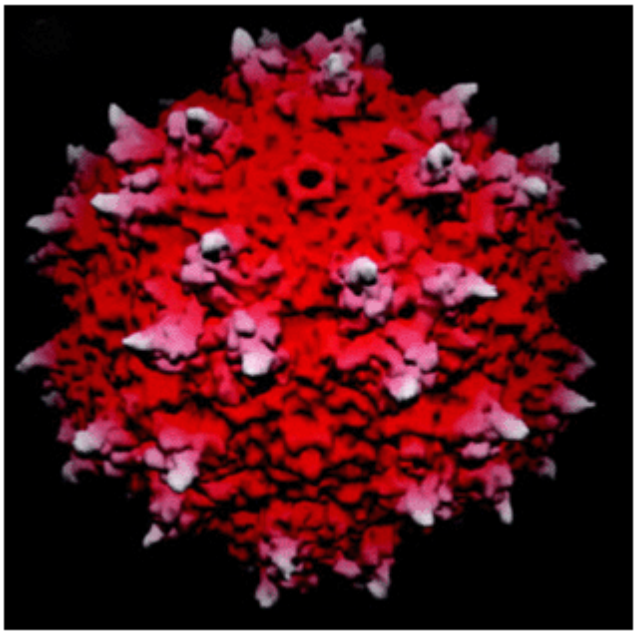

Figure 3: Surface topology of AAV-2 (Xie et al. 2002). *The capsid is colored according distance from the viral center.*

3.2.4 Infection biology of AAV2

Before AAV2 enters a cell, it contacts the cell surface several times and binds to its primary receptor heparan sulfate proteoglycan (HSPG) (Summerford and Samulski 1998; Seisenberger et al. 2001). Residues responsible for HSPG binding have been described to be R484; R487; K532; R585 and R589 (Kern et al. 2003; Opie et al. 2003). For efficient uptake, the virus must also interact with additional secondary receptors. Human fibroblast growth factor receptor 1, hepatocyte growth factor receptor, laminin receptor, the integrins $\alpha_v\beta_5$ or $\alpha_5\beta_1$ have been proposed as secondary receptors (Qing et al. 1999; Summerford et al. 1999; Kashiwakura et al. 2005; Akache et al. 2006; Asokan et al. 2006) but a role of other molecules cannot be excluded.

After binding its receptors, the virus can enter the cell by receptor-mediated, dynamin dependent endocytosis through clathrin coated pits (Duan et al. 1999; Bartlett et al. 2000). The exact events leading to internalization however are not completely understood. After moving along microtubules and microfilaments towards the nuclear area (Douar et al. 2001), AAV2 particles escape from endosomes after their acidification and accumulate in the perinuclear area (Bartlett et al. 2000). Rac1 and the PI3K pathway play an essential role in the efficiency of this step of infection (Sanlioglu et al. 2000).

It is not clear whether only genomes, protein-DNA complexes or whole particles of AAV2 enter the nucleus (Sanlioglu et al. 2000; Lux et al. 2005). Due to their small diameter, whole particles could enter through nuclear pore complexes (NPC) and an interaction of the viral capsid with a nuclear shuttle protein has been reported (Qiu and Brown 1999). However, NPC independent pathways have been suggested by other experimental data (Hansen et al. 2001). It is also important to note that different molecular pathways could be used for infection

depending on cell type, virus load, presence or absence of helper function and kind of helper function (Hansen et al. 2001; Xiao et al. 2002; Lux et al. 2005).

In the absence or helper effects, AAV2 converts its single stranded DNA into a double stranded form and integrates it into the host genome or stay in an episomal state. Integration occurs only in the presence of Rep proteins and mostly at the so called AAV site 1 (AAVS1) on chromosome 19 (Kotin et al. 1992; Nakai et al. 2001) which resides rep binding element and terminal resolution site sequences identical to those of AAV2 (Weitzman et al. 1994; Linden et al. 1996).

After co-infection with helper viruses or in the presence of a helper effect like chemical or physical stress of the cell, integrated provirus can be rescued and initiate a lytic life cycle (Berns and Giraud 1996). In this case, a double stranded genome is produced to induce gene expression and replication (Goncalves 2005).

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3.3 AAV as a gene therapy vector

3.3.1 Production of recombinant AAV2 vectors

In our days, the genomes of AAV2 and other serotypes are available on plasmids. In gutless vectors, the viral genome is replaced by a desired transgene of up to 6 kb, only flanked by viral ITRs that are needed for packaging and gene expression. Producer cells (e.g. HEK293, transgenic for adenoviral genes E1A, E1B) are transfected with plasmids providing AAV rep and cap genes plus additional helper functions. For production of viral libraries or cloning constructs of wtAAV2, the rep and cap genes must be flanked by ITRs. When gutless vectors are produced, only the transgene, added to the transfection mixture on an additional plasmid is flanked by ITRs (see Figure 4)(Laughlin et al. 1983; Rolling and Samulski 1995; Xiao et al. 1998; Collaco et al. 1999; Allen et al. 2000; Grimm et al. 2003).

The viral progeny can be harvested by freeze and thaw cycles and purified on affinity columns (e.g. heparin-columns), iodixanol- or CsCl- gradients (Hermens et al. 1999; Zolotukhin et al. 1999; Anderson et al. 2000). Preparation quality and yield can be quantified by quantitative polymerase chain reaction (qPCR) and enzyme linked immunosorbent assay (ELISA).

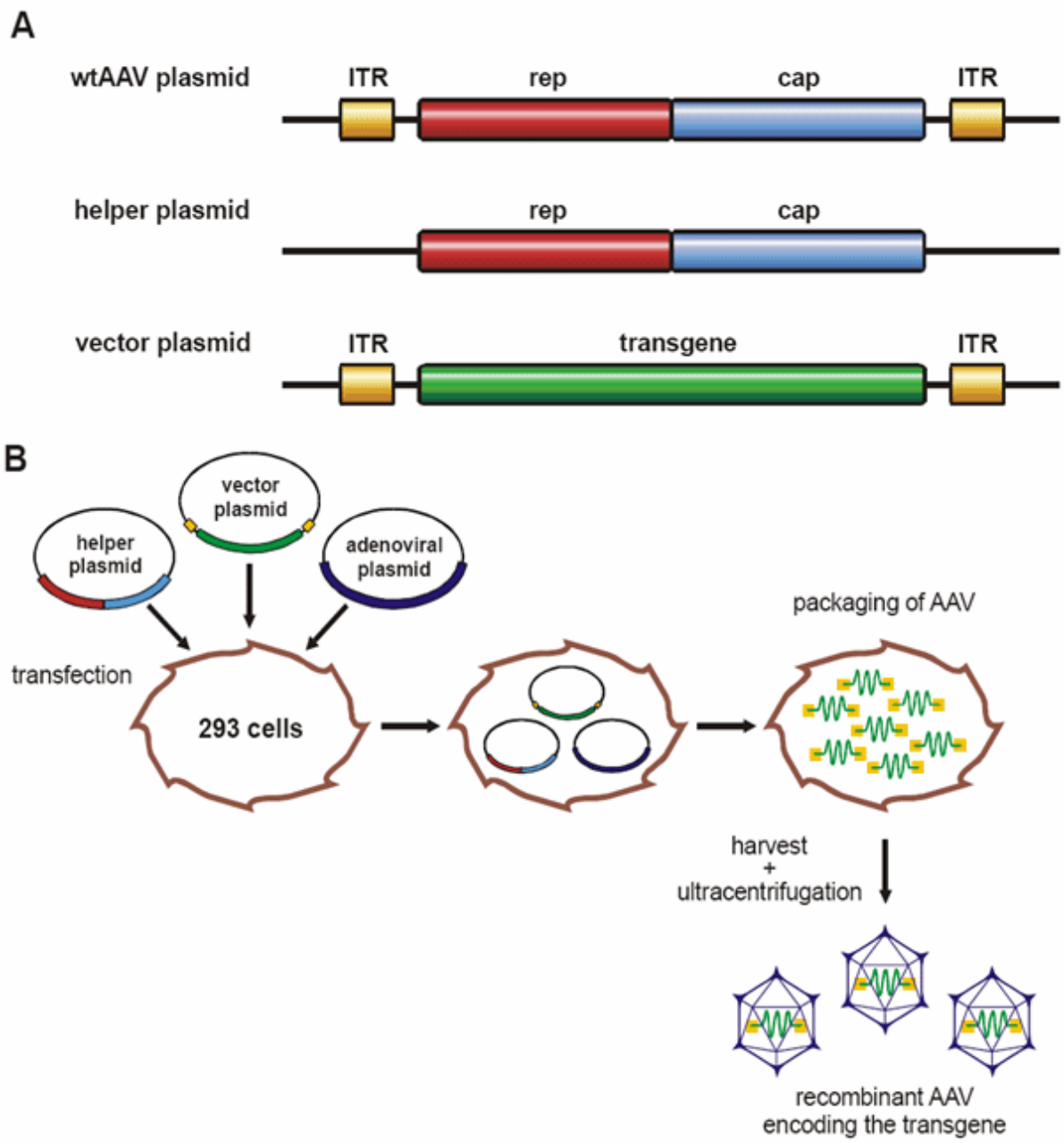


Figure 4: Production of adeno virus free rAAV vectors. *HEK293 cells are transfected with three plasmids carrying rep and cap genes without ITRs in trans on a helper plasmid, adenoviral helper functions on a adenoviral plasmid and the desired transgene flanked by ITRs on a vector plasmid. The virions package within 48 hours and can then be isolated and purified.*

Since second strand synthesis is a limiting step for gene expression, self-complementary genomes have been developed (McCarty et al. 2001), enhancing the expression rate *in vitro* and *in vivo* but limiting the size of the transgene to half the size. Other optimizations have been made to enhance purity and scale of AAV preparations during the last years (Blouin et al. 2004; Van Vliet et al. 2008).

3.3.2 Characteristics of AAV

Depending on the aims of gene therapy trials, requirements for a vector can differ. Therefore, it is hard to judge general advantages and disadvantages of some of AAV´s features. Anyway, I want to point out AAV-specific characteristics in this chapter to reveal its adequacy for gene therapy.

The first and most important requirement for a gene therapy vector is safety. No human disease could be related to AAV serotypes and the only known disease is caused by human parvoviral pathogene iserythrovirus B19 (Brown 2000). AAV has been used in over twenty clinical trials conducted to target a variety of diseases and to date no severe toxicity or immune reactions (see 3.3.3) have been associated with vector subministration. With helper virus free production methods and gutless vectors, AAV vectors are not contaminated with other viruses and cannot replicate themselves (Samulski et al. 1987). They integrate specifically at AAVS1 site *in vitro* if rep is given in trans (Huttner et al. 2003). Integration ath this site does not perturb host cell biology as it can be the case for retroviruses. However, recently some studies have reported occasional events of non-specific chromosomal integration. *In vivo*, stable, circular double-stranded episomes allocate the majority of wild-type AAV DNA (Kotin et al. 1992; Duan et al. 1998; Nakai et al. 2001; Miller et al. 2002; Philpott et al. 2002; Schnepp et al. 2005).

AAV vectors are capable of long term transduction of targeted tissues. Expression of vector transgene was detectable up to over six years after application in some cases (Romano 2005; Warrington and Herzog 2006).

Due to the availability and heterogeneity of different AAV serotypes and variants a broad range of target organs like brain, heart, muscle, liver and lung; dividing and non-dividing cells can be targeted (Flotte et al. 1993; Kaplitt et al. 1994; Podsakoff et al. 1994; Alexander et al. 1996; Kaplitt et al. 1996; Fisher et al. 1997; Manno et al. 2006).

The broad tropism of AAV vectors might be explained by the wide diffusion of their receptors (e.g. HSPG and integrins) on the surface of many cell types. While this allows the use of AAV vectors for a large number of therapeutic applications, it represents a disadvantage for applications requiring specific and selective transduction of target tissues. A typical example is cancer therapy with suicide genes, where unspecific transduction of neighboring tissues would cause severe collateral damage. In addition, unspecific vectors are dispersed in undesired organs and therefore higher doses of the vector must be applied.

Another limitation of AAV vectors is their packaging capacity of 5-6 kb. Recently, different techniques showed that larger transgenes can be split and delivered by so called "trans-splicing vectors" (Dong et al. 1996; Grieger and Samulski 2005; Ghosh et al. 2007).

3.3.3 Immune responses to AAV vectors

Up to 96% of humans are seropositive to AAV2 and up to 60% have neutralizing antibodies (Nabs) against the virus. The prevalence of Nabs is spread worldwide but depends on age, country, and ethnic group generally being highest in Africa for common serotypes (Erles et al. 1999; Calcedo et al. 2009). Although non-hazardous for the patient, neutralization of gene therapy vectors in the blood would prevent transduction of target cells or organs (Scallan et al. 2006; Lin et al. 2008). Some newly isolated, non-human viruses like AAV8 or AAVrh32.33, have a much lower prevalence of neutralizing antibodies (less than 10% in the human population), do not cross react with anti-AAV2 antibodies and have therefore attracted the interest of the scientific community as alternatives to circumvent pre-existing immunity (Gao et al. 2002; Scallan et al. 2006; Calcedo et al. 2009). However, not all serotypes are suitable for human gene therapy due to low infectivity or undesired tropism.

Animal studies also suggest, that even naïve patients would develop antibodies against any serotype or variant after a first treatment. Re-administration of AAV is therefore restricted to serotypes that do not cross-react with antibodies against the serotype used in the first treatment and that can provide sufficient transduction rates in the targeted tissue (Halbert et al. 2000; Moskalenko et al. 2000). For chronic diseases and long-time treatment, immunologic reactions can be suppressed efficiently for every consecutive treatment (Manning et al. 1998). This treatment however is cumbersome, uncomfortable and risky for the patient. Quarantine is required since infection with other pathogens during treatment can lead to serious complications without an intact immune system.

In contrast to other viruses, AAV has minimal inflammatory potential and does not trigger severe immune responses. AAV2 is able to infect dendritic cells but due to an unknown post-entry block, they are not able to efficiently activate a T-cell response (Zhang et al. 2000). The cellular immune response against AAV was disregarded for years until liver inflammation was observed in patients after AAV2 treatment (Manno et al. 2006). Because human is a natural host of AAV2 and liver damage was not described in mice, it was suggested that cellular immune response was triggered by former co-infections of the patients with wtAAV

and immune activating helper viruses that occurred incidentially before the begin of therapy (Mingozzi et al. 2007). However, cellular immune responses against the capsid have been reported in human and animal studies (Manno et al. 2006; Mingozzi et al. 2007; Vandenberghe and Wilson 2007; Wang et al. 2007).

Since gutless AAVs do not express viral capsid or replicative genes, activation of CD8+ T-cells after infection of cells and degradation of the capsid must occur through exogenous antigen presentation. It has been demonstrated that AAV-capsid specific T-cells do not lyse rAAV-infected cells (Li et al. 2007; Li et al. 2007; Wang et al. 2007). In addition, immunologic reactions against endogenous transgenes have not been reported, indicating that effective long-time expression can be reached with AAV vectors by immunosuppression during treatment until the clearance of vector capsid is complete.

3.3.4 Clinical trials with AAV vectors

Being considered a safe and efficient vector with long-time gene expression potential, AAV is being adopted in an increasing number of clinical trials aiming to cure a wide spectrum of diseases like Parkinson, Canavan`s disease, α1-antitrypsin deficiency, rheumatoid arthritis, cystic fibrosis and Hemophilia B (Goncalves 2005).

The first disease that was treated with AAV was a phase I study of patients with mild lung disease of cystic fibrosis transmembrane conductance regulator deficiency (Flotte et al. 1996). The deficiency leads to chronic inflammation and lung damage which lead to early death because of loss of respiratory function. Clinical phase I and II trials have been conducted today with promising outcome for the future but no therapeutically relevant long-time expression has been reported so far.

Interesting results are announced from trials concerning hemophelia B (blood factor IX deficiency), a monogenic defect leading to failure in blood clotting. Tested doses of administered vectors were not causing immunologic side effects but were also too low to raise serum factor IX levels above 1% of normal, the minimal concentration needed for effective blood clotting. Newer results show however that increasing vector doses and switching from intramuscular to intraportal administration leads to liver transduction and therapeutical relevant levels of factor IX expression but also to liver inflammation accompanied by decline of factor IX levels after eight weeks (Manno et al. 2006).

Similar results are reported from lung disease trials against α-1 antitrypsin deficiency (ATT). Transient gene expression was reported, but higher levels are needed for therapeutic treatment (Brantly et al. 2006).

In Leber congenital amaurosis (LCA), a severe retinal dystrophy causing blindness or severe visual impairment before the age of 1 year, AAV therapies have already achieved promising results. One form of the disease, LCA2, is caused by mutations in the retinal pigment epithelium-specific 65-kDa protein gene (RPE65). No severe side effects were diagnosed and patients reported increased sensitivity of sight after AAV-RPE65 treatment (Jacobson et al. 2006; Hauswirth et al. 2008; Maguire et al. 2008; Cai et al. 2009).

Lately, a patient treated with an AAV2 vector in a trial concerning rheumatoid arthritis died but the following investigation allowed to demonstrate that the death was not related to the administered vector but as consequences to anti-TNF therapy drug treatment that caused disseminated histoplasmosis in conjunction with retroperitneal hematoma instead (Kaiser 2007).

Anti-cancer gene therapies studies have not yielded broad success in the clinic. However, since being promising for future therapies, some of the general ideas are listed in the following paragraph. Anti-cancer gene therapies can be classified by different approaches used. Immunotherapies aim at enhancement of tumor cell recognition by the immune system. Malignant cells can be loaded *in vivo* or *ex vivo* with immunostimulatory molecules (e.g. tumor necrose factor α (TNF-α) or CD 40 ligand). Also vaccination of dendritic cells with tumor antigens or *ex vivo* transduction of T-cells with tumor specific T-cell receptors activate the immune system and increase recognition of tumor tissue (Nestle et al. 2005; Morgan et al. 2006; Aiuti et al. 2007; Alexander et al. 2007). Some naturally occurring oncolytic viruses and their laboratory derived relatives selectively replicate in cancer cells, causing cell death and infecting neighboring cells. However, non of the tested viruses was applicable systemically but reported effects were always local (Hu et al. 2006; Aiuti et al. 2007). Suicide gene therapy is a promising approach to effectively abolish tumor cells, especially for metastatic tumors that are hard to treat with irradiation, surgery or local drug application. The concept is to deliver suicide genes exclusively to tumor cells or tumor vascular system cells to directly kill the tumor cells or cut the tumor cells off from oxygen and nutrients with a prodrug that is applied systemically after infection of the target cells with the suicide gene (e.g. herpes simplex thymidine kinase (Hstk) and glanciclovir, a prodrug that becomes toxic

only after being metabolized by Hstk) (Plautz et al. 1991; Niculescu-Duvaz and Springer 2005; Alexander et al. 2007).

In summary, AAV clinical trials for several diseases are at a stage where safety has been proven and vector doses need to be adjusted to gain therapeutic relevant gene expression levels (Romano 2005; Warrington and Herzog 2006). Optimization of vector construction for effective targeting, immune escape and long time transgene expression of the transgene has to be refined and immune modulation during treatment considered.

3.4 Engineering of AAV vectors

The growing understanding of mechanisms underlying the infectious biology of the AAV capsid and its role in the infectious process have paved the way for efforts aiming at engineering viral particles at molecular level in order to tailor them with optimized characteristics required by the envisioned application.

To control packaging efficacy, host immune reactions against AAV, tropism, specificity, infectivity, genome size, intracellular trafficking and gene expression of AAV are major goals that would dramatically contribute to the breakthrough of AAV vectors in clinical routine.

Vector tropism depends on the surface architecture and chemical composition of the capsid surface. The structures presented to the external surface of the viral shell mediate interactions with the humoral immune system and with cellular receptors responsible for the binding and uptake of the particle into the cell. These structures can be chemically or genetically modified to modify the way they interact with the host environment. Viral particles can be engineered by two major approaches: rational approaches and evolutionary approaches.

3.4.1 Rational approaches

In a rational approach, scientists make an educated guess which amino acids or protein domains are to be changed in a defined way. Substitutions, insertions or deletions are chosen on purpose to yield a specific, intended phenotype of the generated mutants.

On the on hand, wtAAV vectors were coated with chemical reagents such as polyethylenglycol or biological molecules, e.g. bispecific antibodies or avidin linked ligands to change their tropism (Bartlett et al. 1999; Ponnazhagan et al. 2002). Although showing

altered tropism *in vitro*, critics argue that binding of coated reagents might not persist *in vivo* and attached molecules could interfere with post entry steps of the vector.

In rational genetic approaches, amino acids have been substituted or peptides have been inserted into the capsid. A single-chain antibody against human CD34 was genetically inserted at the N-terminal region of capsid proteins in an effort to target hematopoietic progenitor cells (Yang et al. 1998). In another case, both genetical modification and chemical coupling were combined: Ried et al. inserted an immunoglobulin (IgG) binding domain at position 587 of the capsid protein to enable rAAV to bind different antibodies via their Fc regions. The Fab region of bound IgGs thus remains free to function as a ligand directed against a specific cell surface receptor (Ried et al. 2002). *in vitro* results show that the tropism of AAV vectors could be expanded to non-permissive cell types by inserting a 14-amino-acid peptide, L14 (QAGTFALRGDNPQG), at position 587 of the AAV2 capsid protein (Girod et al. 1999). In a different approach, single or multiple amino acid residues on the surface of the capsid were substituted or deleted and the resulting phenotype was characterized. Mutation of two crucial arginines led to the disruption of AAV binding to its primary receptor HSPG and therefore detargeting of a number of cell types; other substitutions caused increased resistance against neutralizing serum (Opie et al. 2003; Lochrie et al. 2006). Combination of this detargeting and retargeting strategies were combined to create mutants with new tropism. AAV mutants with inserted RGD-4C peptide at positions 520 and 584 showed loss of Heparin-binding and altered tropism (Shi et al. 2006).

As an additional possibility, capsid proteins of different serotypes were mixed, leading to the formation of chimeric capsids with altered phenotype (Gigout et al. 2005; Wu et al. 2006).

A general drawback of rational design approaches is the unpredictable change of the nature of insertions after integration into the capsid. To build up a viable capsid, the VP protein structure has to fulfill numerous specifications; educated guesses in mutagenesis of the viral shell often leads to non-functional VP protein. Insertions tend to not function in the originally expected way, because the course of capsid assembly and interactions between essential structure domains are not well-enough understood.

3.4.2 Evolutionary approaches

Controversy to rational approaches, evolutionary techniques create a very large number of randomly mutagenised variants of a biomolecule and thereafter screen this “library” for beneficial mutations without trying to predict the specific changes needed for the desired

phenotype. The general advantage of this technique is that it does not require detailed understanding of relationship between protein structure and function. On the other hand, generated libraries must be complex enough to include beneficial mutants and screening methods must be stringent enough to identify the latter ones.

Evolutionary techniques have proven to be useful for protein optimization before being applied to viruses (Stemmer 1994; Vasserot et al. 2003). Directed evolution approaches for viral vectors are based on a general principle: the capsid surface is mutated randomly and large libraries of variants are generated. In this way, viral libraries are constructed carrying mutants with properties differing from those of naturally occurring serotypes or variants. The number of all serotypes and variants known until now, taken together is exceeded more than thousand-fold by the produced number of variants in one such library. Out of this large pool of mutants, beneficial variants can be selected using high-throughput screening methods even if specific knowledge of capsid biology is limited. Packaging-, infection- and replicative-defective or -poor mutants can easily be ruled out during selections and mutants with beneficial phenotype for the desired application can be selected in clonal purity. Different techniques that have been previously applied in the field of AAV vectors are shortly introduced in this paragraph.

Selection of novel peptides from phage libraries and subsequent insertion into AAV: tissue specific targeting peptides are identified by screening phage display libraries and subsequently inserted genetically in the capsid of AAV vectors (Nicklin et al. 2001; Work et al. 2005). Enhanced transduction of vascular endothelia cells was observed after insertion of a 7mer SIGYPLP peptide at position 587 (Nicklin et al. 2001). Grifman *et al.* have incorporated a tumor-targeting peptide previously identified from phage display into rAAV2, which successfully shifted vector tropism (Grifman et al. 2001). However, as for rational design insertions, peptides identified in different viral contexts often lose or abate biological function once inserted in the architectural context of AAV particles. Moreover, AAV virions can lose stability or functionality as consequence of the peptide insertion (Kwon and Schaffer 2008).

Insertion libraries: In this approach, randomized peptides are introduced at specific AAV capsid sites that have been previously proven to tolerate insertions in rational design studies (Girod et al. 1999). Site 587 was used by Perabo et al. to insert a random peptide library and select mutants with up to 100-fold infectivity on M-07e, a human megakaryocytic and a B-cell chronic lymphocytic leukemia cell line (Perabo et al. 2003). Human coronary artery endothelial cells were targeted in an analougous approach (Muller et al. 2003). These

insertion libraries can also be panned to select mutants with immune escaping phenotype (Muller et al. 2003; Perabo et al. 2003; Maheshri et al. 2006; Perabo et al. 2006). In these insertion library approaches, the selected peptides are optimized for functioning in the architectural environment of the AAV capsid.

Random mutagenesis libraries: Long stretches of the viral capsid can be randomly mutagenised, e.g. by error-prone PCR in order to introduce random point mutations. Point mutations were spread all over the viral capsid and immune escaping mutants were isolated out of such libraries (Perabo et al. 2006). These libraries also can be used to select mutants with altered receptor binding, tropism and transduction efficiency (Maheshri et al. 2006). Recently, such a library has been used for *in vivo* selection for tumor and lung targeting mutants (Michelfelder et al. 2009).

Shuffling libraries: Natural biodiversity among AAV serotypes can be exploited by applying shuffling protocols, to swap capsid domains between serotypes via homologous recombination and to generate chimeric capsids. This procedure was used to generate mutants with mixed or novel phenotypes. AAV2 and AAV3 capsids were shuffled together and subjected to a Marker rescue assay, which reports the restoration of gene function by replacement of a defective gene with a normal one by recombination, resulting in chimeric particles with new characteristics such as altered receptor binding and infectivity (Bowles et al. 2003; Hauck et al. 2003). Hauck and colleagues combined capsids of AAV1 and AAV2, obtaining a library they were able to isolate mutants with combined beneficial features of both serotypes out of. A clone containing capsid fragments of serotypes 1, 2, 8 and 9 was isolated from an integrin minus hamster melanoma cell line previously shown to have low permissiveness to AAV. The mutant showed increased transduction rates on this celline and decreased antibody recognition compared to wild type serotypes (Li et al. 2008).

All combinatorial methods can be transferred to other serotypes and vectors, enhancing their phenotypes for the desired purposes.

4 Results I

4.1 Aiming for selectivity

Application of viral vectors with wide and unspecific tropism in clinical applications may translate in dispersion of vector particles in non-target tissue and unleash side effects in unspecifically transduced organs. AAV2-derived vectors that carry suicide genes as transgenes for example can be used in anti cancer therapies only if they specifically target malignant tissue. Combinatorial techniques have already been used for selection of AAV2 capsid mutants targeting refractory cells (Perabo et al. 2003; Yang et al. 2009). Despite showing high transduction rates however, these mutants lacked target selectivity and no protocol for isolation of cell type-specific mutants has been described yet. For technical reasons, it is easier to broaden vector tropism instead of restricting it for more specificity (Buning et al. 2008; Perabo et al. 2008; Michelfelder 2009). Recently, *in vivo* selection protocols have yielded clones with increased selectivity by sceening AAV libraries in mice (Arap et al. 2002; Grimm et al. 2008; Yang et al. 2009) or phage display libraries and eventually incorporating selected peptides in AAV capsids (Work et al. 2006). Since *in vivo* selections in human are ethically not feasible, I aimed to develop a straight forward *in vitro* protocol for the isolation of target cell specific AAV2 mutants. To achieve this goal, I tested the possibility to subject the viral library to positive and negative selection rounds in order to identify clones that efficiently infect a cell type used in the positive selection and inefficiently the cell type used in the negative selection step.

4.1.1 Design of a directed evolution protocol and a viral insertion library

The selection protocol for the isolation of specific mutants from a viral library consists of two steps. In the first step the library is incubated with non-target cells to deplete from the pool mutants that binding to these cells (negative selection). Eventually, the supernatant is transferred to target cells for 48 h. In this second step, the remaining virions can infect the cells and replicate (positive selection). After this step the viral progeny is harvested lysating the infected cells. Repeating this procedure several times, the initial pool is progressively enriched with mutants that do not efficiently bind to non-target cells but efficiently infect and replicate in target cells. After several selection rounds, the biodiversity of the pool is reduced to a level that allows identification of predominant clones (Figure 5).

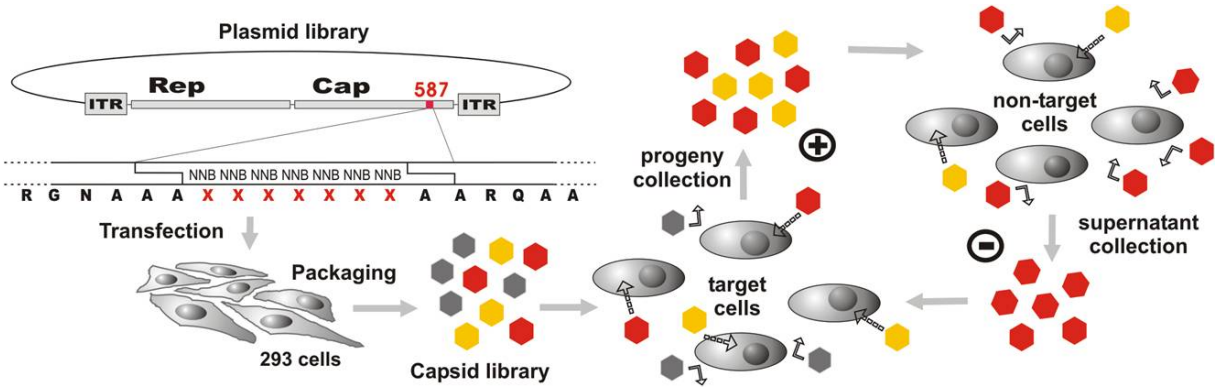


Figure 5: Scheme of the selection protocol. *The viral insertion library is produced in HEK 293 cells. To select BLM specific mutants, the library is incubated with non-target cells (fibroblasts) before the supernatant is used for the infection of target cells (BLM). 48 h post infection, viral progeny is collected, analyzed and eventually introduced to the next selection round.*

As a target cell I used BLM, a human melanoma cell line which is highly permissive for wtAAV2. For the establishment of the protocol I decided for a cell line because homogeneous cell populations and large amounts of material were needed. The use of primary melanoma material might have led to problems in homogeneity of samples and sufficient material supply.

To get rid of mutants that unspecifically infect BLM, a negative selection protocol was needed. I tested fibroblasts and keratinocytes for their ability to trap AAV particles. These cells naturally coexist with melanocytes in the same microenvironment in the body.

The viral library used in this project was obtained by genetic insertion of 7 random amino acids on a peak of the viral capsid protein at amino acid position 587 (VP1-numbering) (Perabo et al. 2003) (see Figure 6). Since being present on all sixty VP proteins that build up the capsid, also the insertion is present sixty times on each capsid.The pool contains approximately $4x10^6$ different clones, expressing each a different type of peptide at the insertion position. Since this position hosts the major receptor-binding site of the AAV2 capsid (Xie et al. 2002), and since it is well exposed on the surface of the viral particle, mutants carrying a 7-mere peptide inserted at this site, depending on the particular peptide should have an altered tropism compared to wtAAV2.

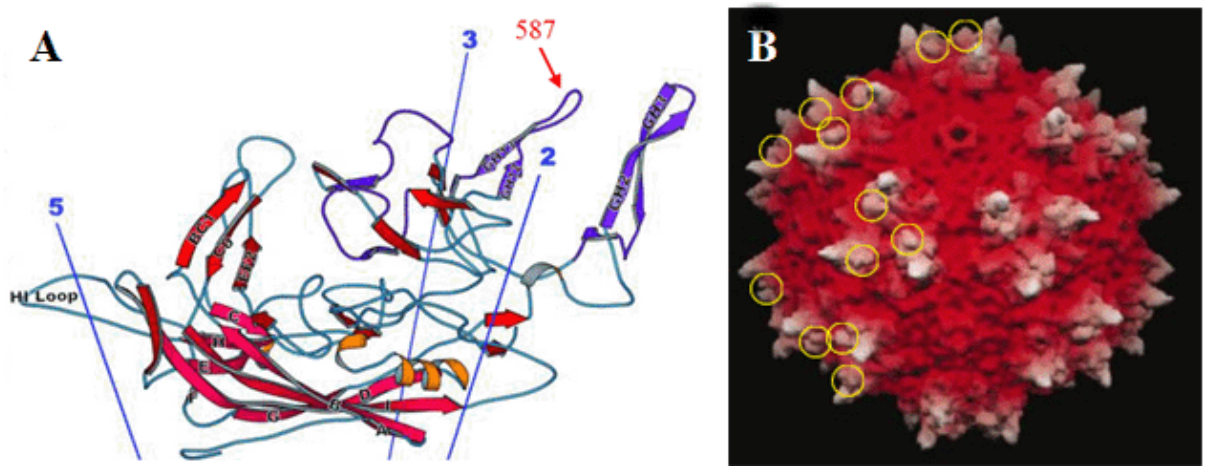


Figure 6A: 3D structure of the VP3 capsid protein *(Xie et al. 2002). The 587 site is indicated by the arrow.* ***B: 3D structure of the AAV2 capsid****, some of the 587 sites are indicated by yellow circles.*

4.1.2 Titration of adenovirus

AAV belongs to the dependovirus family, a group of viruses that need the expression of helper genes in the host cell for replication. Among the proteins that can provide this function, gene products of adenovirus are potent effectors and adenovirus has been the helper agent of choice in all AAV library screening procedure described so far. The amount of adenovirus to be used in each different selection procedure requires exact dosage. If the load of adenovirus is to low, the helper effect provided is sub-optimal and AAV replication will be reduced. Too much adenovirus causes cytolysis and therefore reduces the production of AAV particles. The optimal amount of adenovirus to support AAV replication in BLM cells was identified in a single titration experiment (Figure 7). HeLa cells were infected with 10^3 wtAAV2 particles per cell and increasing amounts of adenovirus. The total viral progeny was measured after 48 h by quantitative PCR (qPCR). The progeny titer increased proportionally to the amount of adenovirus from 0-1400 pfu/cell and reached the highest level ($3.6x10^{10}$ genomic particles) when an adenovirus titer of 2800 pfu/cell was used. Beyond this concentration the production of AAV particles decreased ($1.1x10^{10}$ genomic particles at 5600 pfu/cell). The MOI of 2800 pfu/cell was used for the below described selection protocols.

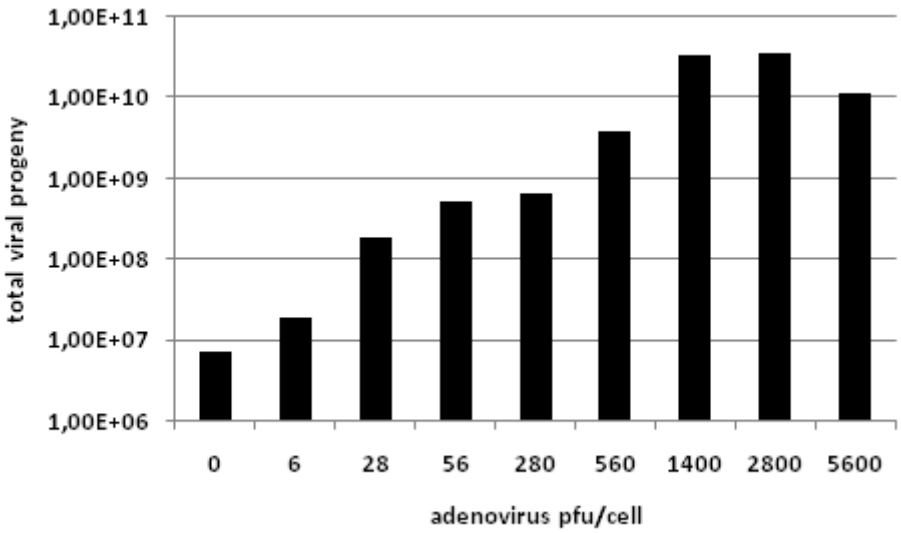


Figure 7: Determination of the optimal adenovirus concentration for the replication of AAV in BLM cells. *Cells were coinfected with same amounts of wtAAV2 and increasing amounts of adenovirus (plaque forming units per cell pfu/cell). AAV progeny was measured by qPCR 48 h post infection (single experiment).*

4.1.3 Adjusting selective pressure

To optimize the selection pressure needed for the negative selection, the decoy ability of primary human fibroblasts and primary human keratinocytes was tested. Fibroblasts effectively depleted viral progeny during the negative selection step. After the following positive selection step the relative viral progeny was more than 100x ($6.6x10^3$) lower as compared to no negative selection ($1.5x10^6$) or negative selection on keratinocytes ($1.2x10^6$) (Figure 8). The reason that prevented keratinocytes to deplete the viral pool was not further investigated. For the successive screening experiments we used exclusively human primary fibroblasts as cellular decoys.

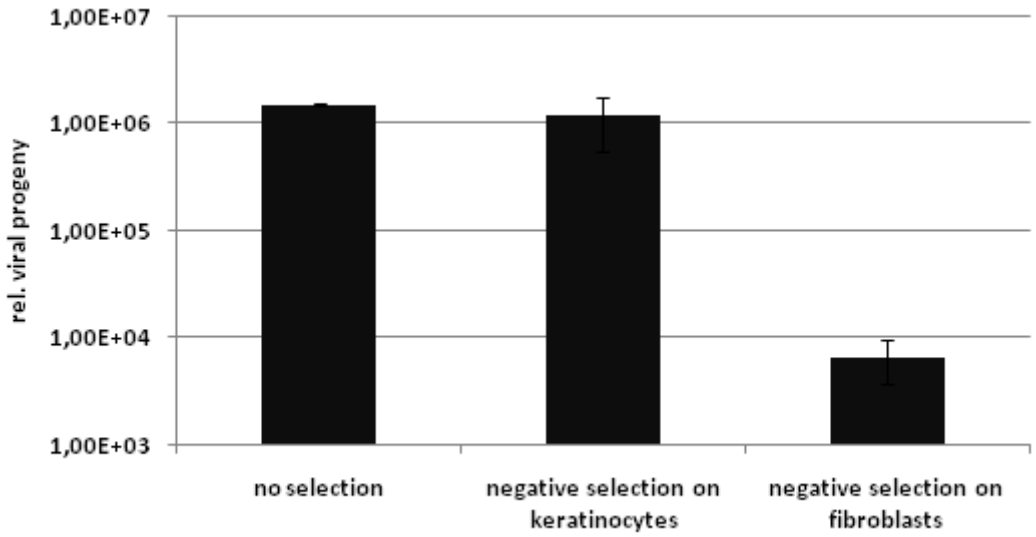


Figure 8: Decoy ability of keratinocytes and fibroblasts. *Same amounts of display library were incubated in 6-wells containing no cells, keratinocytes or fibroblasts. After two hours, the virus-containing medium was transferred to BLM cells. The viral progeny produced of BLM cells was measured by qPCR 48 h post infection.*

A crucial parameter for the success of this kind of selection procedures is the amount of AAV-library to be used. If the initial amount of virions is too low, biodiversity will be poor and the chance of the pool to contain interesting mutants decreases. Conversely, using too many virions per cell could lead to multiple infections of a single cell, resulting in the formation of chimeric particles and therefore in the uncoupling of genotype from phenotype in the viral progeny produced in these cells. The chimeric mutants that are produced in this case would impair the selection process. In addition, the negative selection capacity of cultured cells is limited: cells can only bind a finite number of virions, before surface receptors are oversaturated.

In order to determine the range in which the decoy effect of fibroblasts is effective, I measured the total viral progeny after a test-selection round (consisting of a negative successive a positive selection) either with or without the negative selection step, using different initial amounts of genomic particles (Figure 9).

Increasing the initial amount of AAV from 10^2 to 10^4 genomic particles per cell (GOI) resulted in increasing total viral outcome if no negative selection was carried out. In contrast, viral progeny after negative selection on fibroblasts was efficiently decoyed even when the highest amount of virus was applied. Input of more viral particles increases biodiversity and therefore the chance of the pool to contain interesting clones. Although the negative selection

potential was not yet saturated, no higher amounts of initial virus load were tested in order to avoid formation of chimeric particles that form at multiple infection of the same cell by different insertion mutants. The resulting uncoupling of genotype from phenotype impairs the selection process.

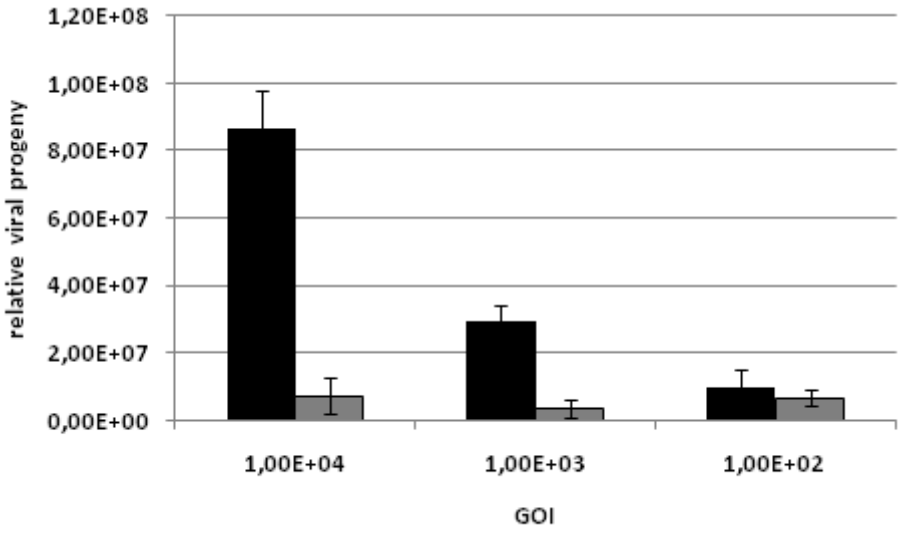


Figure 9: Decoy effect of Fibroblasts cells at different GOIs. *The viral library was amplified on BLM cells without (black bars) or with (grey bars) negative selection on human primary fibroblasts. Relative progeny titers were measured by quantitative PCR 48 h post infection.*

4.2 Selection of BLM specific mutants

To compare the effect of using different initial amounts of viral particles, two selection procedures were performed in parallel.

In one setting, a low number of particles (GOI = 100) was applied and three consecutive selection rounds were performed. Sequencing of the viral pools showed that one mutant G2 (carrying the insertion RSGQSLS) dominated the pool already after the first selection round accounting for more than 50% of the total number of clones. This mutant persisted in the following selection rounds. When tested in an infection assay it showed no increased specificity compared to wtAAV2 (see Table 1). This experiment suggested that the selection pressure had depleted the low biodiversity of the pool too sharply. In this conditions apparently, the evolution of the pool had the characteristics of a genetic drift instead that of a real selection, in which stochastic events led to the surviving of a mutant that showed no selective advantage.

In the second setting, four consecutive selection rounds (each consisting of a negative successive a positive selection) were performed using a GOI of 10^4. Evolution of the pools was monitored by qPCR and sequencing. Twelve clones of the first round´s progeny pool were sequenced to avoid preceding a selection with a pool in which one clone has already ruled out the others (as described above). All sequences appeared only once, suggesting that biodiversity was still high. The pool was amplified on BLM cells to cover the titer needed for the next cycle.

After the second selection round, sequencing of the pool showed that biodiversity was reduced. 12.5% of 24 sequences were represented by three mutants. Titers of the progeny for both initial GOIs were in a range of one log and had again more than doubled (3.2-fold; 2.1-fold), compared to the preceding selection round, indicating the increasing overall fitness of the pool.

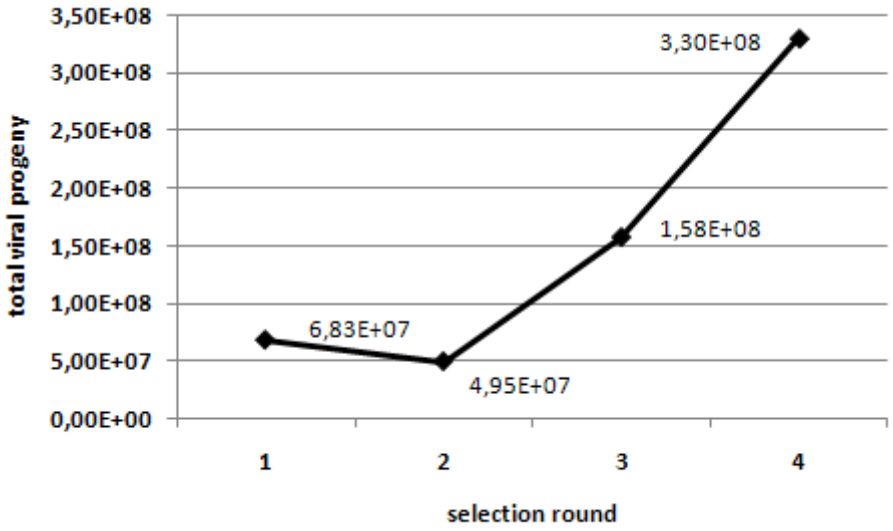


Figure 10: Selection procedure monitored by qPCR.

In order to analyze the pools for accumulated clones a total of 98 samples of all viral pools was sequenced. No redundant clone was found after the first selection round. The insertions found here did also not reappear in the sequences of later selection rounds suggesting that the biodiversity of the pool was still high.

In contrast to this, sequencing of at least 20 clones from each of the later selection rounds showed a gradual decrease in biodiversity and the appearance of clones in multiple copies. The redundancy of clones in the sequenced pools was 0% after the first round, 12.5% after the

second, 33% after round three and 66.7% after selection round 4. The observed increase in viral titers (despite the lower initial amounts of viral particles used in the last two selection rounds), and the concomitant decrease of biodiversity are strong indicators of a successful pressure-driven selection.

45% of the sequenced viral pool after the fourth selection round was composed of five clones (A7, C4, D8, D10, E10) that were packaged with GFP to further analyze their characteristics.

4.3 Characterization of selected mutants

4.3.1 Packaging capacity of selected clones

GFP-expressing vectors carrying the selected 587 capsid insertions were produced and analyzed for their specificity as described in materials and methods. An additional clone that was only found once in the pool after the fourth selection round (A5) was also characterized as an example of a mutant that was not strongly selected (see Table 1). Titers were measured via qPCR showing that mutants A5, C4, D10 and E10 packaged with efficiencies comparable to wtAAV2. Conversely, A7 and D8 viral preparations yielded genomic titers 103- and 211-fold lower than wtAAV2, respectively. This observation could be confirmed after repeating the viral packaging procedure in two additional experiments. Since these low titers allowed performance of infections only at GOIs that failed to yield detectable transduction rates on BLM cells (data not shown), mutants A7 and D8 were not further characterized. The low titer yield of these mutants can be due to ineffective packaging of the transgene or some other difference between selection and packaging that is disadvantageous for the packaging efficiency of A7 and D8. Mutants A5, C4, D10 and E10 were subjected to further characterization.

vector	amino acid sequence of insertion	% of sequenced clones	observed in selection round:	genomic particles/µl
wt	-			$(2.10 \pm 0.26) \times 10^8$
G2*	RSGQSLS	-	1	$(1.91 \pm 0.45) \times 10^8$
A5**	DAANNPR	1x	4	$(8.71 \pm 1.51) \times 10^7$
A7	NDPRKPS	10.5	2, 3, 4	$(2.04 \pm 0.15) \times 10^6$
C4	PRGTNGP	5.8	2, 3, 4	$(1.27 \pm 0.26) \times 10^8$
D8	AGKAGIG	15.1	3, 4	$(9.93 \pm 1.12) \times 10^5$
D10	SRGATTT	8.1	2, 3, 4	$(1.67 \pm 0.18) \times 10^8$
E10	DGSGPTR	5.8	2, 3, 4	$(8.88 \pm 2.64) \times 10^7$

Table 1: Characterization of selected mutants. *Titers were calculated as mean of 3 independent measurements.(*:G2 mutant was selected after using an initial GOI of 100;**: A5 mutant was only observed once in the last selection round)*

4.3.2 Selectivity assay

To estimate the selectivity of the mutans, target and non-target cells were infected with GFP-vectors. GFP is expressed only in the infected cells so that the percentage of transduced cells can be distinguished from non-infected ones by cytofluorimetry 48 h post infection (see Figure 11 A-E). To exclude that results were influenced by the particular amount of genomic particles per cell used in the experiment, triplicate experiments were performed at six different GOIs. For quantification of target cell specificity ratio, three GOIs were chosen that fulfilled the following requirements: more than 25% and less than 75% of BLM cells and more than 3% of fibroblasts had to be infected by the given GOI (marked in Figure 11 A-E by caskets). It turned out that the values of infectivity ratios in the GOI range identified by these parameters were similar (Figure 12) and the final ratio of infected BLM and fibroblast cells was calculated as mean of these three values for each mutant (Figure 11 F).

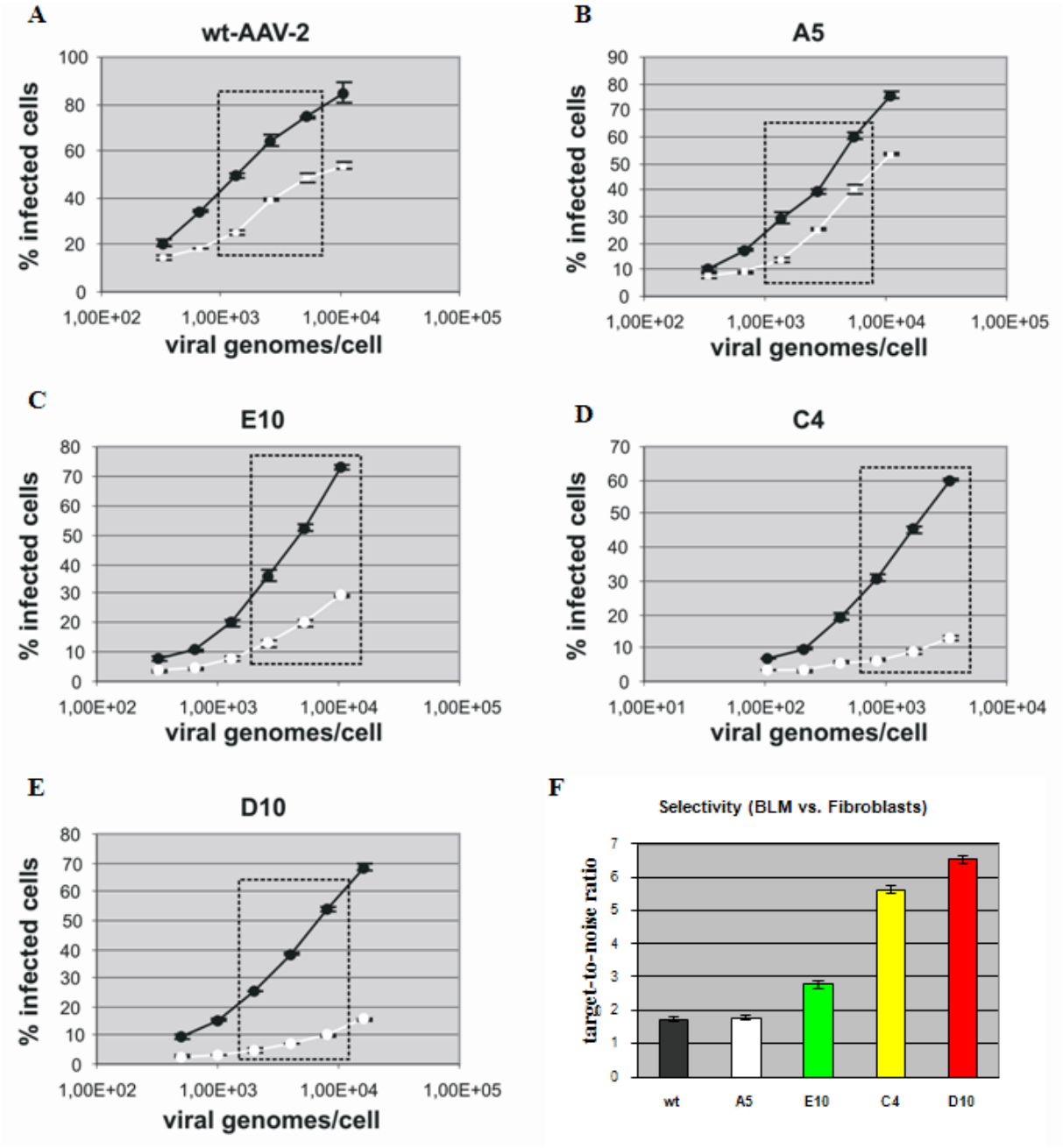


Figure 11: A-E: Transduction efficiencies of wtAAV2 and selected mutants on BLM cells (black) and human primary fibroblasts (white). *Infections were performed with six different multiplicities of infection (x-axis). Transduction rates were calculated as percentage of fluorescent cells. Values are given as average of three independent infection experiments, error bars represent standard deviation.* ***The ratios marked in the caskets were used to quantify specificity ratios of each mutant (F).***

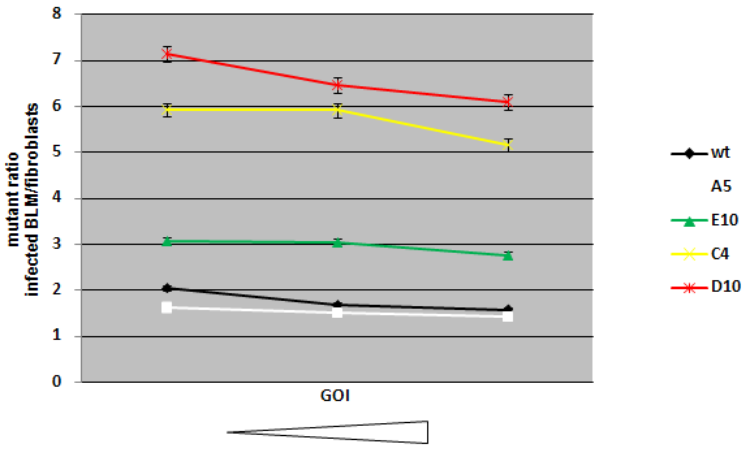


Figure 12 Ratios for all tested mutants of infectivity of BLM and fibroblast cells at three different GOIs that were used to quantify specificity.

The mutants´ infectivity ratios were 1.8, 1.8, 2.8, 5.7 and 6.6 for wtAAV2, mutants A5, E10, C4 and D10, respectively (Figure 12 F). Specificity of the mutants was calculated comparing mutants´ratios with those of wtAAV. The respective values are 1.0, 1.6, 3.2 and 3.7 times higher for A5, E10, C4 and D10 (Figure 13).

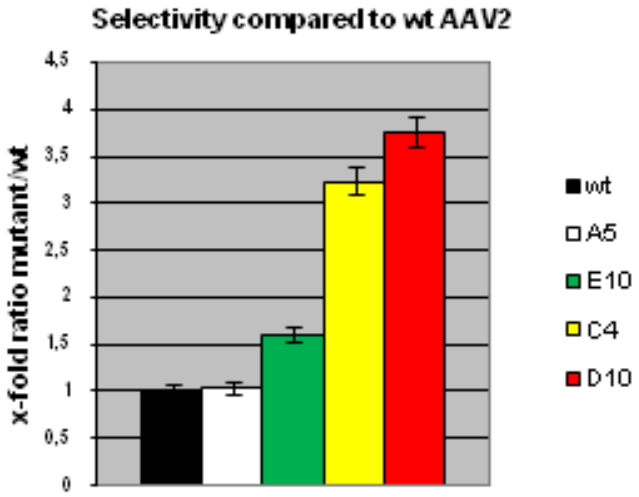


Figure 13: Selectivity of mutants on BLM cells and fibroblasts. *Infection ratios were quantified for wt and mutants and related to specificity of wtAAV2.*

These results indicate that a lower unwanted transduction of non-target cells occurs when using the mutants. This outcome is depicted in Figure 14 comparing directly the curves of D10 and wtAAV infection demonstrating that a virus dose that infects 54% of BLM cells

would collaterally infect 30% fibroblasts when using wtAAV2 but only 8% when using D10 mutant.

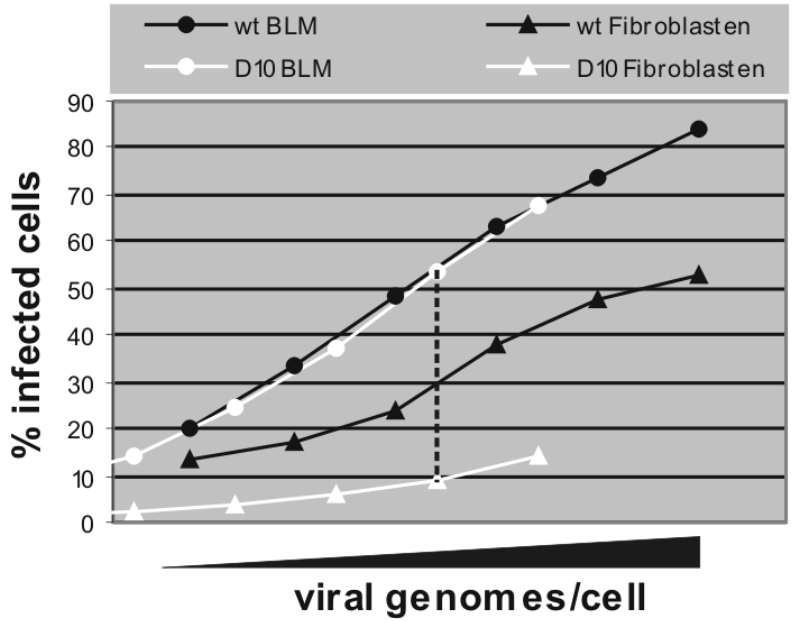


Figure 14: Comparison of transduction efficiencies between wtAAV2 (black) and mutant D10 (white) on BLM cells (circles) and human primary fibroblasts (triangles) with increasing amounts of virus.

4.3.3 Selected mutants are inhibited by heparin

To further characterize the mutants, their ability to interact with AAV2 primary receptor, heparan sulfate proteoglycan (HSPG) was tested. Heparin is the sugar content of HSPG and the soluble form of this molecule can bind to the viral capsid of wt AAV2 suppressing its ability to bind HSPG on the cellular surface in a competitive way. Since the randomized peptides are inserted in the HSPG-binding region of the capsid, depending on the amino acid composition of the peptide, the HSPG binding phenotype of the virions could be altered with important consequences for the tropism of the viral particles (Perabo et al. 2006).

To assess the ability of the selected clones to bind HSPG, GFP-vectors derived from wtAAV2 and from insertion mutants were incubated for 30 min with HeLa cells at a concentration of $6x10^3$ genomic particles per cell in the presence or absence of 85 I.E./ml heparin. 48 h later, the percentage of infected cells was analyzed by cytofluorimetry (Figure 15). All mutants were inhibited by heparin. wtAAV2 and A5 mutant were almost completely blocked (3.1%; 3.5% residual infectivity) whilst E10, C4 and D10 showed higher residual infection rates

(22.5%; 9.3% and 17.2%). The fact that mutants selected after four rounds are not inhibited by soluble heparin as efficiently as wtAAV2 and A5 suggests a reduced binding of these mutants to HSPG, which in turn could explain their reduced infectivity on fibroblasts and other cell types. Retention of BLM tropism could be explained by their ability to use the selected insertion peptide to bind alternative receptors.

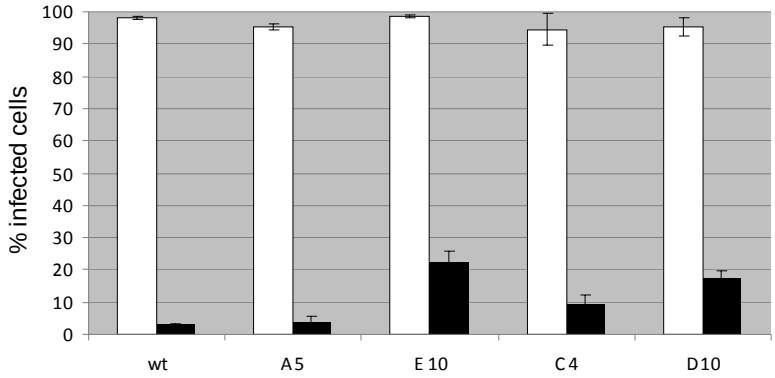


Figure 15: Transduction efficiencies of wtAAV2 and selected mutants in the presence (black bars) or absence (white bars) of soluble heparin. *The percentage of infected cells was quantified 48 h post infection via cytofluorimetry.*

4.3.4 Tropism for other cell types

In order to estimate the specificity of selected mutants with regard to other cell types (not used in the negative selection step), infection assays were conducted on primary keratinocytes, three primary melanoma cell cultures obtained from different patients, a breast cancer cell line and a murine embryonic fibroblast cell line (Figure 16). The BLM-specificity of the mutants was similar on primary fibroblasts (F), primary keratinocytes (K) and primary melanoma samples (M1-3), although some variation was observed. In particular, E10 reached 3-fold increase of BLM selectivity vs. M1 cells. C4 had higher selectivity values than D10 on murine fibroblasts (MF, showing a ratio of 2,8) keratinocytes (K, showing a ratio of 4.5) and breast cancer (B, showing a ration of 6,4, which compared to the 1.4 ratio of D10).

These data show that the BLM-selectivity of the selected mutants is not restricted to the cell type used in the negative selection but appears to regard a wide spectrum of other cell types as well.

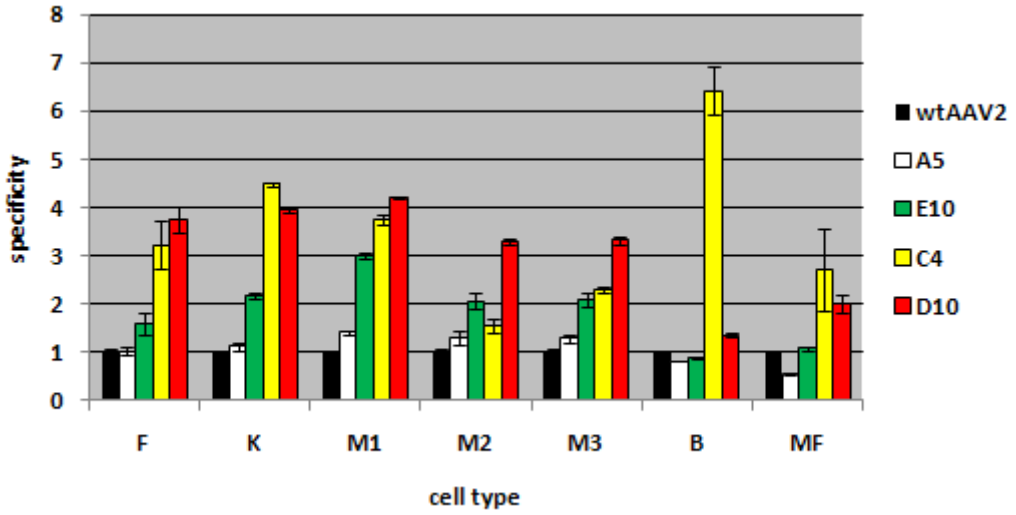


Figure 16: BLM selectivity of capsid mutants in comparison with human primary fibroblasts (F), human primary keratinocytes (K), human primary melanoma cells obtained from three different patients (M1-M3), human breast cancer cells (B) and murine fibroblast cells (MF).

5 Discussion I (Specificity selections)

One of the major problems for safe and efficient AAV-based gene therapy is the lack of tissue-specific targeting vectors. Although effective targeting to AAV non-permissive cells has been achieved by insertion of appropriated ligands in the wt capsid or direct selection from insertional targeting libraries (Girod et al. 1999; Muller et al. 2003; Perabo et al. 2003), these methods mostly generated vectors with extended tropism that however, lacked selectivity. This was due to targeting of widely expressed receptors and because of the existence of important receptor binding domains on the capsid, which remain functional despite the insertions.

I developed in this work an *in vitro* selection procedure that includes positive and negative selection steps for the first time. Applying the new selection protocol to an insertion library that was used to identify mutants with altered tropism but no specificity gain before allowed isolation of AAV2 insertion mutants with up to 3.7-fold increased specificity for the target cells.

Two of the selected clones (A7; D8) were recovered most frequently after the selection process but could not be produced with sufficient titers as GFP-expressing vectors although iterating three times the packaging procedure. The reason for this was not further investigated. One possibility is that the transgene used in my experiments (GFP) is not efficiently packing into these mutants. Another possibility is that these clones have a phenotype that confers a selective advantage during selections but is disadvantageous during the packaging procedure. It was reported before that capsid insertion mutants were not efficiently packaging (Yang et al. 1998). In future trials, mutants with these phenotypes could be ruled out by interposing a packaging step during selections.

Three mutants that were strongly selected (E10; C4 and D10) showed increased selectivity of 1.6, 3.2 and 3.7 times compared to the wildtype for BLM cells that were used for positive selections compared to fibroblast which served as decoys for the negative selections. Selectivity was not due to higher infectivity on target cells but rather to decreased transduction efficiency on non-target cells. A likely explanation of the observed tropism characteristics of the mutants is a reduction of their ability to bind to widely expressed membrane receptors caused by the peptide insertion. Instead, the inserted

peptide could confer the ability to recognize and bind a different receptor that is specifically expressed by BLM cells. Although the differences between wildtype and mutant tropism are not understood on the molecular level, this demonstrates that thanks to the negative selection step, the selection pressure applied in the overall screening procedure was driving the isolation of selective mutants and not of clones that are unspecific but very infectious.

Interestingly, although negative selection rounds were performed exclusively on human primary fibroblasts, primary melanoma cells from three patients and keratinocytes were also infected less efficiently by the three selected mutants than by wtAAV2. This result suggests that the change in viral tropism was exactly targeted towards BLM cells and not generically to melanoma cells. Selected virions interact with surface receptors that are expressed exclusively or more abundant on BLM. Although membrane receptor expression profiles of the tested primary melanoma samples were not analyzed, it is known that they differ between individual cases (Sekulic et al. 2008); so it is likely that differences in membrane receptors composition between BLM and primary cells accounted for the BLM-specificity of the selected mutants. A partial exception was mutant C4 which infected patient melanoma cells (M1) almost as efficient as BLM cells, suggesting that this capsid variant is able to recognize one or more receptors present on both cell types.

The results demonstrate the potential of combinatorial techniques to isolate cell type specific capsid mutants from viral insertion libraries and provide the first proof of principle that negative selection is a suited method to restrict viral tropism to a defined cell type, reducing collateral infection of other cell types.

Interestingly, the two most specific mutants identified in these experiments carry very different peptide insertions (C4: SRGATTT; D10: PRGTNGP) and accordingly show different tropisms. C4 for example, is less BLM-selective than D10 when compared on fibroblasts and primary melanoma cells but it is more selective on keratinocytes, breast cancer cells and murine fibroblasts compared to BLM. This suggests that the biodiversity present in the library and the described selection method can yield mutants that carrying different insertion, achieve the goal of increased specificity by targeting distinct receptors, although molecular mechanisms have to be resolved to proof that.

During the selection process, insertion peptides with similar sequences have accumulated leading to different variants that share analog properties. Remarkably, all 98 sequenced

mutants (including those from the original library) carry at least one positively charged amino acid in the insertion peptide and all of the selected mutants carry an arginine; C4 and D10, the most specific ones carry an overall positively charged XRGXXXX peptide. A positive charge seems to be essential for the virions to survive the selection procedure. The pattern of peptides showed no commonly known binding motive, but the XRGXXXX motive could mimic a binding motif of wtAAV2, that also carries an RG motif at position 585-586. This could on one hand preserve the tropism necessary to still successfully infect BLM cells, and on the other hand introduce enough architectural modifications to decrease affinity for receptors that allow infection of fibroblasts. Interestingly, this RG motif could be part of an unknown integrin binding motif. RGD peptides are well known binding motives for cellular integrins which are especially involved in the oncogenic transformation on many cancer cells (Eble and Haier 2006). Noteworthy in this respect, RGD motives were selected in previously described panning procedure of the same library used here (Perabo et al. 2003). Exploiting the full potential of peptide variability, directed evolution strategies might allow finding new binding motives that are either unknown or do not exist in nature.

The A5 mutant was not significantly differing in phenotype from the wt virus, showing that unspecific mutants remained in the pool even after four selection rounds. The insertion of A5 seems to be neutral for the tested phenotypic characteristics, showing that it is important to optimize the selection protocol in order to enable clones with beneficial phenotype to prevail against neutral clones. To reach this aim, viral pools could be passaged multiple times over non-target cells before proceeding to the next selection step. This technique could also be used to apply more complex negative selection pressure and even more specific selection by using multiple distinct non-target cells. Otherwise, the results show that the mutants tropism was restricted specifically to BLM and they were not efficiently infecting primary melanoma samples. This is also a hint that therapy vectors e.g. for melanoma had to be selected for each individual case of disease since the cell surface characteristics e.g. on melanoma samples can be different from case to case (Sekulic et al. 2008).

Since selected mutants maintained their heparin binding abilities and since secondary receptor binding domains of the capsid might be located apart from the library insertion site, the pool screened in these experiments might have been biased with mutants that mostly retain their natural tropism, limiting the potential for discovery of selective clones.

Combining the insertion of randomized peptides with mutations aimed at disrupting natural receptor binding domains could push the selection towards the isolation of more selective mutants that use alternative cell entry routes.

The here described mutants were selected an tested in an *in vitro* system and have not been tested *in vivo*. In the future selected mutants will be tested in a BLM mouse model. This will allow the characterization of selectivity in a situation where the mutants are challenged by a higher number of different cell types at the same time, but with the addition that this model system is not directly transferrable to human. Recently, *in vivo* selections in mice have been applied using a peptide insertion library. However, selected mutants were not specific (Michelfelder et al. 2009). The results of *in vivo* selections in mice can not automatically be transferred to the human system. Since humans are AAV2´s natural host, animal models

Since different cell types could be targeted by similar methods and the protocol can be adapted to other viral platforms, these results open new perspectives for the engineering of patient- and cell-specific gene therapy vectors. However, to engineer a selective vector suitable for *in vivo* application, detargeting and targeting strategies can be combined. The mutation of more than one site might be required to generate a mutant with all desired characteristics.

6 Results II

6.1 Aiming for antibody evasion

A major issue in the development of viral vectors for human gene therapy concerns the host´s immune response to the virus. AAV2 derived vectors do not trigger severe immune responses in humans (Warrington and Herzog 2006). Recent surveys however have shown that cellular immune response mechanisms can induce mild liver inflammation and lead to shut off transgene expression (Manno et al. 2006). Additionally, depending on ethnic group and age up to 80% of individuals have been naturally infected by AAV2 and carry antibodies that can neutralize virus or vector particles, reducing or completely abolishing the therapeutic efficacy of gene transfer (Calcedo et al. 2009).

For chronic diseases and long-time treatment, immunologic reactions can be suppressed efficiently for every consecutive treatment (Manning et al. 1998). This treatment however is cumbersome, uncomfortable and risky for the patient. An alternative to immune suppression of the patient during treatment is the use of vectors that are not recognized by pre-existing antibodies. The discovery of a large number of natural AAV variants has provided a large spectrum of capsid alternatives that can be employed to circumvent neutralization by the immune system. However, due to marked differences in capsid composition, these serotypes substantially differ from AAV2 tropism, and are often not suited for use in therapeutic application requiring AAV2 like tropism and infectivity rates. Furthermore, patients treated with this alternative serotypes would likely produce neutralizing antibodies after the first application (Halbert et al. 2000).

For these reasons, immune escaping vectors are of great interest for clinical application since they could supersede immunosupression and offer the possibility to treat patients with lower vector doses. An attractive idea is to engineer novel variants that escape neutralization from pre-existing antibodies but maintain the desired tropism. Even if these variants would also trigger the production of neutralizing antibody after their application, the availability of a pool of *ad hoc* engineered immune-escaping capsid alternatives would provide the possibility to successfully administrate therapeutic vectors for consecutive treatments.

Genetic engineering by rational design or directed evolution has been recently used to generate capsids that escape antibody neutralization and has led to identify several amino

acid residues of the capsid proteins that can be mutated in order to decrease antibody recognition. Despite providing an important proof of principle however, near-to-physiological range concentrations of antibodies could still neutralize transduction of these vectors (Lochrie et al. 2006; Maheshri et al. 2006; Perabo et al. 2006; Perabo et al. 2006).

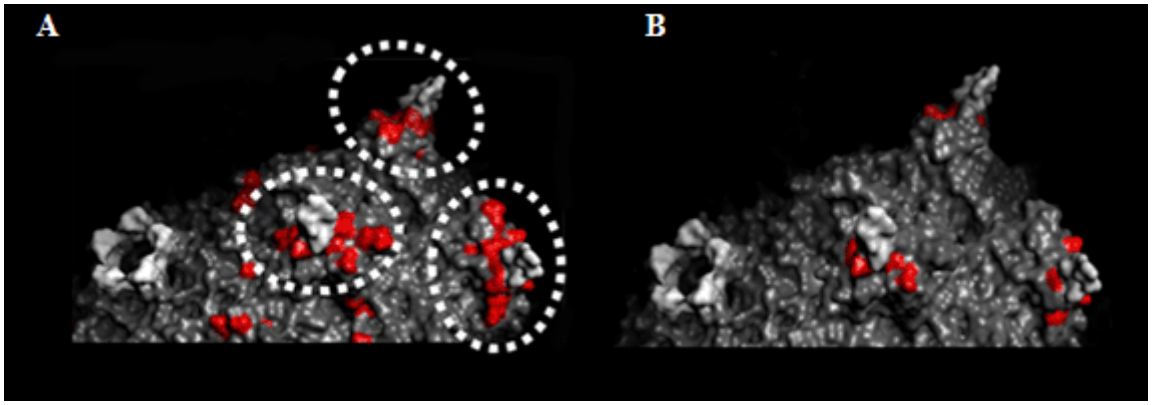


Figure 17 A: Described positions of capsid mutations that yield in an immune escape phenotype (red). *White circles mark the clustering of beneficial mutations around the three-fold symmetry spike of the viral capsid.* ***B: Positions chosen for the pdegen5Lib library.***

To optimize the protocol, all relevant data published to date was analyzed. These data can be summarized in Figure 17 A, depicting approximately 20 positions which - if mutated yield in immune-escaping capsid variants. The positions cluster on the 3-fold symmetry spikes of the AAV2 capsid. All trials conducted so far used labor-intensive cloning strategies, educated guesses or combinatorial techniques in which large capsid regions were mutated randomly. We hypothesized that the limitations of former trials was mainly due to two reasons. First, a complete immune-escaping phenotype requires combination of more antigenic domains concomitantly. Combinatorial libraries screened in these studies have been obtained by capsid randomization by error prone PCR. Since amino acid substitutions are likely to generate defective particles, the mutation rate has to be kept relatively low at each selection round to avoid excessive modification of the capsid topology. This results in a slow, progressive selection of mutations that requires multiple re-iterations in order to combine beneficial mutations. Even if DNA shuffling has been used in the study of (Perabo et al. 2006) to accelerate accumulation of mutations, it is possible that the selection rounds were stopped before optimal mutants had been generated. Another possibility is that the amino acids used to substitute the original residues at the antigenic sites were not the most appropriate to confer a stealth phenotype. The effect of

the kind of amino acids substitutions of selected immune escape variants on the phenotype was never further analyzed. However, since various amino acids have different chemical and structural properties, also their effect on a crystal-like structure like the viral capsid protein should differ. My working hypothesis was that substitutions of similar amino acids on unimportant sites show no or low phenotypic effect compared to exchanges at positions relevant for antibody recognition and substitution with more distinct amino acids. In an ideal approach, all beneficial positions described in former trials should be substituted. However, a complete randomization of twenty amino acids would require the availability of a library with 20^{20} different clones, which is technically impossible to obtain. Currently, technical limitations allow the production of combinatorial libraries of AAV capsids containing approximately 10^8 different clones. Therefore, only one or two amino acids would be represented at each position after randomizing 20 positions.

With this in mind, I restricted mutagenesis to five positions previously described as being important for antibody recognition (see Figure 17 B). Through reduction of randomized positions from twenty to five I decreased the number of possible variants from 20^{20} to 20^5 ($3{,}2x10^6$).

6.1.1 Generation a complete randomization library (pdegen5Lib)

A library of the AAV2 capsid mutants was generated, randomizing amino acid positions 449, 458, 459, 493 and 551 (VP1-numbering) by PCR using primers containing the NNS codon that gives rise to all 20 amino acid residues and minimizes the frequency of stop codons for randomization of triplets in each position. After cloning, randomization was verified by analyzing the sequencing profile of the obtained pool (see Figure 18) and by sequencing of 41 clones (see Figure 21). No redundant mutant or a strong bias towards predominant amino acids was observed. A statistical evaluation of the diversity of the pool allowed quantifying that the obtained library consisted of approximately $6x10^6$ variants and therefore statistically represents all possible combinations of amino acids at the chosen sites (see 10.2.6 for technical details).

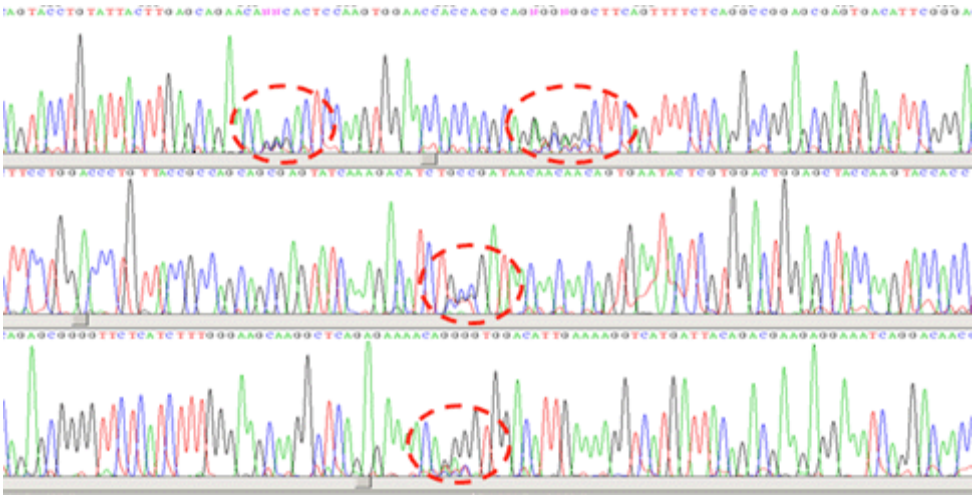


Figure 18: Sequencing profile of the viral library *(Chromas software). The randomized positions are indicated by red circles.*

6.2 Selection of immune escaping mutants

To screen the library for immune escaping mutants I used a selection protocol that was previously described (Figure 19)(Perabo et al. 2006). Before infecting HeLa cells the viral library was incubated with neutralizing human serum that was obtained with informed consent from the Klinikum Großhadern in Munich and tested for the ability to neutralize AAV2 infection (Huttner et al. 2003). After two days, viral progeny was harvested and analyzed via qPCR and sequencing before being applied to additional selection rounds. The procedure favors the accumulation of mutants that despite the presence of neutralizing antibodies can infect the cells and replicate themselves - all valuable attributes for a gene-therapy vector.

6.2.1 Selection procedure

Four consecutive selection rounds were performed starting with the pdegen5Lib library applying a total of 10^9 genomic particles (GOI = 100) in the first three rounds and 10^8 genomic particles (GOI = 10) in the last selection round. To monitor selective pressure and antibody evasion phenotype of the pools at every selection round viral progeny titers were analyzed by qPCR. In addition, control selections were performed without serum and using wtAAV2 instead of the libraries in parallel. The pools that were exposed to the highest selective pressure in each round were used for subsequent rounds (see Table 2).

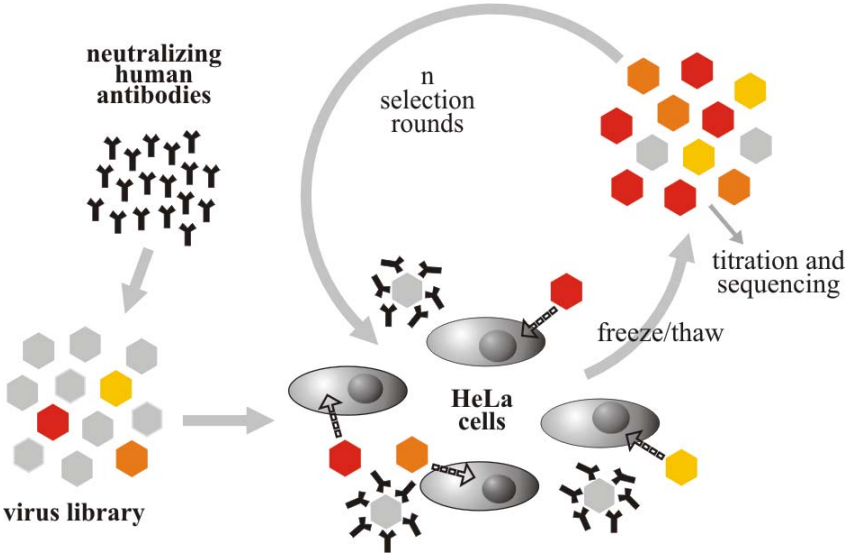


Figure 19: Scheme of the protocol used for immune escape selections. *The viral library is incubated with neutralizing human antibodies before infection of HeLa cells. 48 h post infection, viral progeny is harvested, analyzed and eventually introduced to another selection round.*

In the absence of serum, or with serum concentration of 5%, progeny titers obtained from infection with wtAAV2 or with the pools were comparable in all selection rounds, indicating that no selective pressure was active and that wtAAV2 and library clones are similarly efficient in producing progeny on HeLa cells. In contrast, incubation with 33% serum led to a drop of 99.5% and 99.9% (compared to the titers obtained without serum) for the library and for wtAAV2 respectively. Therefore, 33% serum (and 0% as control) was used in the second selection round.

In the second round the yield of total library pool progeny after incubation without serum was increased 6.8 fold. However, incubation with 33% serum diminished the progeny production by more than 99.99% (compared to the titers obtained without serum) for both wtAAV2 and pool progeny. Despite higher progeny loss, absolute pool progeny titers harvested were higher than after the first round.

Hence, during the third round 50% serum was applied in parallel to using 33% serum in an additional experiment to apply even more stringent selective pressure. Wt was only treated with 0% and 33% serum and showed a decrease of progeny titer of 99.9% compared to only 90.8% of the pool. Selective pressure applied by 50% serum incubation reduced pool progeny titers by more than 99.9% but a progeny titer of $(1.11 \pm 0.18) \times 10^7$ could be recovered and was sufficient for the next selection round.

For the fourth selection round, GOI was reduced to 10 for the pool to increase the antibodies per virus ratio and therefore the selective pressure. Strikingly, despite the GOI reduction and the use of 50% serum, the yield of progeny was 71% of the total progeny recovered when the initial library was applied without serum and 142 fold higher than the progeny obtained after challenging the initial library with 33% serum, suggesting that the pool was evolving towards a population of viral clones with increased ability to infect cells despite the presence of AAV2 neutralizing antibodies.

Another way to demonstrate evolution of the viral pool is to calculate the relative viral progeny of the pools and the wt in every selection round, since they were treated equally and the same GOI was applied (except for the fourth round). The results clearly indicate that the fitness of the library was increased during the selection rounds (see Figure 20). Relative progeny titer is constantly rising from 3.8-fold to 975.6-fold compared to wt.

selection round		**progeny titer of pools/µl**		**Relative progeny**
	% serum		**progeny titer of wt/µl**	**pools/wt**
1	0	$(1.68 \pm 0.07) \times 10^{10}$	$(1.63 \pm 0.08) \times 10^{10}$	1.0 ± 0.09
1	5	$(1.12 \pm 0.12) \times 10^{10}$	$(1.50 \pm 0.07) \times 10^{10}$	0.7 ± 0.12
1	**33**	**$(8.43 \pm 0.53) \times 10^{7}$**	$(2.20 \pm 0.26) \times 10^{7}$	3.8 ± 0.69
2	0	$(1.15 \pm 0.04) \times 10^{11}$	$(1.19 \pm 0.16) \times 10^{10}$	9.7 ± 1.61
2	**33**	**$(1.58 \pm 0.16) \times 10^{8}$**	$(8.01 \pm 0.43) \times 10^{5}$	197.3 ± 30.4
3	0	$(1.85 \pm 0.21) \times 10^{11}$	$(4.60 \pm 0.30) \times 10^{10}$	4.0 ± 0.72
3	33	$(1.70 \pm 0.10) \times 10^{10}$	$(5.90 \pm 0.40) \times 10^{7}$	288.1 ± 36.7
3	**50**	**$(1.11 \pm 0.18) \times 10^{7}$**	n.d.	-
4	**50**	**$(1.20 \pm 0.04) \times 10^{10}$ GOI = 10**	$(1.23 \pm 0.04) \times 10^{7}$ GOI = 100	975.6 ± 70.7

Table 2: Selection monitoring. *Serum concentrations, viral progeny titers of pools and wt, relative progeny (pools/wt) of all tested pools in all selection rounds. The pools in bold letters were applied to the next selection rounds and used for sequencing.*

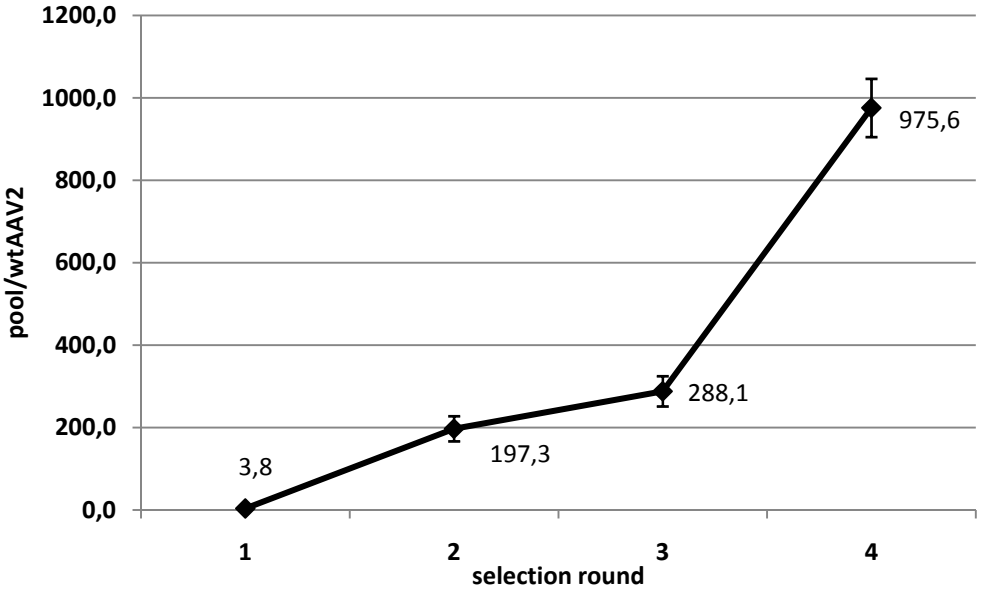


Figure 20: Comparison between viral progenies obtained from wtAAV2 and evolving pool during selection rounds. *Values in the Y axis are expressed as ratio between progeny obtained from library infection and wtAAV2 infection.*

6.2.2 Selection results

14 clones were sequenced after the 2nd round. No redundant clone was found and no bias for predominant amino acids observed, indicating that biodiversity was still too high to isolate predominant clones. Interestingly, only one charged amino acid (Asp) was found (mutant A11, position 551).

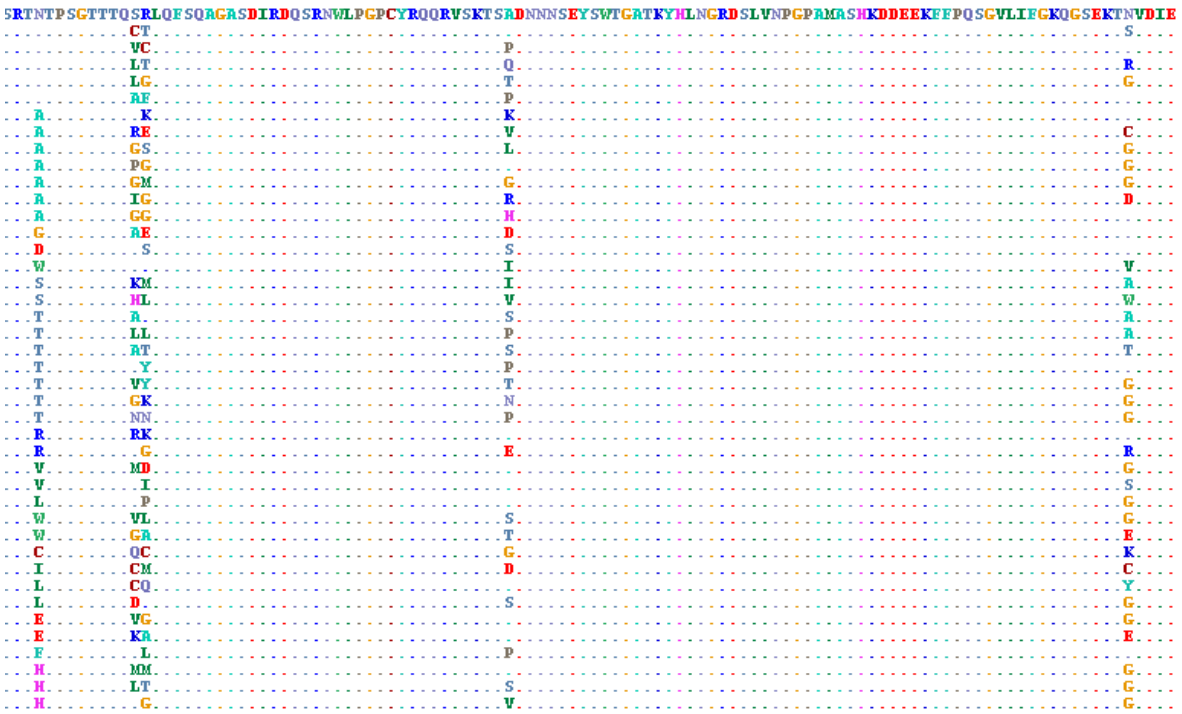
43 clones of the progeny harvested after the fourth selection round were sequenced (see Figure 21). No predominant clone was observed and only one mutant appeared twice, which could be due to the cloning procedure and is not a result demonstrating adequate selection advantage of that single clone. However, the overall number of different amino acids found in the five randomized positions decreased substantially for all positions, suggesting that the biodiversity had been reduced by the selective pressure.

Evaluation of the sequenced sample of the pool showed that small hydrophilic amino acids were positively selected at all five positions while the proportion of big, hydrophobic amino acids had all-over reduced (see Table 3). A possible explanation is the general advantage of small, hydrophilic amino acids to fulfill better the needs of the virus surface to be water-soluble and not disturb protein folding. Interestingly, no proline was found at positions 449, 459 and 551 and only a proportion of 5% at site 493 after selection. In

contrast, the proportion of proline was increased from 2% in the library to 19% in the selected pool at position 449 indicating a positive stabilizing or folding effect at this specific position. Alkaline amino acids were only found at positions 458, 459 and 551 after selection. Acidic amino acids were completely lost at positions 449, 458, 459 and 493 but the proportion was doubled at position 551. A beneficial immune escaping phenotype of negative amino acid at this site was reported before (Perabo et al. 2006). The results demonstrate the potential to narrow down the kinds of amino acids beneficial for capsid biology at specific sites without identification of a predominant clone and may help to better understand the structural requirements of each site on the virus surface. This can lead to a reduction of trial and error procedures in future approaches.

Since a clear reduction of biodiversity was observed together with an overall enhanced phenotype of the pool after the fourth selection round 16 clones were picked and packaged with GFP to test their packaging capacity and immune escaping phenotype.

Library:



Pool after 4th selection round:

Figure 21: Sequences of the viral library before selection (upper panel) and the viral pool after the fourth selection round (lower panel). *(Bioedit software; wt sequence on top)*

Amino acid	Position 449	Position 458	Position 459	Position 493	Position 551
wt amino acid	N	S	R	A	N
wt sequence observed (%)	12/7	22/9	7/7	24/28	22/37
different amino acids observed	16/7	15/10	17/9	15/7	13/8
small, hydrophilic (%)	40/87(wt)	46/55 (wt)	42/65	32/62	70/74 (wt)
big, aromatic, hydrophobic (%)	41/12	37/21	34/23	39/33 (wt)	15/9
proline (%)	0/0	2/19	2/0	15/5	0/0
alkaline (%)	12/0	12/5	15/12 (wt)	7/0	7/2
acidic (%)	7/0	2/0	7/0	7/0	7/14

Table 3: Number of different amino acids observed in total and percentage distribution in the library (n=41) and the selected pool (n= 43). *(Values were rounded to full numbers)*

6.3 Characterization of selected mutants

6.3.1 Packaging of GFP- rAAV vectors

Eight mutants, representing diverse chemical compositions of the mutations at the five randomized positions were chosen for further characterization. In addition, four clones of the unselected library and four of the pool after the second selection round were characterized to evaluate the influence of selection on virions phenotype.

Vector	Amino Acids at positions 449, 458, 459, 493 and 551	Genomic titer/µl	Capsid titer/µl	Capsids/ genome
wt	NSRAN	$(1.90 \pm 0.05) \times 10^8$	1.18×10^9	6,2
AAV1	-	$(2.91 \pm 0.17) \times 10^7$	n.d.	-
K4	NSKAD	$(2.05 \pm 0.06) \times 10^7$	$4{,}61 \times 10^8$	23
B6	AGSLG	$(1.43 \pm 0.06) \times 10^7$	n.d.	-
B8	TARSA	$(1.14 \pm 0.10) \times 10^7$	$1{,}60 \times 10^8$	14
C4	CQCGK	$(5.55 \pm 0.09) \times 10^7$	$1{,}03 \times 10^9$	19
C7	ICMDC	$(2.24 \pm 0.05) \times 10^6$	n.d.	-
A2*	**ASCMG**	**$(4.63 \pm 0.21) \times 10^7$**	**$9{,}44 \times 10^8$**	**20**
A11*	**AYGGD**	**$(5.10 \pm 0.40) \times 10^7$**	**$8{,}18 \times 10^9$**	**16**
B2*	**AGGGQ**	**$(1.32 \pm 0.08) \times 10^8$**	**$1{,}01 \times 10^9$**	**8**
B10*	**AGTTG**	**$(5.74 \pm 0.19) \times 10^7$**	**$2{,}29 \times 10^8$**	**3,9**
C6**	**QQVSA**	**$(4.50 \pm 0.35) \times 10^8$**	**$1{,}74 \times 10^9$**	**4**
E3**	TPQSD	$(1.15 \pm 0.12) \times 10^7$	$2{,}38 \times 10^8$	21
E4**	AYGTE	$(5.17 \pm 0.58) \times 10^8$	$2{,}90 \times 10^9$	6
E10**	THCGE	$(1.91 \pm 0.12) \times 10^7$	$3{,}06 \times 10^8$	16
F8**	GNGTA	$(3.59 \pm 0.16) \times 10^8$	$1{,}86 \times 10^9$	5
H6**	**SGKSG**	**$(1.56 \pm 0.06) \times 10^8$**	**$1{,}57 \times 10^9$**	**10**
H8**	TPQTK	$(4.73 \pm 0.18) \times 10^8$	$1{,}38 \times 10^9$	3
H11**	SASPG	$(4.54 \pm 0.19) \times 10^8$	$1{,}59 \times 10^9$	4

Table 4: Amino acid substitution, genomic and capsid packaging titers of the selected clones and ratio of empty to full capsids of packaged GFP-vectors *(*:pool after second selection round; **:pool after fourth selection round)*

To check the immune escaping phenotype of individual clones, six mutants A2, A11, B2, B10, C6 and H6 were chosen (carrying non-wt amino acids at all five positions) and subjected to detailed characterization. wtAAV1, wtAAV2 and K4 mutant (being the best mutant isolated by the same selection protocol out of an error prone PCR library in our lab) were used as controls to be compared with the selected mutants due to their immune

escape behavior. Being a different serotype that due to over 100 amino acid mutations in comparison to AAV2, AAV1 does not cross-react with anti AVV2 antibodies it was chosen as control to evaluate the immune-escaping phenotype of the selected mutants.

Mutants packaged efficiently (see Table 4), the two clones isolated in the fourth selection round yielding the highest titers. The ratio between empty and full capsids varied from 4 to 16 for the selected mutants which is in the same range as wtAAV2 (6.2).

This demonstrates that, apart from the immune-escaping properties (characterized in the following paragraphs), the selection protocol selects mutants that efficiently package in the *in vitro* conditions of the screening procedure.

6.3.2 Infection assays

GFP-expressing vectors were analyzed for their immune escaping behavior. Increasing amounts of serum were incubated with GFP-vectors. HeLa cells were infected 2 h later and the percentage of transduced cells was measured 48 h later by cytofluorimetry.

To make sure that the results were not influenced by differences in capsid or genomic titers, or by differences in iodixanol concentrations, these experiments were conducted with same amounts of genomic particles, total capsids and volume of iodixanol.

In addition to the serum that was used for selections, mutants were tested on another neutralizing patient serum and on a highly concentrated intravenous immunoglobulin pool (IVIG) derived from more than thousand people. This IVIG pool represents an immunologic average of the human population.

Serial dilutions of the sera were used to measure the neutralization curves of the vectors (see Figure 22). Since IVIG is more concentrated (50mg/ml IgG) than human serum (8 mg/ml IgG), vectors were neutralized at lower concentrations, while 20% of serum 1 was not sufficient to neutralize the vectors completely.

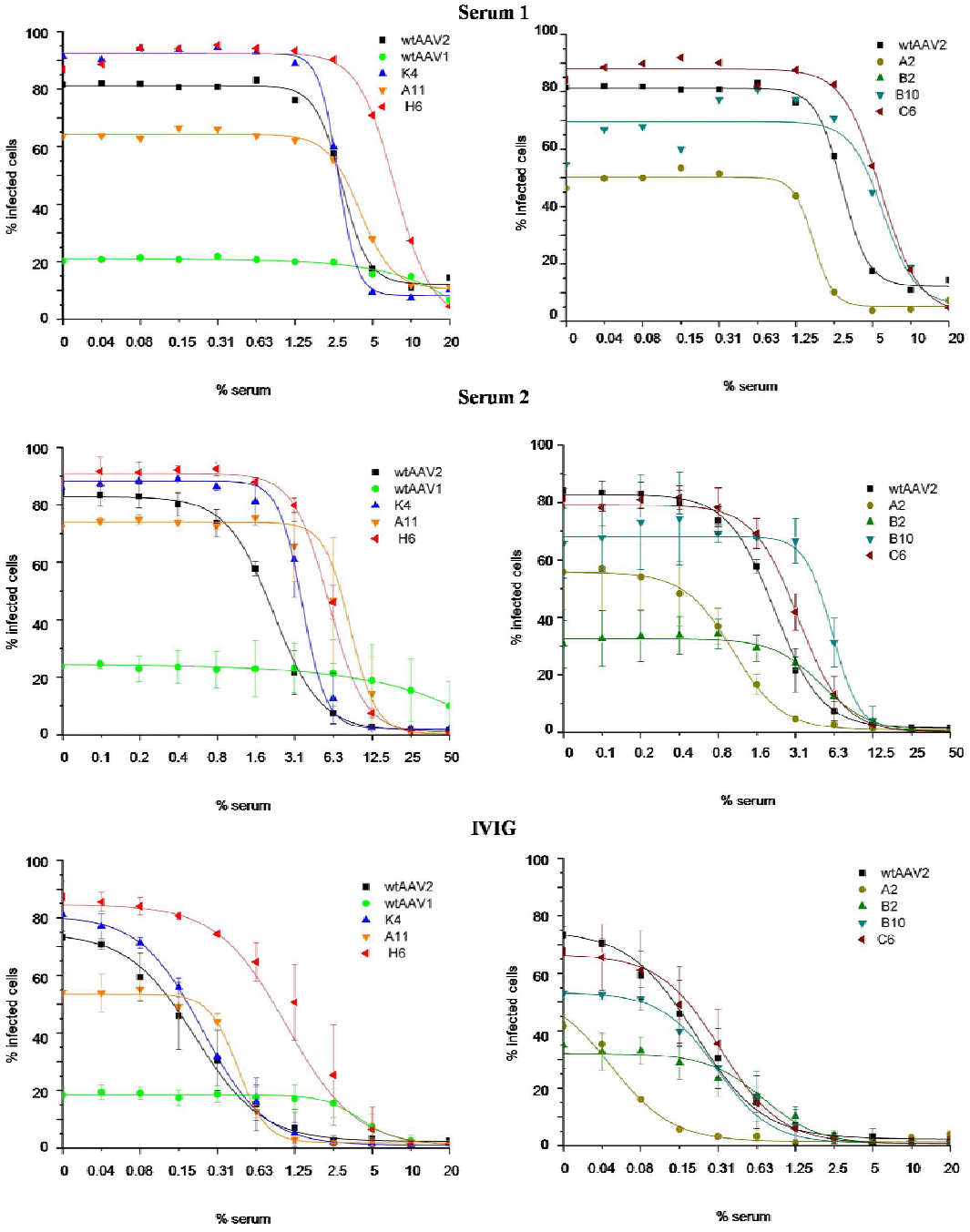


Figure 22: Neutralization assays. *GFP-vectors were incubated with increasing amounts of serum 1 (upper panel), serum 2 (middle panel) or IVIG (lower panel) before infection of HeLa cells. The percentage of transduced cells was estimated 48h post infection by cytofluorimetry.. In the figures on the left, wtAAV2 (black) is illustrated together with wtAAV1 (green), K4 mutant (blue), A11 mutant (orange) and H6 mutant (red). On the right, wtAAV2 is shown with A2 (dark yellow), B2 (olive), B10 (cyan) and C6 (dark red).*

Each mutant showed a characteristic phenotype on all three tested sera. The measurements with serum 1 were performed only as single experiment and without mutant A2 because

the serum stock was exhausted. However they are in accordance with those obtained for serum 2 and IVIG.

To quantify the immune-escaping ability of the mutants, the amount of serum needed to halve the number of transduced cells (N50) was calculated. N50, relative N50 values (N50 value of mutants divided by N50 of wtAAV2) and relative infectivities (infectivity of mutants divided by infectivity of wtAAV2) of the vectors are summarized in Table 5. B2 mutant could not be tested on serum 1 due to serum stock exhaustion.

vector	wtAAV2	wtAAV1	K4	A2*	B2*	B10*	C6**	A11*	H6**
N50 (serum 1)	2.89	14.51	2.70	1.72	n.d.	6.08	5.81	4.02	7.45
relative N50 (serum 1)	1	5.0	0.9	0.6	n.d.	2.1	2.0	1.4	2.6
relative infectivity (serum 1)	1	0.26	1.14	0.62	n.d.	0.86	1.08	0.79	1.14
N50 (serum 2)	2.16	37.78	3.61	1.02	5.02	6.06	3.29	8.48	5.79
relative N50 (serum 2)	1	17.5	1.7	0.5	2.3	2.8	1.5	3.9	2.7
relative infectivity (serum 2)	1	0.30	1.07	0.67	0.39	0.82	0.96	0.89	1.10
N50 (IVIG)	0.21	4.05	0.24	0.20	0.05	0.70	0.33	0.45	1.08
relative N50 (IVIG)	1	19.3	1.1	0.1	0.2	3.3	1.6	2.1	5.1
relative infectivity (IVIG)	1	0.25	1.08	0.73	0.43	0.71	0.89	0.72	1.13

Table 5: N50 values, relative N50 values and relative infectivities without serum (compared to wtAAV2) of the tested vectors on patient sera 1, 2 and IVIG *(*: mutant from pool after second selection;**: mutant from pool after fourth selection round).*

Taken together, the results reported in Figure 22 and Table 5 show that wtAAV2 is more infectious than wtAAV1, B2, A2, B10 and A11, equally infective as C6 and K4 and less infective than H6 in the absence of serum. N50 values demonstrate that A2 is more sensitive to neutralization by all three tested sera in comparison to wt AAV2. All other mutants escape neutralization better than wtAAV2. The only exceptions are K4 on serum 1

and A11 on IVIG. Mutants B2 and A11 show variations in their immune escaping phenotype when tested on different sera (N50: 0.2, 2.3 for B2; 1.4, 2.1 and 3.9 for A11). The H6 mutant shows highest transduction rates with and without serum. (The N50 of B10 is 1.05-fold better on serum 2 but due to lower infection rate, the total amount of infected cells is higher for H6. The N50 of B10 is much lower on IVIG compared to H6. At high concentrations of serum 2, wtAAV1 is infecting more cells than H6.) wtAAV1 is showing highest N50 values on all three sera, varying between 5-fold and 19.3-fold superior to wtAAV2.

6.3.3 Decoy assays

Mutants A11, which showed best the best N50 value on serum 2, and mutant H6, which was the best on serum 1 and IVIG, were further investigated for their ability to bind human antibodies and to escape neutralization independently from their generic infectivity, packaging efficiency and efficiency of transgene expression.

Same amounts of wtAAV2 were mixed with increasing amounts of wt, A11 or H6 empty particles (empty particles can be produced by a standard packaging method by transfecting 293 cells without the ITR containing plasmid, for details see Materials and Methods section) and incubated with increasing amounts of serum 2 and IVIG. In this setting, antibodies contained in the serum are bound by viral particles in a measure that is directly proportional to their affinity for the capsids. If empty particles of one particular mutant are decoyed efficiently by serum antibodies, transduction rates determined by full wtAAV2 particles will be higher.

This experiment showed that at high serum concentrations, adding wt empty particles proportionally increased transduction rates more than addition of H6 and A11 empty capsids. Infection rates measured in the absence of serum were not affected by the addition of empty particles, indicating that no competition between empty and full capsids on cellular receptors responsible for the capsid internalization process had taken place.

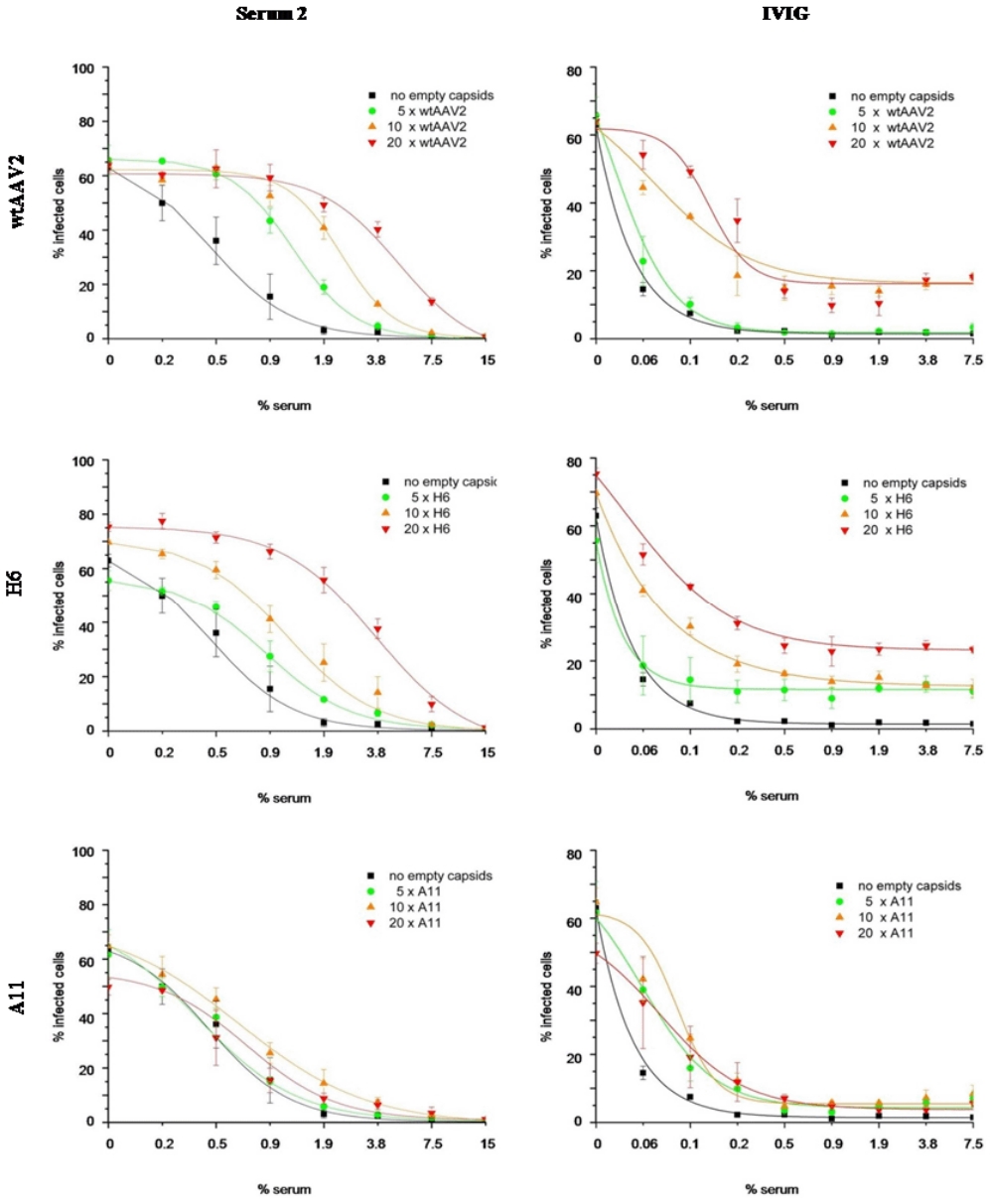


Figure 23: Decoy assay for wtAAV2, A11 and H6 mutant. *Same amounts of wtAAV2 were mixed with 5-, 10- and 20-fold empty particles of wtAAV2 (top), H6 (middle) or A11 (bottom). After incubation with serial dilutions of serum 2 (left graphs) and IVIG (right graphs), Hela cells were infected and transduction rates measures 48 h post infection.*

To quantify the results of Figure 23, N50 values of serum 2 obtained with wtAAV2, H6 or A11 particles were compared and are shown in Table 6. While the addition of wtAAV2 empty capsids increased N50 values proportionally in a capsid dose-dependent manner (11.51 fold after adding 20-fold more capsids), the addition of empty H6 capsids had a reduced effect (8.53-fold) and the addition of A11 capsids resulted in no significant increase (1.63-fold). Strikingly, when the experiment was done with IVIG, the addition of

10x and 20x wt or H6 empty particles fully rescued AAV2 infectivity even at the highest IVIG concentrations. Despite preventing us from being able to calculate N50 values, this is a clear demonstration that while wt and H6 capsids can bind anti-AAV2 antibodies, A11 capsids can efficiently escape recognition and therefore are inefficient decoys.

In conclusion, showing a reduced decoy effect for H6 and almost no decoy effect for A11 these results demonstrate that amino acid substitutions at the 5 randomized positions dramatically decrease the recognition by anti-AAV2 antibodies. Importantly, the amount of decrease changes considerably depending on the specific kind of amino acid substitution.

Additional empty capsids	**5x**	**10x**	**20x**
wtAAV2	2.95	5.48	11.51
H6	2.08	2.70	8.53
A11	0.88	1.46	1.63

Table 6: Analysis of the decoy efficiencies of wtAAV2, H6 and A11 empty particles on serum 2. *The x-fold increases of N50 values after addition of empty capsids are given.*

6.3.4 Multiple cell infection and Heparin inhibition assay

To find out more about the mutants characteristics, I tested their infectivity on human and murine cell lines from different tissues and compared it to wtAAV2 and wtAAV1. Same amounts of GFP-vectors were applied to the cells and transduction rates were analyzed 48 h post infection (see Figure 24).

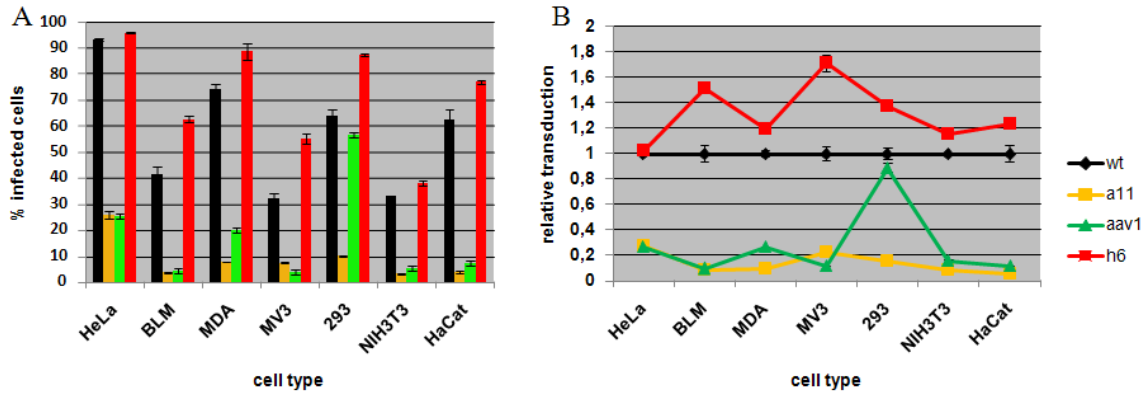


Figure 24: Total (B) and relative (B) infectivities of wtAAV2, A11, wtAAV1 and H6 on various cell types. *Same numbers of genomic particles were used to infect cells. Transduction rates were measured 48 h post infection via cytofluorimetry. For relative infectivities, wtAAV2 was normalized to 100% on each cell type.*

The mutant A11 showed always lower infectivity, compared to wt varying between 6% on Hacat and 28% on HeLa cells. H6 was always superior to wtAAV2, while wtAAV1 was showing only 10% of wtAAV2 infectivity on HeLa but 89% on HEK293 cells. Being less infective in most cell types, AAV1 and wtAAV2 showed almost same infection rates on HEK293, suggesting a radically different tropism of these natural AAV serotypes. This hypothesis is in agreement with the results obtained infecting HeLa cells in the presence or absence of soluble heparin, an analogon of HSPG, the primary receptor of AAV2 (see Figure 25).

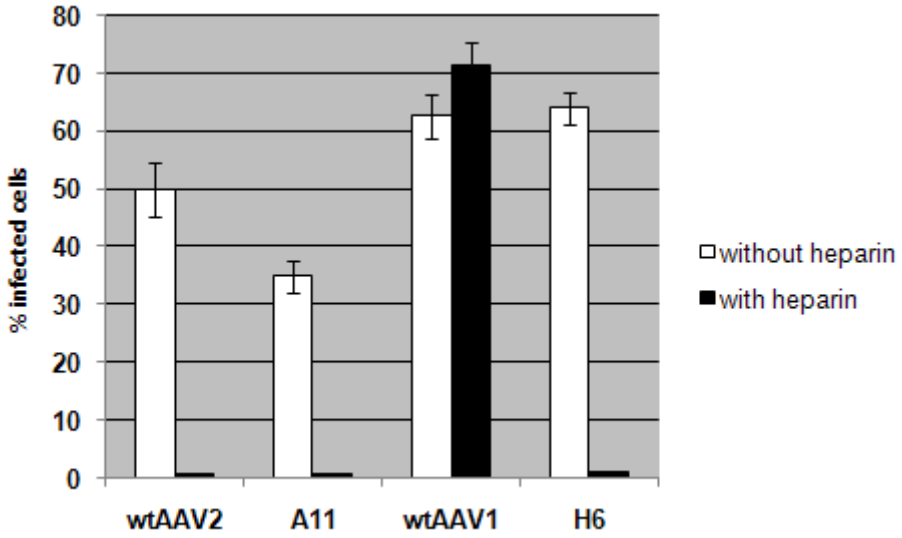


Figure 25: Transduction efficiencies of wtAAV2 mutant A1, wtAAV1 and mutant H6 in the presence (black bars) or absence (white bars) of soluble heparin. *The percentage of infected cells was quantified 48 h post infection via cytofluorimetry.(The tenfold dose of wtAAV1 was used to achieve comparable levels of infection)*

These results demonstrate that while AAV1 does not use the primary AAV2 receptor, the selected mutants do so, indicating that the five amino acid substitutions do not disrupt the original ability of the capsid to interact with natural receptors, maintaining an AAV2-similar tropism.

7 Discussion II (Immune escape selections)

Since a large majority of the human population is seropositive for AAV2 and other serotypes are not having the desired characteristics for some applications, me and others intend to engineer the AAV2 capsid in a way that disrupts binding sites of prevalent antibodies. Mutations introduced to redirect the viral tropism have been shown to decrease binding and neutralization by pre-existing antibodies (Wobus et al. 2000; Huttner et al. 2003). This principle has been eventually exploited to generate *ad hoc* immune-escaping viral mutants by amino acid substitutions at the viral capsid surface by rational design or by directed evolution approaches (Lochrie et al. 2006; Maheshri et al. 2006; Perabo et al. 2006). These studies identified several amino acid residues whose substitution decreased binding of human or rabbit antibodies. Mutants that retained infectivity of more than 1% after incubation with 6.7% serum (Maheshri et al. 2006), up to 10-fold increased infectivity on IVIG (Lochrie et al. 2006) and 5.5-fold increase on diluted serum (Perabo et al. 2006). Absolute data cannot be compared because different sera and read out systems were used, but all-over outcome of the formerly conducted trials was: Despite providing an important proof of principle however, near-to-physiological range concentrations of antibodies could still neutralize transduction of these vectors. Therefore, I intended to create a new kind of viral library that would allow to enhance immune escaping properties of AAV2 derived vectors.

Rational approach studies have shown that specific sites of the capsid are especially important for antibody recognition (Moskalenko et al. 2000). Until now, application of evolutionary methods for the generation of immune-escaping AAV vectors has employed libraries obtained by randomization of large portions of the viral capsid (Maheshri et al. 2006; Perabo et al. 2006). Due to technical limitations these libraries can represent a maximum of one or two different amino acids at each position. These technical drawback and the results of my experiments underline the necessity to combine rational design and combinatorial methods to restrict regions to randomize and allow a complete screening of possible mutations. However the total biodiversity of libraries still is a limiting factor, indicating that consecutive iterations of selection procedures and combination of different kinds of libraries will be needed to further enhance mutants´ phenotype.

The observation that single amino acid differences are responsible for different aspects of the viral phenotype (packaging efficiency, infectivity and antibody recognition) demonstrates that the concept of complete randomization libraries is relevant not only for the generation of immune-evading mutants, but for any engineering goal.

In the course of experiments it turned out that AAV1, differing in more than hundred amino acids from AAV2 had the highest N50 values on all three sera but transduction rates were low on the tested cells. This points out that although alternative natural serotypes usually are strongly immune-escaping, the dramatic structural differences responsible for the immune-escaping phenotype determine considerable tropism-differences at the same time. This is limiting the possibility to use them to replace AAV2 vectors for the treatment of seropositive patients. The tropism profile obtained on 8 different cell types and by the analysis of heparin binding confirmed these differences in tropism. Both these experiments showed that conversely to AAV1, the selected mutants retain an AAV2 like tropism.

The immune-escaping phenotype of the mutants was tested on two AAV2 seropositive patients sera and on IVIG, a pooled IgG-stock (immunoglobuline G) which represents the immunologic average of a human population. Phenotypes of the tested mutants showed some quantitative serum dependent variation but qualitatively it could be demonstrated that mutations that decrease neutralization tend to be effective independently from the kind of antibodies used.

Mutant A11, which showed the best N50 value on serum 2, and mutant H6, which was the best on serum 1 and IVIG of the selected, were further investigated for their ability to bind human antibodies and to escape neutralization independently from their generic infectivity, packaging efficiency and efficiency of transgene expression by a decoy assay. The relative N50 values measured after addition of 20 x empty capsids of either wtAAV2, A11 or H6 mutant demonstrated that binding of wt capsids to neutralizing antibodies of wt was stronger than for H6 mutant and A11 mutant respectively. The N50 values compared to infection without additional empty capsids were 1.35 fold higher for wtAAV2 than with H6 empty capsids and 7.06 higher than with A11 empty capsids, meaning that H6 mutant is binding to anti AAV2 antibodies less efficiently than wt but especially A11 has lower antibody recognition phenotype. H6 was showing enhanced immune escape although having a relatively high decoy capacity. This might be explained with binding of the antibodies without being as efficiently neutralized as wtAAV2.

Finally, it is important to note that after performing four selection rounds, the surviving pool of mutants was not dominated by redundant clones. Despite the presence of a number of identical or similar clones appearing in multiple copies, the biodiversity of the pool was still remarkable, suggesting that further selection rounds or increased selection stringency during the screening procedure could lead to the isolation of further optimized mutants, which for example combine high infectivity with low antibodies recognition. After optimization of the here mutated amino acid residues of the viral capsid is achieved, codon randomization could be extended to additional residues in order to improve the immune-escaping phenotype.

To yield an immune escaping mutant that is not at all recognized by patient serum would need Further understanding of antibody binding sites, optimization of more residues, combination of different libraries and consecutive evolution steps can in future further enhance selection results.

Even if neutralization is not completely abolished, lower recognition can be useful for therapy vectors and may be combined with targeting mutants to optimize vectors for specific applications. Bringing together vector variants of with different beneficial characteristics would lower the needed doses for therapy, diminish unwanted side effects and lead to a more efficient delivery of the genetic cargo to the desired target tissue.

The results gathered within this work demonstrate that for the generation of immune-escaping mutants, besides identifying immunogenic residues of the viral capsid it is fundamental to optimize the amino acid substitutions at these sites. The specific chemical characteristics of each single residue have dramatic consequences on structural and biological properties of the capsid.

8 Conclusions and outlook

Despite promising results of first clinical trials with gene therapy vectors they are not suitable for routine application. The breakthrough of such applications in clinical routine depends on the availability of efficient and safe gene delivery vector. Although natural viruses are inspiring elegant technical solutions, characteristics ideally required of gene therapy viral particles often differ from those of original structures. Therefore, depending on the envisioned utilisation, biological viruses demand different levels of manipulation before they can be successfully employed. For example it is desirable to enhance production yields, stability, control tropism and avoid neutralization by pre-existing antibodies. Incomplete understanding of capsid biology and of the correspondence between protein architecture and function currently limits the development.

Application of modern molecular biology techniques is contributing to understand the principles of particle assembly, immunogenicity, genome encapsidation and capsid infection biology. Rational design and combinatorial approaches are used to relate particle architecture to function and eventually alter it. Understanding how capsid proteins interact with each other, with the viral genome and with host cells will benefit a broad range of disciplines, from clinical virology to gene therapy and other diverse biotechnology applications of viral nanoparticles.

Engineering techniques like those described in my work allow the exploitation of the potential and plasticity of protein chemistry to tailor vectors for the specific requirements of different applications. Pushing back the boundaries of molecular engineering of viral nanoparticles, further refinement and use of these methods promise to provide solutions that transcend technical limitations imposed by natural systems and classical modification approaches. Eventually, this will facilitate the transition to a phase in which nanoparticles devices can be manipulated at will, overcoming drawbacks of systems that too closely resemble the original.

In the future, especially the combination of rational design and combinatorial techniques and more knowledge about capsid biology promises to enable scientists to develop new vectors with even more patient- and application-specific phenotypes.

9 Materials

9.1 Chemicals, solutions and enzymes

Substance	Supplier
Agarose	Sigma, Deisenhofen, Germany
Ampicillin	Sigma, Deisenhofen, Germany
Aqua bidest.	Millipore, Billerica, Germany
Benzonase	Merck, Darmstadt, Germany
Bovine Serum Albumine	AppliChem, Darmstadt, Germany
D-MEM medium	Invitrogen Corporation, Germany
Etidium bromide	Roth, Karlsruhe, Germany
Fetal Calf Serum	Invitrogen Corporation, Karlsruhe, Germany
Heparin	B. Braun Melsungen AG, Germany
HEPES	Roth, Karlsruhe, Germany
Iodixanol	Sigma, Deisenhofen, Germany
MassRuler DNA Ladder Mix	MBI Fermentas GmbH, St. Leon-Rot, Germany
Methanol	Roth, Karlsruhe , Germany
PBS	Invitrogen Corporation, Karlsruhe, Germany
Penicillin/Streptomycin	Invitrogen Corporation, Karlsruhe, Germany
Phusion DNA-Polymerase	Finnzymes, Keilaranta, Finland
Proteinase K	Merck, Darmstadt, Germany
RPMI 1640 medium	Invitrogen Corporation, Germany
Sodium Chloride	Riedel-de Haën, Seelze, Germany
Sodium Dodecyl Sulfate (SDS)	Merck, Darmstadt, Germany
Tris	Merck, Darmstadt, Germany
TritonX-100	Sigma, Deisenhofen, Germany
Trypsin-EDTA	Invitrogen Corporation, Karlsruhe, Germany
Tween 20	Merck, Darmstadt, Germany

If not mentioned the chemicals were bought from Sigma-Aldrich, Taufkirchen, Germany; Merck, Darmstadt, Germany or Carl Roth GmbH & Co., Karlsruhe, Germany.

DNA-restriction and -modifying enzymes not listed were bought from MBI Fermentas, St. Leon-Rot, Germany

9.2 Standard kits

DNeasy® Tissue Kit	Qiagen, Hilden, Germany
EndoFree® Plasmid Kits	Qiagen, Hilden, Germany
Gel Extraction Kit	Qiagen, Hilden, Germany
PCR Purification Kit	Qiagen, Hilden, Germany
Qiaex II Gel Extraction Kit	Qiagen, Hilden, Germany
Light-Cycler-FastStart DNA Master SYBR Green I	Roche, Mannheim, Germany

9.3 Plasmids

p587Lib7: insertion-library plasmid, contains the AAV-2 Rep and Cap encoding regions flanked by the viral ITRs

pdegen5Lib: degeneration library plasmid, randomised at positions 449, 458, 459, 493 and 551 of VP1 counting, contains the AAV-2 Rep and Cap encoding regions

pRC: AAV based helper plasmid containing the AAV-2 Rep and Cap coding regions but lacking the viral ITRs (Girod, Ried et al. 1999)

pRC"Kotin": AAV based helper plasmid containing the AAV-2 Rep and Cap coding

pXX6-80: kindly received as gift from J. Samulski and encoding for the Adenoviral genes VA, E2A and E4 (Grimm, Kern et al. 1999) regions but lacking the viral ITRs, containing SnaBI and BsiWI cloning sites

scGFP: GFP gene controlled by human cytomegalo virus promoter; a deletion in the terminal resolution sites interferes with strand displacement resulting in a self complementary genome, which is packaged into viral capsid (Hacker, Wingenfeld et al. 2005)

wtAAV-2: contains the AAV-2 Rep and Cap encoding regions flanked by the viral ITR's (Girod, Ried et al. 1999).

9.4 Primers

Oligonucleotides manufactured by Metabion, Martinsried, Germany are listed in 5'→3′ direction.

Primers for wtAAV:

3201-forward

5' – GGTACGACGACGATTGCC – 3'

4066-reverse

5' – ATGTCCGTCCGTGTGTGG – 3'

Primers for rAAV (eGFP):

EGFP-1 forward

5' – GCTACCCCGACCACATGAAG – 3'

EGFP-1 reverse

5' – GTCCATGCCGAGAGTGATCC – 3'

Primers for Amplification of selected clones:

BsiWI- forward

5' – TACCAGCTCCCGTACGTCCTCGGC – 3'

New SnaBI-reverse

5' –CGCCATGCTACTTATCTACG– 3'

Primers for the generation of pdegen5Lib:

449,458,459 degen-forward

5′-GCAGAACANNSACTCCAAGTGGAACCACCACGCAGNNSNNSCTTCAGTTT-3′

449,458,459 degen-reverse

5′-AAACTGAAGSNNSNNCTGCGTGGTGGTTCCACTTGGAGTSNNTGTTCTGC-3′

493 degen-forward

5′-GACATCTNNSGATAACAACAACAGTGAATACTCG-3′

493 degen-reverse

5′-CGAGTATTCACTGTTGTTGTTATCSNNAGATGTC-3′

551 degen-forward

5′-GGCTCAGAGAAAACANNSGTGGACAATG-3′

551 degen-reverse

5′-TCCACSNNTGTTTTCTCTGAGCCTTGC-3′

9.5 Antibodies and sera

- A20 (AAV2-capsid antibody, IgG3), monoclonal mouse hybridoma supernatant (DKFZ Heidelberg, PD Dr. J. Kleinschmidt)
- B1 (VP3-specific antibody), monoclonal mouse hybridoma supernatant (DKFZ Heidelberg, PD Dr. J. Kleinschmidt)
- Biotin conjugate Streptavidin (Dianova)
- Biotin-SP-conjugated rabbit anti mouse antibody (Dianova)
- Intratect (pooled human IgG stock, 50mg/ml, Biotest)
- Serum 1 patient Serum
- Serum 2 patient serum

9.6 Bacteria strains

For chemical transformation: *E.coli* DH5α: F-, lac1-, recA1, endA1, hsdR17, Δ(lacZYA-argF), U169, F80dlacZΔM15, supE44, thi-1, gyrA96, relA1; (Hanahan 1983)

For electrical transformation: Electroten-Blue Electroporation Competent Cells: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1, gyrA96 relA1 lacKanr [F′ proAB lacIqZΔM15 Tn10 (Tetr)], Stratagene, La Jolla, USA

9.7 Eukaryotic cells

BLM, MV3: human melanoma cell line; RPMI with 10% FCS, 100 units/ml of Pen/Strep and 1% of non-essential amino acids

Fibroblasts: primary human Fibroblasts; isolated from healthy female skin tissue; DMEM with 10% FCS and 100 units/ml of Pen/Strep

HaCat: human keratinocyte cell line; spontaneously immortalized; DMEM with 10% FCS and 100 units/ml of Pen/Strep

HEK293: human kidney cells; transformed with adenovirus 5 DNA, containing E1A and E2; D-MEM with 10% FCS and 100 units/ml of Pen/Strep

HeLa: human cervix adenocarcinoma; D-MEM with 10% FCS and 100 units/ml of Pen/Strep

Hom01; MelA, M001: primary human melanoma cells; DMEM with 10% FCS and 100 units/ml of Pen/Strep

Keratinocytes: human primary cells; DMEM with 10% FCS and 100 units/ml of Pen/Strep

MDA: human breast cancer cell line, DMEM with 10% FCS and 100 units/ml of Pen/Strep

NIH3T3: murine embryonic fibroblast cell line, DMEM with 10% FCS and 100 units/ml of Pen/Strep

9.8 Data treating software

Microsoft Office; Roche Molecular Biochemicals Lightcycler Software v3.5; Elisa Reader SofMax Pro v1.2.0; Origin 8; Adobe Photoshop CS2; Freeware: BioEdit; pDraw; Chromas

9.9 Laboratory equipment, disposables

Equipment	Supplier
48well Multiwell (polystyrene)	Beckton-Dickinson GmbH, Heidelberg
5-6B Centrifuge	Beckman Instruments GmbH, München, Germany
Balance Adventure Pro	Ohaus Corporation, NJ, USA
Spectophotometer	
BioRadSmartSpec3000	Bio-Rad laboratories GmbH, München, Germany
Cell culture plastic ware	Beyer GmbH, Düsseldorf, Germany
Centrifuge 5415 D	Eppendorf AG, Hamburg, Germany
Centrifuge 5810 R	Eppendorf AG, Hamburg, Germany
Dialysisfilter VS (0.0025 µm)	Milipore AG, Zug, Switzerland
Electrophoresis Unit	
Sub-Cell GT Gel	Bio-Rad laboratories GmbH, München, Germany
Emax Precision Microplate Reader	Molecular Devices GmbH, München, USA
FACSCalibur	Beckton-Dickinson GmbH, Heidelberg
Gene Pulser II	Bio-Rad laboratories GmbH, München, Germany
Heater/Magnetic Shaker	
Heidolph MR 3001	Heidolph Instruments, Schwabach, Germany
Hera -80° freeze	Heraeus, Sepatech GmbH, Osterode, Germany
Incubator Shaker Innova 4430	New Brunswick Scientific, Inc, NJ, USA
Light Cycler	Roche Diagnostics GmbH, Mannheim, Germany
Light Microscope Axiovert 25 CFL	Carl Zeiss Jena GmbH, Göttingen, Germany
Mamalian Incubator Hera Cell 150	Heraeus, Sepatech GmbH, Osterode, Germany
Neubauer Haematocytometer	LO Laboroptik GmBH, Friedrichsdorf, Germany
pHmeterSevenEasy	Mettler-Toledo GmbH, Schwerzenbach, Switzerland
Polyesterol tubes 50 ml	Beckton-Dickinson GmbH, Heidelberg
Pump P-1	Amersham Pharmacia Biotech, Freiburg, Germany
Sorval Ultracentrifuge Combi	DuPont GmbH, Bad Homburg
Thermomixer Comfort	VWR International GmbH, Darmstadt, Germany
Therocycler T3000	Biometra, Göttingen, Germany
Vortex Genie2	Scientific Industries, Inc, NY, USA
Waterbath Medingen W6	Medigen GmbH, Martinsried, Germany

Electroporationcuvettes, 0,1 cm	Bio-Rad laboratories GmbH, München, Germany
Sterile filters 0.22µm, 0.45µm	Schleicher&Schuell GmbH, Dassel, Germany

General laboratory ware was bought at VWR International GmbH, Darmstadt, Germany.

10 Methods

10.1 Bacteria culture

10.1.1 Cultivation of bacteria

Bacteria were grown at 37°C with LB medium overnight while vigorously shaking.
For growing the bacteria on plates, 15 g/l Agar was added to LB medium for solidification.
For transformed bacteria with resistance to ampicillin, 50 mg/l ampicillin was added to the medium.

LB medium (1 l):
10 g Tryptone
5 g Yeast extract
5 g NaCl
ad 1 l H_2O

10.1.2 Glycerol stocks

1ml of overnight culture was spun down at 2500 rpm for 1 min in a table centrifuge and resuspended in 1ml LB containing 50% glycerol before freezing at -80°C.

10.1.3 Preparation of chemically competent bacteria

Bacteria strain DH5α was grown at 37°C and vigorous shaking (200 rpm) overnight in 8 ml of LB medium. 10 hours later 1 ml was transferred into 30 ml of LB medium and the bacteria were grown until an optical density (OD_{600}) of 0,7. Incubation on ice was performed for 30 min followed by centrifugation at 3200 rpm for 15 min at 4°C. Pellet was re-suspended in 10 ml of cold TFBI buffer. Incubation on ice was done and followed by a centrifugation which was performed as before. The pellet was re-suspended in 3 ml of cold TFB II.

TFB I buffer (200 ml):
30 mM potassium acetate
100 mM $CaCl_2$
15 % glycerol
ad 190 ml H_2O
Autoclaved, than addition of sterile filtered:
50 mM $MnCl_2$

TFB II buffer (50 ml):
10 mM MOPS
75 mM $CaCl_2$
10 mM KCl
15 % glycerol
ad 50 ml H_2O

10.1.4 Chemical transformation of bacteria

200 µl of competent bacterial suspension was transferred into the reaction tubes already containing the plasmid which should be introduced (coding for resistance to ampicillin). After vortexing the suspension was incubated for 45 sec at 42°C. Afterwards, 1 ml of LB medium was added cells were shook at 200 rpm and 37°C for 30 min. Bacteria were plated onto ampicillin containing LB-plates (50 µg/ml) and incubated overnight at 37°C.

10.1.5 Preparation of electrocompetent bacteria

Pipet tips, centrifuge tubes and reagents were precooled to 4°C. 2 l baffled flasks with 400 ml prewarmed YENB were used to grow bacteria. A colony of freshly streaked Electroten blue was inoculated with 100 ml YENB medium overnight without letting it grow to full density.
The next day, 2 ml of overnight culture were inoculated in 400 ml YENB and shook until reaching OD_{600} of 0,7. This culture was split into four additional flasks and grown again. Directly after reaching OD_{600} of 0,7, bacteria were cooled in ice water and centrifuged 10 min at 4000 x g. Pellets were washed twice with 200 ml water and one additional time with 50 ml

10 % glycerol. After a last centrifugation, cells were resuspended in 1ml of 10 % glycerol, split into 40 µl aliquots and frozen in liquid nitrogen before being transferred to -80°C.
(YENB medium: 0.75% Bacto yeast extract; 0.8% Bacto nutrient broth (salt free))

10.1.6 Electrical transformation of bacteria

Library ligation DNA was dialyzed against water for at least 2h before transformation. Pipet tips and cuvettes were cooled to 4°C, SOC medium was prewarmed to 37°C before starting. 40µl of chemically competent bacteria were thawed on ice, mixed with ¼ of the library ligation and cautiously pipetted into the cuvette. The electroporation was performed at 1,85kV, 600Ω and 25µFD, resulting in a time constant between 11 and 13. 1 ml SOC medium was added directly and the cells were shook for 1h at 37°C, before transfer of the cells to 500ml of LB medium and plating a defined dilution of the reaction to estimate the biodiversity. A glycerol stock was done and the DNA was extracted the next day using a Qiagen maxi kit.

SOC medium:
20 g Tryptone
5 g Yeast extract
5 g NaCl
Ad 1 l Water
Add 10 ml of filter-sterilized 1 M $MgCl_2$, 10 ml 1 M $MgSO_4$, 20 ml 20% (w/v) glucose

10.2 DNA techniques

10.2.1 Plasmid amplification and extraction, DNA cleanup

For plasmid amplification, extraction and DNA cleanup suitable Qiagen kits were used following the manufacturers protocols.

10.2.2 DNA quantification

DNA concentration was measured using a BioRadSmartSpec 3000 spectrophotometer. Quantification is made by measuring absorbance at 260nm. Low concentrated DNA-probes were analyzed on agarose gels.

10.2.3 Restriction enzyme digest

Digestion with restriction enzymes was performed according to the manufactures instructions in a 20 µl solution containing 1 µg of DNA, 1-10 U of restriction enzyme per 1 µg of DNA and 1x buffer.

10.2.4 Agarose gel electrophoresis

Stock solution for agarose gels was prepared by adding the desired percentage of agarose to the TBE Buffer mixed with ethidium bromide (0.25 µg/ml).TBE buffer was then added to the chamber. Electrophoresis was performed at 75 V and 200 mA.

TBE Buffer (10x; 5 l):
540 g Tris Base
275 g Boric Acid
200 ml of 0.5 M EDTA pH 8.0
ad 5 l H_2O

10.2.5 Sequence analysis

Sequencing of bacterial clones was performed (Qiagen, Hilden, Germany) after DNA extraction and PCR amplification from viral preparations (viral libraries and viral pools after selection) using primer 4066. Single viral genomes were obtained by amplification of viral DNA (Primers: New SnaBI-reverse; BsiWI-forward), cloning amplified DNA into pRC"Kotin" backbone, transformation into bacteria and picking single colonies of the plated cultures.

1x Standard PCR-premix:

1 µl dNTPs (200µM each)

10 µl 5x Phusion reaction buffer

2 µl New SnaBI-reverse (10 µM)

2 µl BsiWI-forward (10 µM)

5 µl template DNA (DNeasyeluate)

Ad 49.5 µl water

0.5 µl Phusion Polymerase

Standard amplification PCR-cycling NS1/Bb (3°C/sec):

Denaturation	98°C	30´´	
Amplification	98°C	10´´	34 x
	56°C	20´´	
	72°C	30´´	
Elongation	72°C	10´	
Cooling	4°C	∞	

10.2.6 Cloning of the pdegen5Lib library

The pwtAAV2 Plasmid (2 µg/µl) was used as a template for the following PCRs. When PCR-products were used as templates, they were purified by gel extraction before. All PCR steps were programmed with a slope of 3°C/sec. The PCR-process was executed in the order given below.

449,458, 459-493premix:

1 µl dNTPs (200µM each)

10 µl 5x Phusion reaction buffer

2 µl 449,458,459 degen-forward (10 µM)

2 µl 493 degen-reverse(10 µM)

2 µl wtAAV2 (1:10)

Ad 49.5 µl water

0.5 µl Phusion Polymerase

PCR-Cycling NS1/Bb 5 sec elongation:

Denaturation	98°C	30´´	
Amplification	98°C	10´´	34 x
	56°C	20´´	
	72°C	5´´	
Elongation	72°C	10´	
Cooling	4°C	∞	

493-551 premix:

1 µl dNTPs (200µM each)

10 µl 5x Phusion reaction buffer

2 µl 493 degen-forward (10 µM)

2 µl 551 degen-reverse (10 µM)

2 µl wtAAV2 (1:10)

Ad 49.5 µl water

0.5 µl Phusion Polymerase

PCR-Cycling NS1/Bb 5 sec elongation

449-551 premix

1 µl dNTPs (200µM each)

10 µl 5x Phusion reaction buffer

10 µl 449,458,459 degen-forward (10 µM)

10 µl 551 degen-reverse (10 µM)

1 µl 449-493 PCR-product

1 µl 493-551 PCR-product

Ad 49.5 µl water

0.5 µl Phusion Polymerase

PCR-Cycling NS1/Bb 5 sec elongation

BsiWI-449 premix

1 µl dNTPs (200µM each)
10 µl 5x Phusion reaction buffer
2 µl BsiWI-forward (10 µM)
2 µl 449degen-reverse (10 µM)
1 µl wtAAV2 (1:10)
Ad 49.5 µl water
0.5 µl Phusion Polymerase

Standard amplification PCR-cycling NS1/Bb

551-NewSnaBI premix

1 µl dNTPs (200µM each)
10 µl 5x Phusion reaction buffer
2 µl 551degen forward (10 µM)
2 µl New SnaBI-forward (reverse)(10 µM)
1 µl wtAAV2 (1:10)
Ad 49.5 µl water
0.5 µl Phusion Polymerase

Standard amplification PCR-cycling NS1/Bb

449-NewSnaBI premix

1 µl dNTPs (200µM each)
10 µl 5x Phusion reaction buffer
2 µl 449 degen forward (10 µM)
2 µl New SnaBI-reverse (10 µM)
1 µl 449-551 PCR-product
1 µl 551-New SnaBI PCR-product
Ad 49.5 µl water
0.5 µl Phusion Polymerase

Standard amplification PCR-cycling NS1/Bb

BsiWI-NewSnaBI premix

1 µl dNTPs (200µM each)

10 µl 5x Phusion reaction buffer

2 µl BsiWI-forward (10 µM)

2 µl New SnaBI-reverse (10 µM)

2 µl BsiWI-449 PCR-product

1 µl 449-New SnaBI PCR-product

Ad 49.5 µl water

0.5 µl Phusion Polymerase

Standard amplification PCR-cycling NS1/Bb

The insert was digested with BsiWI and SnaBI and ligated into pwtAAV2 backbone (s. library ligation). After ligation, the pdegen5Lib was directly dialyzed and cells were transformed (s. Electrical Transformation of Bacteria). Virus was packaged on HEK293 cells as described above. The viral library was sequenced and the chromas file was checked for complete randomization. A biodiversity of $5.5x10^6$ was measured by extrapolation of the number of bacterial colonies after transformation. The diversity limiting step in the library generating procedure is the efficient ligation and transformation of PCR-generated library variants into plasmids and afterwards bacterial cells. The number of total bacteria transformed is estimated by streaking out samples of the bacterial library and equalizing the number of clones to the number of different variants existing in the library. This is feasible due to the enormous number of DNA-variants compared to the number of bacterial clones.

10.2.7 Library ligation

300 ng of wt-backbone (wtAAV2-plasmid, digested with SnaBI and BsiWI) was mixed with 200 ng insert and heated to 50°C for 10 min before adding 100 µl T4 ligation mix, containing 1µl of T4 Ligase. The reaction mixture was incubated at 16°C overnight. The reaction was stopped by heat inactivation at 65°C for 10 min.

10.3 Eukaryotic cell culture

10.3.1 Cultivation of cells

Cells were cultivated at 37°C and 5% CO_2 in humid atmosphere.
Cells were grown with antibiotic Penicillin/Streptomycin.

10.3.2 Trypsinisation

Cells were washed with PBS, trypsin (0.5 g/l) was added in an amount that covered the bottom of the plates, followed by an incubation at 37°C (duration depending on the cell line). When the cells detached from the plate reaction was stopped with medium containing 10% FCS.

10.3.3 Seeding / passaging

Cells were transferred into a new plate or flask containing fresh medium that was warmed up to 37°C. Agitation of the plate with movements in cross were made in order to spread the cells homogeneous.

10.3.4 Freezing and thawing cells

Cells were trypsinized, gathered in a falcon tube and pelleted at 1200 rpm. Supernatant was removed and cell pellet was resuspended with freezing solution. 1 ml of cell suspension was added to each freezing vial. Cells were then directly transferred to -80°C in an isopropanol containing freezing carrousel. The next day, cells were transferred to liquid nitrogen.
For thawing the cells, the freezing vials were taken out of liquid nitrogen storage and the suspension let to thaw at 37°C, after which it was taken to a flask or plate with prewarmed medium.

Freezing solution:
90% FCS
10% DMSO

10.3.5 Cell counting

After trypsinizing and diluting the cells, 10 µl were transferred into a hematocytometer. The number of cells in each of the four squares was counted and an average was made for more precise determination. The number of cells (n) determined equals n x 10^4 per ml.

10.4 Production of AAV2-vectors

10.4.1 AAV-vector packaging

Five 15 cm petri dishes were used for seeding 7.5 x10^6 HEK293 cells/plate in 25 ml DMEM with Glutamax plus 10% FCS and 1% P/S.
24 hours later (cells showed approximately 80% confluence) fresh medium (10% FCS and 1% P/S) was added.
The transfection was performed 2 hours later using 37.5 µg of DNA per plate.

For five plates rAAV (scGFP):

5 ml	250 mM $CaCl_2$
37.5 µg	pRC
37.5 µg	scGFP
112.5µg	pXX6-80
5 ml	HBS-Puffer

For wtAAV/AAV-libraries:

5 ml	250 mM $CaCl_2$
75 µg	wtAAV2 or AAV library plasmid
112.5	µg pXX6-80
5 ml	HBS-Puffer

After adding HBS buffer, the solution has to be vortexed directly.

HBS-buffer

Weighted:

5.95 g HEPES

8.18 g NaCl

1.5 ml 0.5M NaP (pH 7.29)

ad 400 ml with bought SIGMA water (adjust pH to 7.1)

Exactly 2 minutes after vortexing the solution was dropped onto the cells. 24 hours later (approximately 100% confluence), medium was exchanged to 20ml DMEM with Glutamax plus 2% FCS and 1% P/S.

The cells were harvested 48 h post transfection by scraping them from of the plate, collecting them in a conic centrifuge tube and centrifuging them at 3000 rpm for 15min. The supernatant was wasted and the pellet resuspended in 7.5 ml of lysis buffer (150 mM NaCl; 50 mM Tris/HCl, pH 8.5). The cells were 3 times submitted to thermal shock by using liquid nitrogen and a water bath at 37°C.

To get rid off cellular DNA or RNA the suspension was treated with 50 U of Benzonase per ml of suspension at 37°C during 30 min. The falcons were centrifuged for 30 min at 4°C and 4000 rpm; the solution was transferred into a new falcon (pellet was discarded) and filled up with PBS, centrifuged again for 30 min at 4°C and 4000 rpm. The outcome of this procedure is called crude lysate and was used for selections.

10.4.2 Iodixanol gradient purification

An iodixanol step gradient centrifugation was performed using four different iodixanol solutions (see below for the composition of each solution) and PBS with magnesium and potassium for equilibration of the tubes. A needle reaching the bottom of the tube was used to slowly add (using an “Amersham Biosciences Pump P-1”) all the solutions starting with lowest density solution. The centrifugation was performed at 63000 rpm for 1h at 18°C and subsequently the 40% iodixanol phase harvested.

15% Iodixanol :		25% Iodixanol :	
10xPBS	5 ml	10xPBS	5 ml
2 M MgCl2	25 µl	2 M MgCl2	25 µl
1 M KCl	125 µl	1 M KCl	125 µl
5 M NaCl	10 ml	Optiprep	20 ml
Optiprep	12.5 ml	0.5% phenol red	100 µl
0.5% phenol red	75 µl	Sigma water	ad 50 ml
Sigma water ad 50 ml			

40% Iodixanol:		60% Iodixanol:	
10xPBS	5 ml	2 M MgCl2	25 µl
2 M MgCl2	25 µl	1 M KCl	125 µl
1 M KCl	125 µl	Optiprep	50 ml
Optiprep	33.5 ml	0.5% phenol red	25 µl
Sigma water ad 50 ml			

10.4.3 Virus titration

Genomic titer

For extraction of the DNA from the viral particles a “QiagenDNeasy Tissue Kit” was used and the protocol for “Isolation of Total DNA from Cultured Animal Cells” was performed. 10 µl of virus were diluted in 200 µl of PBS. The genomic titer was then determined by quantitative real time PCR (qPCR) using a “Roche LightCyclerFastStart DNA Master SYBR Green I” kit. The probes were prepared as described below.

a) wtAAV genomic titration

Primers: 3201-forward (sense); 4066-reverse (anti-sense)

Volumes per probe:

LCFastStart DNA Master	2 µl
Sense (100 µM)	0.1 µl
Antisense (100 µM)	0.1 µl
$MgCl_2$ (25 mM)	3.2 µl
H_2O	12.6 µl
2 µl DNA extracted from 200µl of viral preparation	

Cycling:

Denaturation	95°C	15´	
Amplification	95°C	10´´	34 x (20°C/sec)
	60°C	3´´	
	72°C	35´´	
Melting curve	68°C→95°C		0.05°C/sec

b) rAAV (eGFP) genomic titration

Primers: EGFP-1 forward (sense); EGFP-1 reverse (anti-sense)

Volumes per probe: s. wtAAV genomic titration

Cycling:

Denaturation	95°C	10´	
Amplification	95°C	10´´	50 x (20°C/sec)
	63°C	3´´	
	72°C	20´´	
Melting curve	67°C → 95°C		0.1°C/sec

By comparison with the standards the program calculates the amount of DNA in the probes. The standards were diluted from according plasmid stocks with photometrical detected DNA concentration.

10.4.4 Capsid titer (ELISA)

The determination of the capsid titer was performed using the anti AAV A20 antibody. Based on the genomic titer and the fact that the capsid titer is normally 10x higher, eight serial dilutions were made in order to obtain concentrations in the linear range of the ELISA (between 107and 1010 capsids/µl).

100 µl of PBS, serial dilutions of Control (empty AAV capsids), and specimen (diluted in PBS) were pipetted separately into the wells of a 96-well plate. The plate was sealed with parafilm and incubated for 1h at 37°C or at 4°C overnight.

A wash step was performed 3 times: 3 times the contents of the 96-well plate were emptied and each well was filled with 200 µl wash buffer, incubated for approximately 5 sec and then the contents emptied again. Afterwards the wells were incubated with 200µl of blocking buffer for 1h at 37°C.

Then, wells were incubated with 100 µl of A20 antibody (1:10, Hybridoma supernatant) in blocking buffer for one hour. Three washing steps were repeated as described above.

The second antibody (1:25000, α-mouse IgG-Biotin conjugate) was pipetted into each well, and incubated for 1h at 37°C. Another 3 washing steps were performed just as described before and 100 µl of streptavidin peroxidase conjugate were pipetted into each well and incubated for 1h at 37°C.

After another 3 washing steps and two additional washing steps with 200 µl of water, 100 µl of ready-to-use TMB- substrate was activated with 30% H2O2and added into each well. Incubation was performed for 5-15 min at room temperature. Color reaction was then stopped by adding 50µl of 1M H2SO4 into each well and the intensity of the color reaction was measured using a photometer at 450 nm.

Wash buffer: PBS, 0,05% Tween 20
Blocking Buffer: Wash Buffer containing 3% BSA, 5% Sucrose

10.5 Infection assays and selection procedures

10.5.1 Titration of adenovirus on BLM

$2.5x10^5$ BLM cells were seeded in a 6-well plate and infected 24 h later with various amounts of Adenovirus and $2x10^3$ genomic particles per cell wtAAV2. 48 h post infection, viral progeny was harvested and measured by qPCR.

10.5.2 Decoy assay on fibroblasts and keratinocytes

$2.5x10^5$ BLM cells, $1x10^5$ primary human fibroblasts or $5x10^5$ primary human keratinocytes were seeded in 6-well plates. 24 h later, 10^6 viral genomic genomic particles of the display library were incubated in 1 ml of medium for 2 h either in empty wells, above fibroblasts or keratinocytes. BLM cells were infected in the meanwhile with 2800 pfu/cell of adenovirus. After 2 h, the adenovirus-containing medium was wasted and the library-containing medium was transferred from empty-, fibroblast or keratinocytes wells to BLM cells. 48 h post infection, viral progeny was harvested and relative amounts per µl were measured by qPCR.

10.5.3 Selection protocol for BLM-specific mutants

2.5×10^5 human primary skin fibroblasts and BLM cells were seeded in 6-well plates and fibroblasts infected 24 h later with 10^4; 10^3 genomic particles of AAV display library. The culture supernatant was harvested 2 h post infection and used to infect BLM cells. At the same time, BLM cells were superinfected with 2800pfu/cell of adenovirus type 5. 48 h post infection, cells were harvested by trypsinisation and viral progeny was obtained by three cycles of freeze-thaw and spin-down of the cell debris. Adenovirus was heat-inactivated at 60°C for 30 minutes. After each selection round, the genomic titer of these preparations was monitored by qPCR using the "wtAAV genomic titration" protocol and eventually used for further selection rounds. Selection pressure was adjusted by variing the initial amount of virus that was applied to the cells. Between the selection rounds, viral pools were eventually amplified using 15cm dishes with $5x10^6$ BLM seeded 24h post infection. Cells were preinfected with 2800 pfu/cell adenovirus 2 h prior to AAV infection and viral progeny harvested 48 h post infection.

Amplifications were performed on a 15 cm dish, using no more than GOI=100 for amplifications, to avoid uncoupling of genotype and phenotype through multiple infections of single cells.

10.5.4 Immune escape selection protocol

$5x10^6$ HeLa cells were seeded on 150 mm Petri-dishes 24 h before infection. Various amounts (0%; 5%, 33% or 50%) of human neutralizing serum and 100 viral particles per cell were incubated 24 h after seeding. Low GOIs and many cells were used increasing the possibility that efficient clones could really infect at least one cell while simultaneously reducing the chance of generating chimeric viruses with no correspondence between genotype and phenotype. The incubations were performed for 2 h at 37°C in a total volume of 15µl (filled up with PBS). The serum was obtained with informed consent from the Klinikum Großhadern, Munich, Germany, and tested for its ability to neutralize AAV-2 (Huttner et al. 2003).

Simultaneously, HeLa cells were preinfected with adenovirus (700 pfu/cell) in 10 ml of DMEM/0% FCS. After two hours, the adenovirus-containing medium was wasted and cells were infected with the AAV-library. 48 h post infection cells were collected by centrifugation and resuspended in 500 µl of lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.5). Cells were lysed by three cycles of freeze/thaw and cell debris was removed by centrifugation. Adenovirus was heat inactivated (60°C, 30 min) and supernatant containing the viral progeny was applied to further selection rounds. After each round, viral DNA was extracted from 10µl of the lysate and genomic titer of progeny was monitored by qPCR using the "wtAAV genomic titration" protocol. Accordingly, a dilution of the viral progeny equaling GOI = 100 was introduced into the next selection round.

The selective pressure was increased in a selection by increasing the percentage of serum. In the first two selection rounds, the serum concentration was kept lower (33%) than in the following two (50%).

10.5.5 Infection assay (BLM cell specificity)

A total of 4×10^4 BLM/Fibroblast cells were seeded in 48-well plates and infected 24 h later with same amounts of genomic particles of wt or capsid modified GFP expressing vectors. After 48 h, cells were harvested and GFP expression was measured by Cytofluorimetry analyzing at least 5000 cells. Target-to-noise ratios were obtained by dividing the percentage of infected BLM by the percentage of infected fibroblasts. To compare selectivity of the mutants with selectivity of wt, mutant target-to-noise ratios were divided by wt target-to-noise ratio.

10.5.6 Infection assays (Immune escape mutants)

$2x10^4$ HeLa cells were seeded in 48 well plates 24 h before infection. Identical numbers of transducing and total particles ($6.5x10^8$, adding A11 empty particles when needed) in the same volume (adding 40% iodixanol if needed) were incubated with serial dilutions (0,05%-15% in PBS) of human serum or IVIG (Intratect©, a pooled human IgG stock) for 2 h at 37°C in a total volume of 20µl. Before addition to the cells, 100 µl of PBS was added to each sample. At least 5000 cells were analyzed by Cytofluorimetry 48 h post infection to determine the amount of GFP expressing cells.

Mixture of full and empty particles with iodixanol was titrates after mixture by qPCR and ELISA assay.

N50 was calculated using hill sigmoidal curve fit (origin 8):

y = START + (END - START) * x^n / (k^n + x^n)

(START: max. y-data, END: min. y-data, n=2, k=x at y50)

10.5.7 Decoy assay

For determination of the decoy effect of wt and mutant capsids, $2x10^4$ HeLa cells were seeded in 48 well plates 24 h before infection. 10^5, $2x10^5$ or $4x10^5$ of either wt or mutant empty particles were added to10^4 wt genomic particles per cell. Incubation, infection and Cytofluorimetry were performed as described above (Infection Assays (Immune Escape Mutants)).

10.5.8 Heparin inhibition assay

24 h prior to infection $2x10^4$ HeLa cells per well were seeded in 48 well plates. $6x10^3$ genomic particles of wt or capsid modified GFP expressing vectors per cell ($6x10^4$genomic particles per cell for AAV1) were incubated in a total volume of 500 µl of DMEM/10% FCS in the presence or absence of 1.7% (85 I.E./ml) soluble heparin (5000I.E./ml, B. Braun Melsungen AG, Germany) at 37°C for 30 min.

This solution was then used to infect cells. 48 h post infection, the percentage of transduced cells was determined by Cytofluorimetry.

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12 Abbreviations

Amino acids

A	**alanine**
C	**cysteine**
D	**aspartate**
E	**glutamate**
F	**phenylalanine**
G	**glycine**
H	**histidine**
I	**isoleucine**
K	**lysine**
L	**leucine**
M	**methionine**
N	**asparagine**
P	**proline**
Q	**glutamine**
R	**arginine**
S	**serine**
T	**threonine**
V	**valine**
W	**tryptophan**
Y	**tyrosine**

Bases

A	**adenine**
C	**cytosine**
G	**guanine**
N	**A, C, G or T**
S	**C or G**
T	**thymine**

Abbreviations

AAV	**adeno-associated virus**
Ad	**adenovirus**
bp	**base pair**
CD	**cluster of differentiation**
CFTR	**cystic fibrosis transmembrane conductance regulator**
d	**day**
DNA	**deoxyribonucleic acid**
e.g.	**for example**
ELISA	**enzyme-linked immunosorbent assay**
FACS	**fluorescence-activated cell sorting**
GFP	**green fluorescent protein**
GOI	**“genomicity of infection”; viral genomes applied per cell**
h	**hour**
Hstk	**herpes simplex thymidine kinase**
HSPG	**heparan-sulfate proteoglycan**
I.E.	**international units**
IgG	**Immunoglobulin G**
IL	**Interleukin**
ITR	**inverted terminal repeat**
IVIG	**intravenous immunoglobulin**
Kb	**kilobases**
kDa	**kilo Dalton**
min	**minute**
µl	**micro liter**
ml	**milliliter**
N50	**amount of serum needed to halve the number of transduced cells**
Nabs	**neutralizing antibodies**
Nm	**nanometer**
NPC	**nuclear pore complex**
ORF	**open reading frame**
pfu	**plaque forming units**
qPCR	**quantitative polymerase chain reaction**
RBS	**Rep binding site**

rpm	**rounds per minute**
s	**second**
TGF□	**transforming growth factor □**
TLR	**toll-like receptor**
TRS	**terminal resolution site**
VP	**viral protein**
w/o	**without**
wt	**wild typ**

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Frau Dagmar Mörsdorf betreut worden.

Köln, den ....

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