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ADENO-ASSOCIATED VIRUS DISPLAY: IN VITRO EVOLUTION OF AAV RETARGETED VECTORS

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aus

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Erklärung:

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Büning H., Ried M., **Perabo L**., Gerner F., Huttner N., Enssle J. and Hallek M. *Receptor targeting of adeno-associated virus vectors*. Gene Therapy (2002) 10:1142.

CHAPTER I

INTRODUCTION

1.1. GENE THERAPY IN PERSPECTIVE

The development of suitable vectors for human gene therapy has been a challenging goal over the past decade, as the enormous potential of this approach has attracted the attention of increasing numbers of scientists (Fig. 1). To date, a wide variety of inherited as well as methabolic disorders is being targeted by clinical and pre-clinical trials in which several classes of viral and non-viral vectors are being exploited as tools for delivery of therapeutical genetic information into cells (Fig. 2 and Tab.1).

Although remarkable advances have been reported over the years, the ultimate success of gene therapy will depend on the ability of researchers to develop vectors that address a number of still unsolved problems. Every specific application field presents differentiated issues and problems, however, some of these are general and represent a common struggle for the scientific community engaged to develop such systems.

In the case of viral vectors, a major concern is the issue of safety. Three recent nephast events mined the field raising criticism among scientists and media, leading to a grinding halt for several viral gene therapy clinical trials in a number of different countries.

In 2001, Jesse Gelsinger, an 18 years old Ornithine Transcarbamylase (OTC) deficiency patient died in Pennsylvania, USA, after treatment with an adenoviral vector containing a normal copy of the OTC gene. The following investigation allowed to conclude that the subministred vector triggered an immunoreaction that lead to a multiple organ system failure and to the death of the patient. The host immunoresponse to the vector and to the carried transgenes is currently a major worry for gene therapists (Somia and Verma, 2000).

In 2002, in a clinical trial for X-linked Severe Combined Immunodeficiency, 2 out of 11 patients have developed T-cell Leukemia as a consequence of the administration of a retroviral vector containing the common γ-chain gene for the cytokine receptors IL-2R, IL-4R, IL-7R, IL-9R and IL-15R. Although 9 of the patients experienced significant restoration of their immune system, the 2 adverse events reminded the scientific community of the risks of manipulating the human genome with agents capable of inserting foreign DNA sequences in the host chromosomes. As a result, similar clinical trials exploiting retroviruses as gene delivery vectors have been stopped in several countries including Germany. More details are available at http://www4.od.nih.gov/oba/RAC/Fact_Sheet.pdf

As a way to reduce some of the risks connected with the use of viral agents for gene therapy, the goal of generating tissue specific vectors has attracted considerable intellectual and financial resources, representing not only a safety issue, but also a way to increase the

efficiency of the therapy. A vector with the ability to infect and transduce only its target cell type, not only minimizes the risk associated with the transfer of potentially dangerous genes into other tissues, but also increases the concentration of the therapeutic gene product delivered to the ill tissue, maximizing the effect of the therapy and requiring lower doses of the vector.

Adeno-associated virus of type 2 (AAV-2) based vectors hold the potential to successfully adress many issues. AAV-2 is a non pathogenic infectious agent, it does not elicit a strong cellular immune response and does not integrate randomly in the host genome after infection. Moreover, previous studies demonstrated the potential for successful redirection of the tropism of these vectors (Bartlett *et al.*, 1999; Girod *et al.*, 1999b; Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Rabinowitz *et al.*, 1999; Ried *et al.*, 2002; Shi *et al.*, 2001; Shi and Bartlett, 2003; Wu *et al.*, 2000).

The goal of this work has been the establishment of a novel combinatorial approach for the generation of adeno-associated virus (AAV) vectors that infect target cells in a receptor specific manner.

On the basis of the efficacy and the potential suggested by our data, we anticipate that this technology will facilitate the development of gene transfer systems for clinical application.

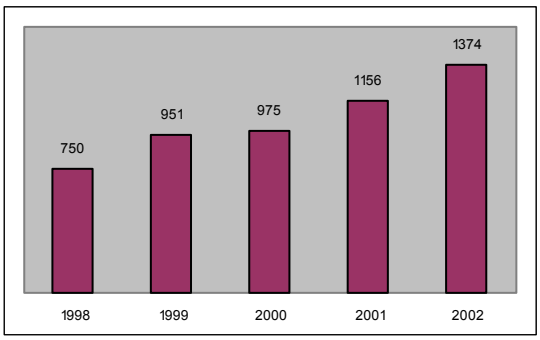


Fig.1: *Number of abstracts presented at the annual American Society of Gene Therapy Meetings.*

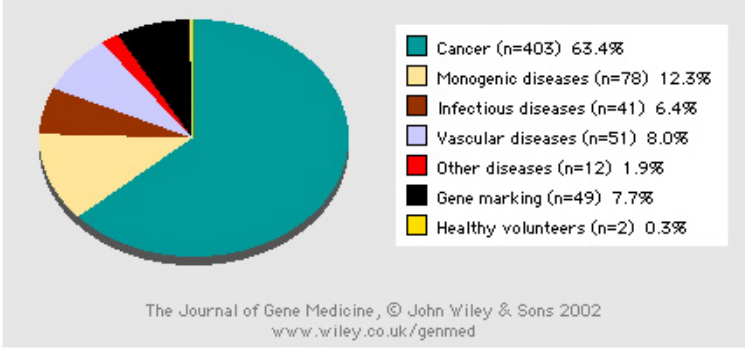


Fig. 2: *Diseases targeted by gene therapy protocols worldwide.*

Table 1. *Gene therapy clinical trials worldwide.*

Vector	Trials[1]	Example of diseases	pros / cons
Viral			
Adeno-associated virus	**15 (2.4%)**	**Cystic fibrosis, hemophilia B prostate cancer, neurological disorders, muscular dystrophy**	**+ high efficiency, stable gene expression (AAV, retroviruses)** **– low selectivity, risk of immunogenicity or toxicity, limited coding capacity (except HSV)**
Adenovirus	**171 (26.9%)**	**Many cancers, peripheral artery disease, cystic fibrosis, Canavan disease**	
Herpes simplex virus	**5 (0.8%)**	**Brain tumor, colon carcinoma**	
Poxvirus	**39 (6.1%)**	**Many cancers**	
Retrovirus	**217 (34.1%)**	**Many cancers, AIDS, SCID, rheumatoid arthritis, graft-versus-host disease, multiple sclerosis, osteodysplasie, hemophilia**	
Nonviral			
Gene gun[2]	**5 (0.8%)**	**Melanoma, sarcoma**	**+ high safety, unlimited coding capacity** **– low efficiency, low selectivity, transient gene expression**
Lipofection[3]	**77 (12.1%)**	**Many cancers, cystic fibrosis, coronary artery disease, restenosis**	
Naked DNA	**70 (11.0%)**	**Many cancers, peripheral artery disease, coronary artery disease, peripheral neuropathy, open bone fractures**	
RNA transfer	**6 (0.9%)**	**Many cancers**	
Other	**25 (3.9%)**		

[1] Number of open clinical trial world wide

[2] DNA coated on small gold particles and shot with a special gun into target tissue

[3] Includes liposomes and various packages of lipid, polymer, and other molecules

Source: http://www.wiley.co.uk/genmed/clinical/; www4.od.nih.gov/oba/rdna.htm

1.2. ADENO-ASSOCIATED VIRUSES

Adeno-associated viruses belongs to the family of parvoviridae. This family groups viruses with a linear, single-stranded DNA genome of approximately 5 kb and a non-enveloped, icosaedrical capsid with a diameter of 18-30 nm (Siegl *et al.*, 1985). This makes of parvoviruses the smallest known DNA viruses. Within this group, the adeno-associated viruses are classified in the genus of the Dependovirus (Lat. dependere: to depend), as they require exogenous factors for their replication. This distinguishes them from autonomous parvoviruses. Dependoviruses and autonomous parvoviruses infect vertebrates. Another group of parvoviruses, the densoviruses, infects insects and replicates autonomously.

To date, eight AAV serotypes (AAV-1, 2, 3, 4, 5, 6, 7 and 8) are known that share different levels of DNA sequence homology and display different tropism (Gao *et al.*, 2002; Lukashov and Goudsmit, 2001).

AAV-2 was discovered in 1965 as contamination of adenovirus preparations and therefore its name (Atchison *et al.*, 1965). Early findings indicated that concomitant adenovirus infection was required for AAV-2 to replicate its genome and give raise to a productive infection. Later other viruses (herpesviruses, vaccinia and papillomaviruses) were identified that could provide AAV-2 with this ability, as well as several chemical or physical factors like carcinogenic compounds, UV or γ-irradiation and heat shock (Berns, 1990; McPherson *et al.*, 1985; Sanlioglu *et al.*, 1999; Schlehofer *et al.*, 1986; Thomson *et al.*, 1994; Walz *et al.*, 1997; Yakinoglu *et al.*, 1988; Yakobson *et al.*, 1987; Yalkinoglu *et al.*, 1991).

In the absence of such helper factors, the viral DNA integrates stably into the host cell genome after the infection giving raise to a latent infection (Berns and Linden, 1995). In the presence of the Rep viral protein, the integration of the AAV-2 genome takes place site specifically in the q arm of human chromosome 19 (Kotin *et al.*, 1991; Kotin *et al.*, 1990; Linden *et al.*, 1996a; Linden *et al.*, 1996b; Ponnazhagan *et al.*, 1997a; Samulski *et al.*, 1991; Weitzman *et al.*, 1994).

In the presence of helper factors, quiescent AAV genomes integrated in the host chromosomes can be excised and lead to a productive infection cycle (Berns *et al.*, 1975; Cheung *et al.*, 1980; McLaughlin *et al.*, 1988).

The mechanism of assembly of AAV particles is not known in detail. The newly synthesized VP proteins assemble to form empty capsids that associate with viral DNA. These intermediate structures become mature infectious virions in a few hours (Myers *et al.*, 1980; Wistuba *et al.*, 1997b).

The production of single stranded viral DNA (ssDNA) in the host cell is coupled to the synthesis of capsid proteins. In the absence of these proteins, double stranded (ds) AAV replication intermediates can be detected in the cell but no ssDNA molecules can be produced (de la Maza and Carter, 1980). ssDNA excised from the cellular genome is copied to a ds replicative form (RF1) that gives raise to ssDNA or to a higher replictive form (RF2).

Integration of the viral DNA or production of virions (respectively in the absence or presence of helper factors) can be reproduced after transfection of plasmids encoding the viral genome (Laughlin *et al.*, 1983; Samulski *et al.*, 1982), creating the basis for the production of AAV in laboratory and for the generation of AAV based vectors.

1.3. GENOME ORGANIZATION OF AAV

The 4.7 kb DNA genome of AAV can be divided in three functional regions (Fig. 3):

- The terminal inverted repeats (ITRs) of 145 bp each, serve as replication start (Berns, 1990). Moreover, they are reported to play roles in the regulation of viral gene expression and in the integration and excision of the AAV genome in and from the cellular chromosomes (Labow and Berns, 1988; McLaughlin *et al.*, 1988).

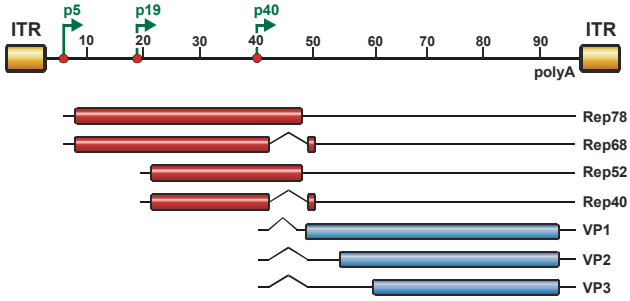


Fig. 3: *Organization of the AAV genome and gene products.*

- The 5' located *rep* open reading frame codes for a family of four multifunctional non-structural proteins (Rep). The mRNAs coding for Rep78 and its splicing variant Rep 68 start at the p5 promoter and are 4.2 and 3.9 kb long respectively. The mRNAs coding for Rep52 and its splicing variant Rep40 start at promoter p19 and are 3.6 and 3.3 kb long respectively. (Lusby and Berns, 1982; Marcus *et al.*, 1981). Proteins Rep78, 68, 52 and 40 consist of 621, 537, 397 and 313 amino acids respectively (Mendelson *et al.*, 1986).
- The 3' located *cap* open reading frame codes for the three structural capsid proteins VP1, VP2 and VP3 that are transcribed from the p40 promoter and expressed at a 1:1:8 ratio (Kronenberg *et al.*, 2001). The efficiency of the translation is regulated by the alternative splicing of the VP1 coding intron and by the use of an unusual initiation codon (ACG) for VP2 that leads to a 10-fold reduced translation of the

protein in comparison to the AUG intiation codon of VP3 (Becerra *et al.*, 1988; Laughlin *et al.*, 1979). All three capsid proteins use the same stop codon. The molecular weights of VP1, VP2 and VP3 are 90, 72 and 60 kDa. The half-life of the viral proteins is of approx. 15 hours while that of the mRNAs is of approx. 4-6 hours (Carter and Rose, 1974).

1.4. STRUCTURAL AND FUNCTIONAL PROPERTIES OF THE PARVOVIRUS CAPSID PROTEINS

Recently the atomic structure of AAV has been determined to 3 Å resolution by x-ray crystallography (Xie *et al.*, 2002). It was the first structure of a dependovirus to be determined. While the atomic structure of related autonomous parvoviruses, including canine parvovirus (CPV), feline panleukopenia virus (FPV), minute virus of mice (MVM), Aleutian mink disease parvovirus (ADV), and the human parvovirus B19, has been resolved during the past decade, the three-dimensional structure of the AAV capsid remained still unknown (Agbandje *et al.*, 1994; Agbandje-McKenna *et al.*, 1998; Chang *et al.*, 1992; Chapman and Rossmann, 1993; Chipman *et al.*, 1996; McKenna *et al.*, 1999; Strassheim *et al.*, 1994; Tsao *et al.*, 1991). Instead, alignments of these related parvoviruses with AAV had led to hypothetical models of the AAV capsid. Random and systematic mutagenesis approaches helped to map functional sites on the capsid, e.g. putative binding sites for the primary receptor HSPG, immunogenic epitopes and flexible loop regions at the capsid surface that accept the insertion of targeting ligands (Girod *et al.*, 1999b; Rabinowitz *et al.*, 1999; Wobus *et al.*, 2000b; Wu *et al.*, 2000). Now that the crystal structure has been resolved, function can be mapped to the structure.

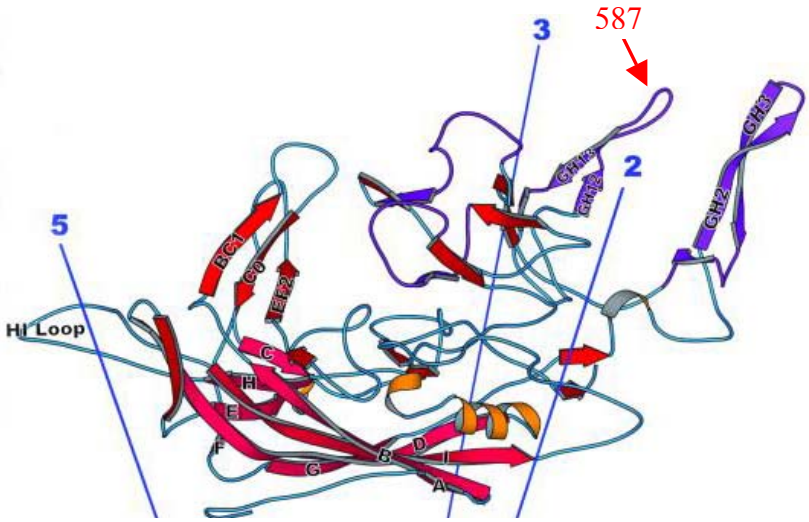


Fig. 4: *3D structure of the VP3 capsid protein (Xie et al., 2002). See text for details. The 587 site is indicated by the arrow (see "Targeting of viral vectors " paragraph).*

Striking similarities but also notable differences exist between AAV and related autonomous parvoviruses. Each viral capsid is composed of 60 subunits arranged with T=1 icosahedral symmetry (Xie *et al.*, 2002). The three structural proteins VP1, VP2, and VP3,

which share overlapping sequences and differ only at their N-termini, build the AAV capsid with a relative stoichiometry of about 1:1:8 (Kronenberg *et al.*, 2001). Each subunit (Fig. 4) has a β-barrel motif consisting of two anti-parallel β-sheets, which is highly conserved among parvoviruses. This β-barrel motif forms elongated smooth lumps at the inner surface of the capsid at the 2-fold symmetry axis (Kronenberg *et al.*, 2001).

Between the strands of the β-barrel core large loop insertions are found that share only low similarity among the parvovirus family (Fig. 5). These loops comprise two-thirds of the capsid structure and constitute the capsid surface features that interact with antibodies and cellular receptors. These surface features include a hollow cylinder at the 5-fold axis of symmetry which is surrounded by a circular depression (canyon), and a depression spanning the 2-fold axis (dimple). The most prominent features of the capsid are the 3-fold-proximal peaks, which cluster around the 3-fold symmetry axis. The peaks are not derived from one capsid subunit protein but from the interaction of two adjacent subunits. The sequences that compose these structures belong to the GH loop (between the β-sheets G and H) which is, with approximately 220 amino acids, the longest loop insertion. Other interactions between loops of neighboring subunits are found at the 5-fold cylinder, where amino acids from the HI loop interact with residues from the BC and EF loop. The N-terminal unique regions of VP1 and VP2 could not be resolved by X-ray crystallography, because of low electron density. Kronenberg and coworkers, who performed cryo-electron microscopy assumed that globular

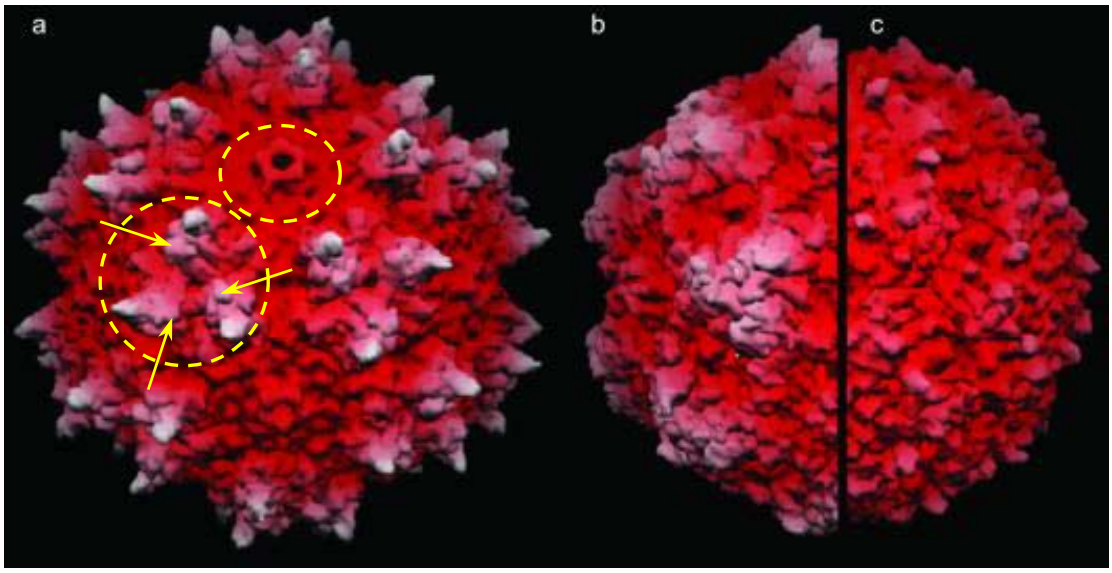


Fig. 5: *Comparison between surface topologies of AAV-2 (a), CPV (b) and insect densovirus (c) (Xie et al., 2002). The capsids are drown to scale and colored according to distance from the viral center. The circles highlight the 3 (larger circle) and 5-fold (smaller circle) simmetry regions. The arrows indicate the position of the 587 site*

structures at the inner surface of the capsid at the 2-fold axes of symmetry, which are attached to the smooth lumps, represent the positions for the N-terminal extensions of VP1 and VP2.

At the 3-fold proximal peaks, in the valleys separating the three peaks of one 3-fold axis are located clusters of positive charges (Fig. 6), which are implicated in receptor binding. Although no definitive HSPG binding motif has been found on the capsid surface so far, mutational analyses have identified these locations being involved in binding to the primary receptor HSPG (Wu *et al.*, 2000). Especially the basic aa R487, R585, R588 and H509, which are at the side of the peak, seem to play a crucial role (numbers refer to VP1 numbering) (Grifman *et al.*, 2001; Wu *et al.*, 2000; Xie *et al.*, 2002). Interestingly, the separation between these clusters at the side of the peaks is 20 Å, consistent with binding neighboring disaccharides (Margalit *et al.*, 1993), whereas the separation between threefolds is ~ 70 Å, commensurate with the spacing of highly sulfonated regions of the carbohydrate (Fry *et al.*, 1999). Regions of negative charges are mainly found at the top of the 5-fold cylinder and at the sides of the 2-fold dimple facing the 3-fold region. The role of the threefold-proximal region as receptor binding site is supported by the fact that the neutralizing monoclonal antibody C37-B, which inhibits binding of AAV to the host cell, has its epitope adjacent to these residues in the three-dimensional structure, namely between aa 492 and 503 at the shoulder of a peak (Wobus *et al.*, 2000b). The epitope of another neutralizing antibody, A20, could also be mapped to the 3-fold spike region. It is situated in the valley between the peaks of one 3-fold axis (Wobus *et al.*, 2000b). In contrast to C37-B, A20 does not block receptor binding but neutralizes AAV infection at a post-binding step (Wobus *et al.*, 2000b), possibly by interfering with internalization, endosomal release or viral uncoating. These data suggest 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 additionally located at this prominent feature.

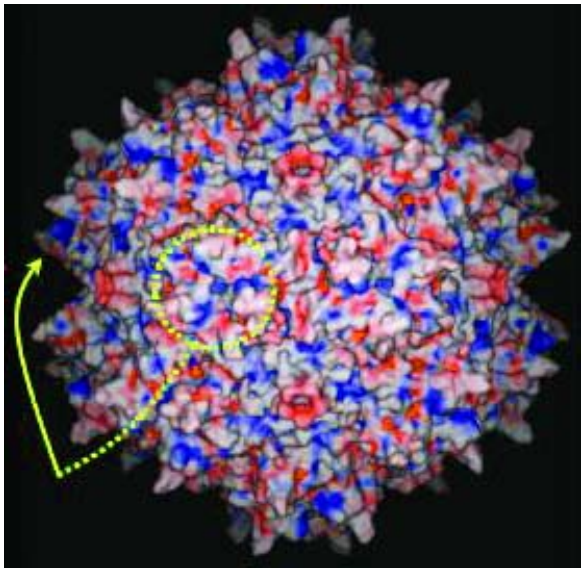


Fig. 6: *Electrostatic surface potential of AAV-2 capsid (Xie et al., 2002). The potential runs from negative values (red) to positive (blue). Putative receptor binding sites are positively charged patches on the side of each threefold-proximal peak and are indicated edge-on by the arrow or head-on by the circle.*

1.5. INFECTION BIOLOGY OF AAV-2

The adeno associated virus of type 2 infects the host cell through a mechanism that involves at least two initial steps:

- Binding of the virion to the cellular membrane
- Internalization of the virion through clathrin coated pits endocytosis

These two steps of the infection process seem to be mediated by two different classes of cellular receptors. A primary receptor is responsible for the virus recognition of the target cell and it's binding. A secondary receptor triggers the cascade of events leading to endocytosis and internalization of the viral particles.

Heparan sulfate proteoglycan (HSPG) has been identified as primary receptor for AAV-2 (Summerford and Samulski, 1998). HSPG is a widely expressed molecule among human cell types, explaining the broad tissue range of AAV-2.

As secondary receptors the $\alpha_v\beta_5$ integrin, the human Fibroblast Growth Factor Receptor (hFGFR1) and nucleolin have been described (Mizukami *et al.*, 1996; Qing *et al.*, 1999; Qiu and Brown, 1999a; Summerford *et al.*, 1999). While integrin is involved in endocytosis, hFGFR1 seems to additionally enhance the attachment process. However, there have been also conflicting results about the contribution of these co-receptors (Qiu and Brown, 1999b).

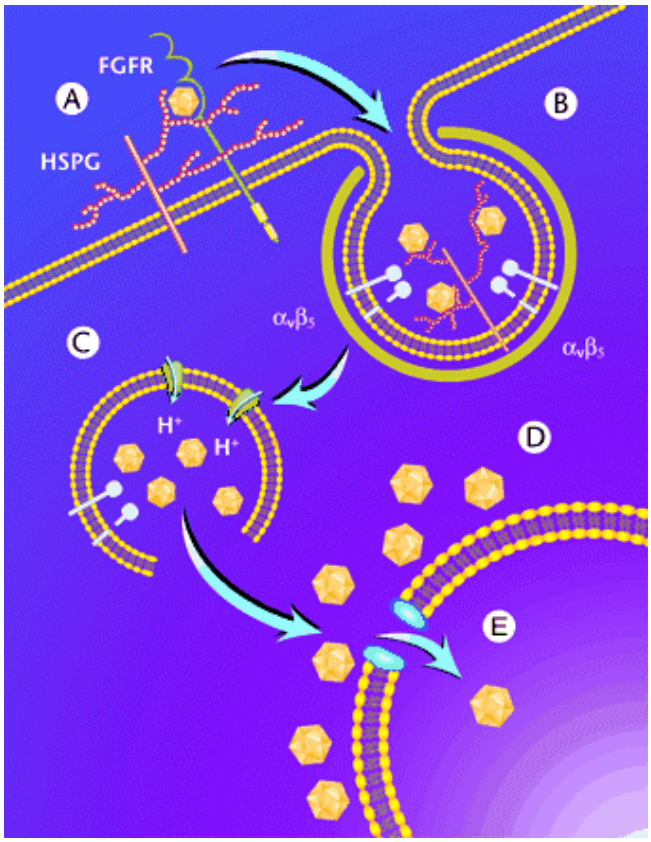


Fig.7: *Proposed model for the infection of AAV-2 (Bartlett et al., 2000b). See text for details.*

A correlation between the permissivity of a given cell type to AAV-2 and the ability of the virus to bind to the cell has been reported, so that if binding does not occur, AAV-2 is not able to infect the cell even in the presence of secondary receptors (Ponnazhagan *et al.*, 1997b).

This observation may explain the inability of AAV-2 to infect many hematopoietic cell types. AAV-2 fails to bind on those cells

(Ponnazhagan *et al.*, 1997b; Russell and Kay, 1999), probably due to the lack of HSPG on their surface. Receptor mediated endocytosis via clathrin coated pits seems to be the predominant, but not exclusive, AAV entry pathway. A possible model postulates that $\alpha_v\beta_5$ integrin clustering facilitates the localization of virus particles to coated pits, similarly to adenovirus. In the subsequent internalization process dynamin, a 100 kDa cytosolic GTPase, is involved (Bartlett *et al.*, 2000b; Duan *et al.*, 1999; Wang *et al.*, 1998). Specifically, oligomerization of dynamin into a ring structure is required for the formation of clathrin coated vesicles and subsequent pinching of coated pits from the cell membrane (Hinshaw, 2000; Hinshaw and Schmid, 1995; Sever *et al.*, 2000). Additionally, integrin clustering has been shown to activate Rac1, a GTP binding protein, which facilitates internalization. Subsequently, activation of Rac1 leads to activation of PI3K signaling pathways (PI3K, phosphatidylinositol-3 kinase) which are required for efficient trafficking of virus containing endosomal vesicles along microfilaments and microtubules to the nucleus (Sanlioglu *et al.*, 2000). To exit the endosomal compartment a low pH is required (Douar *et al.*, 2001). In contrast to its natural helper virus, adenovirus, AAV particles are delivered to the late endosome before they are released into the cytoplasm (Douar *et al.*, 2001). The maturation of endosomes involves a progressive decrease of their internal pH. This acidic milieu may trigger conformational changes of the viral capsid, exposing domains which disrupt the endosomal membrane. Such a model is consistent with observations made for other viruses where confirmational change of the capsid following acidification of the endosome have been reported (Root *et al.*, 2000; Zadori *et al.*, 2001).

Interestingly, the unique region of the AAV VP1 protein contains a phospholipase A_2 (PLA_2) motif (HDXXY) which is conserved among parvoviruses (Girod *et al.*, 2002; Zadori *et al.*, 2001). Although nothing is known about the structural localization of this domain nor about its functional properties, the PLA_2 group together with other functions located in the VP1 unique region (DNA binding, nuclear localization) may be required for endosome exit and transfer of the viral genome to the nucleus.

Relatively little is known of how the virus enters the nucleus and where viral uncoating occurs. Several studies have observed a perinuclear accumulation and subsequent slow nuclear entry of fluorescent labeled viral particles (Bartlett *et al.*, 2000b; Sanlioglu *et al.*, 2000). In contrast, Seisenberger and coworkers observed by single molecule imaging a very quick transfer of viral particles to the nuclear area and no perinuclear accumulation (Seisenberger *et al.*, 2001). If viral particles localized in the nucleus are partially uncoated or otherwise modified remains unclear. Transport across the nuclear envelope has been

extensively studied for many cellular proteins and some viruses. Most models include a role for the nuclear pore complex (NPC) for transit into and out of the nucleus. The NPC allows macromolecules < 30-40 kDa to freely diffuse across the nuclear envelope and, in certain cases, it can open to accommodate particles as large as 28 nm (Kasamatsu and Nakanishi, 1998). For AAV transport across the nuclear envelope seems not to depend on active transport through nuclear pore complexes (NPC) (Xiao *et al.*, 2002). Moreover, all cellular factors required for uncoating and second-strand synthesis are contained within the nucleus (Hansen *et al.*, 2001).

1.6. PRODUCTION OF RECOMBINANT AAV VECTORS

The structural properties of the AAV capsid allow the production of recombinant viral particles that carry an up to approximately 4.5 kb long DNA molecule flanked by ITR sequences (Tal, 2000).

The production protocol takes advantage of the ability of the two viral gens (*rep* and *cap*) and of the ITR sequences to accomplish their role in the replication of the viral DNA and in the packaging of mature virions even when provided to the host cell in trans on exogenous plasmids (Laughlin *et al.*, 1983; Samulski *et al.*, 1982).

Therefore, transduction in a permissive cell of two plasmid species, one encoding the two viral genes (rep/cap plasmid), and the other coding for an exogenous DNA sequence flanked by ITR sequences (vector plasmid), leads to the production of viral particles containing a DNA molecule coding for the exogenous sequence flanked by the ITRs (Fig. 8). Given the absence of the packaging sequences on the rep-cap coding plasmid, no contamination of wt virus will be present in the final viral preparation. Another requirement of this procedure is the concomitant action of a helper factor for the replication of the viral genome. This can be provided co-infecting the cells with adenovirus. In this case however, the resulting viral preparation will contain adenovirus progeny.

Adenovirus free AAV preparations can be obtained by providing the helper function transfecting into packaging cells a third plasmid that contains the essential Ad helper genes (E4, VA and E2a) but lacks the Ad structural and replication genes (Collaco *et al.*, 1999; Grimm *et al.*, 1998; Xiao *et al.*, 1998).

AAV viral progeny can be harvested 48 h p.i. from the lysate of the transfected cells and purified to high titers (up to 10^{14} particles/ml) by one of several described protocols (Allen *et al.*, 2000; Anderson *et al.*, 2000; Auricchio *et al.*, 2001; Collaco *et al.*, 1999; Gao *et*

al., 2000; Gao *et al.*, 1998; Grimm and Kleinschmidt, 1999; Inoue and Russell, 1998; Kapturczak *et al.*, 2001; Liu *et al.*, 1999; Monahan and Samulski, 2000a; Monahan and Samulski, 2000b; Rolling and Samulski, 1995; Tamayose *et al.*, 1996; Vincent *et al.*, 1997; Xiao *et al.*, 1998; Zolotukhin *et al.*, 1999).

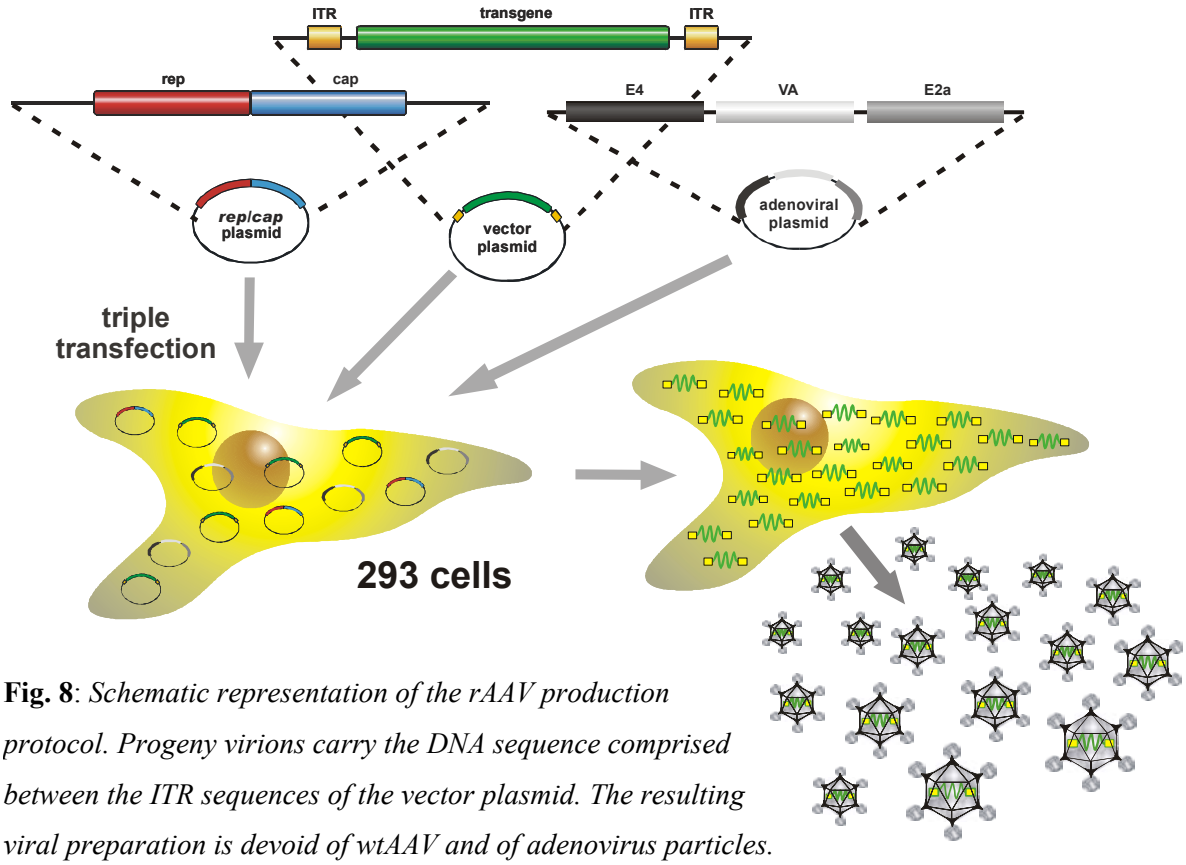


Fig. 8: *Schematic representation of the rAAV production protocol. Progeny virions carry the DNA sequence comprised between the ITR sequences of the vector plasmid. The resulting viral preparation is devoid of wtAAV and of adenovirus particles.*

1.7. AAV AS VECTOR FOR GENE THERAPY

In recent years, AAV-2 based vectors have received increasing attention as candidates for gene therapy of many genetic disorders (Grimm and Kleinschmidt, 1999; Hallek and Wendtner, 1996; Kotin, 1994; Monahan and Samulski, 2000a; Monahan and Samulski, 2000b; Tal, 2000; Wendtner *et al.*, 2002).

One of the main reasons is the safety of this vector: no known pathology has been related to AAV infection. Moreover, AAV vectors are replication deficient and fail to unleash cellular immune response (Hernandez *et al.*, 1999; Jooss *et al.*, 1998; Monahan and Samulski, 2000b). This has been attributed to the poor ability of AAV to infect dendritic cells, as well as to the lack of viral genes in the vector's genome. Hypothetical risk factors that are still not ruled out are the potential ability of AAV ITR sequences to activate cellular genes or

promoters following the integration of the viral DNA in the cellular genome (Blacklow *et al.*, 1971; Kotin, 1994) and the potential of AAV vectors to transduce the germinal line of the host (Burguete *et al.*, 1999; Rohde *et al.*, 1999).

The latter threat arises however from a characteristic of AAV that could be exploited to the advantage of gene therapists: infection of cells with AAV in the presence of the Rep protein, leads to site specific integration of the vector's genome into a locus on human chromosome 19 that does not seem to influence the biology of the cell (Hallek and Wendtner, 1996; Monahan and Samulski, 2000a; Monahan and Samulski, 2000b; Samulski *et al.*, 1991).

The *rep* gene is deleted from AAV vector's DNA to make space for the therapeutic gene, but several undergoing studies aimed to restore the site specific integration ability by providing the Rep protein in trans (Huttner *et al.*, 2003; Kotin *et al.*, 1990; Owens, 2002; Philpott *et al.*, 2002; Young *et al.*, 2000a; Young and Samulski, 2001; Young *et al.*, 2000b).

Another advantage is the ability of AAV vectors to lead to long term expression of the transgenes. Previous studies could show expression of the vector's genome after more than one year (Fisher *et al.*, 1997). Moreover, AAV vectors are able to infect non-dividing and post-mitotic cells.

AAV capsids are relatively stable, allowing high titer purification of the virions. Recent optimization of the vector's production technology allows nowadays to obtain recombinant viral preparations that contain up to 10^{10}-10^{11} infectious particles/ml (Grimm and Kleinschmidt, 1999).

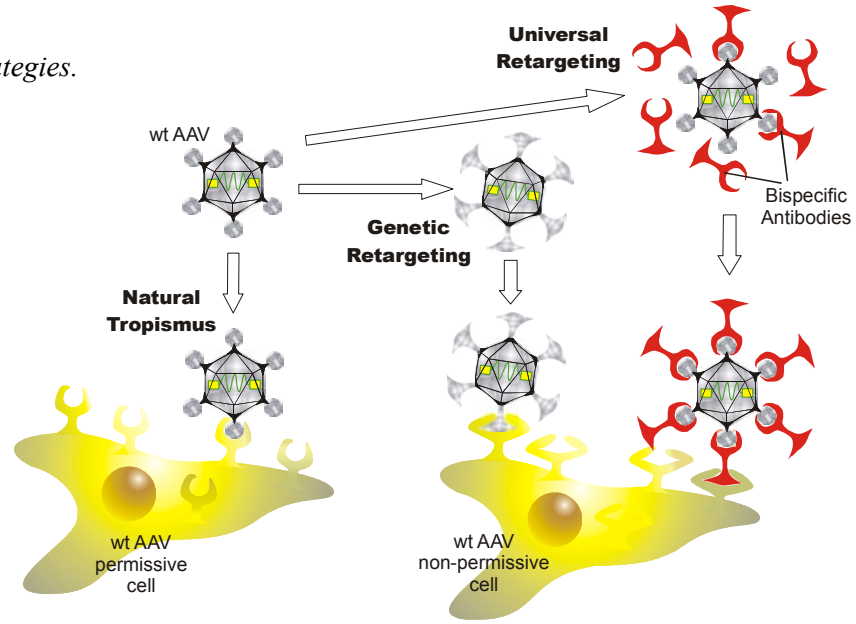
The small size of AAV capsids is responsible for the limited DNA packaging capacity. Traditional vectors can host DNA molecules not longer than approx. 4.5 kb. Recent reports however exploited the production of more capable vectors by the use of "splicing vectors". Briefly, a longer DNA sequence is splitted in two parts contained in two different viral particles. Appropriate splicing sequences drive the reconstitution of the intact DNA molecule in the infected cell's nucleus (Chao *et al.*, 2002; Duan *et al.*, 2001; Duan *et al.*, 2003; Halbert *et al.*, 2002).

1.8. TARGETING OF AAV VECTORS

Controlling the tropism of gene vectors (targeting) represents a critical goal in gene therapy to increase both the efficiency and safety of the gene transfer. This goal can be pursued by providing the virions with the ability to recognize and interact with specific receptor molecules that are present only on the surface of the targeted cells. This process is known as "vector re-targeting".

Two main strategies have been used to retarget AAV in previous reports: a) chemical cross-linking of bifunctional antibodies to the viral capsid; b) genetic modification of the viral capsid (Fig. 9).

Fig. 9: *Targeting strategies.*



Bartlett and co-workers targeted AAV-2 non-permissive human megacaryoblasts with a viral preparation that was preincubated with bifunctional antibodies that are able to bind both the capsid and the surface receptor $\alpha_{IIb}\beta_3$ integrin (Bartlett *et al.*, 1999). However, the levels of transduction of target cells were unsatisfactory to fulfill the expectations for an eventual clinical application. The lack of covalent binding between the capsid and the targeting molecule is responsible for the low stability of the capsid-antibody complex. At the same time, the interaction between the viral particle and the antibody is prone to affect negatively the overall functionality of the virion.

Studies conducted in our laboratory by Anne Girod and Martin Ried (Girod *et al.*, 1999b) have opened the road for the genetic re-targeting of AAV based vectors. Since the crystal structure was not known by that time, they aligned the sequence of the AAV-2 VP1 protein with the homologous protein of CPV (for which the structure was known) and other parvoviruses. Six capsid sites that were highly variable among parvoviruses and were believed to be expressed in flexible regions exposed toward the outer surface of the capsid, were tested as retargeting sites by the insertion of an L14 motif which contains an RGD sequence and is known to bind to $\alpha_V\beta_1$ integrins (Aumailley *et al.*, 1990). This studies led to the description of a locus on the *cap* gene where insertion of an exogenous ligand provides the resulting virus with the ability to infect cell types that are non-permissive to wt AAV infection but express on their surface the receptors recognized by the inserted ligand. This site

is located at amino acid position 587 of the capsid protein. In the last three years this locus has become the site of choice for the insertion of re-targeting sequences in the AAV-2 capsid (Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Ried *et al.*, 2002; Shi *et al.*, 2001; Shi and Bartlett, 2003; Wu *et al.*, 2000).

The choice of appropriate ligands to be inserted at this site for the production of receptor-specific vectors relies on our knowledge of peptides with affinity for the targeted receptors. However, for many clinically interesting cell types, no cell type specific receptors and ligands are known.

Several groups reported in the last two years the application of the phage display technology to sort sequences with desired biological properties out of combinatorial peptides libraries, and subsequently introduced these sequences in the re-targeting site of the vector (Engelstadter *et al.*, 2000; Grifman *et al.*, 2001; Nicklin *et al.*, 2001). Although some positive results were reported, this approaches suffer from an intrinsic limitation: re-targeting molecules that display attractive biological properties in an exogenous environment, or selected in the architectural context of a phage particle, when introduced in the physical structure of the final vector are prone to destabilize the capsid or to lose their biological properties, leading to a loss in the vector's titer in the first case and to low transduction rates in the second case.

To circumvent all these problems we established a new technology, which we called "AAV Display", for the selection of re-targeting molecules directly in the vector's architectural context and cellular milieu where the molecules will be required to work. This approach does not require any information about the receptors expressed on the surface of the target cells and can be theoretically adapted for any type of viral vector.

CHAPTER II

SPECIFIC GOALS OF THIS WORK

The primary goal of this work was to demonstrate that efforts to re-target AAV vectors could take considerable advantage of the potential of combinatorial approaches.
This goal has been achieved through the successful accomplishment of the following steps:

- Production of a combinatorial library of AAV capsid variants through random insertional mutagenesis. A pool of randomly synthesized molecules was inserted at amino acid position 587 of the VP1 capsid protein as this site has proven to be suitable for the retargeting of AAV. The viral library was generated and purified by conventional AAV-production procedures and characterized statistically to assess the biodiversity and the random nature of the insertions.

- Establishment of an experimental procedure to screen this library for clones with desired tropism characteristics. The protocol has been adapted for the selection of mutants that were able to infect and produce viral progeny in wtAAV non-permissive target cells. Briefly, this procedure comprehends the infection of target cells with the viral library, the harvest of the eventually produced viral progeny and the infection of a new batch of target cells with this progeny (second selection round). The re-iteration of these infection/harvest rounds drives the selection of the capsid variants initially present in the library, that are better able to undergo any step of a productive infection (binding, entry, escape from the endosome, transport to the nucleus, nuclear internalization, replication and packaging of the progeny DNA into newly synthesized capsids) in the target cells system.

- Application of this procedure for the identification of viral clones with the ability to infect, in a receptor-specific manner, cell types that are non-permissive to wilde type AAV infection. As cellular targets, two hematopoietic cell lines were used (M07e and Mec1 cells). For each cell line, two 587 site-inserted sequences were identified.

- Characterization of the selected mutants and demonstration of the retargeting. The identified sequences were cloned into plasmids for the production of Green Fluorescent Protein expressing rAAV. Viral clones that expressed GFP as marker and that carried the retargeting sequences were produced and used to assess transduction rates on target cells, demonstrating the achievment of highly efficient transduction of these non-permissive cell type in a receptor-specific manner.

The results of these experiments were accepted for publication on March 22nd 2003 [**Perabo L**., Büning H., Kofler D., Ried M., Girod A., Wendtner C., Enssle J. and Hallek M. *In vitro selection of viral vectors with modified tropism: the adeno-associated virus display*. Mol. Ther. (2003) 8:151-157]. The description of the experimental procedure, results and their discussion are provided in this thesis in the form of the original publication.

One of the re-targeting viral clones (Mec-A) identified by screening of the AAV Display on Mec1 cells demonstrated the ability to escape neutralization by human serum antibodies [Huttner N., Girod A., **Perabo L**., Edbauer D., Büning H. and Hallek M. *Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies*. Submitted to Gene Ther. (2003)].

Preliminary experiments to assess the permissivity of B-CLL cells and B-CLL cell lines to infection by AAV have contributed to investigate the potential of AAV vectors for application into gene therapy of B-cell Chronic Lymphocytic Leukemia [Wendtner C.M., Kofler D.M., Theiss H.D., Kurzeder C., Buhmann R., Schweighofer C., **Perabo L**., Danhauser-Riedl S., Baumert J., Hiddemann W., Hallek M. and Büning H. *Efficient gene transfer of CD40 ligand into primary B-CLL cells using recombinant adeno-associated virus (rAAV) vectors*. Blood (2002) 100:1655-61].

The state of the art of in the field of AAV retargeting is summarized in a review published by our group during my PhD training [Büning H., Ried M., **Perabo L**., Gerner F., Huttner N., Enssle J. and Hallek M. (2002). *Receptor targeting of adeno-associated virus vectors*. Gene Ther. (2002) 10:1142].
Data regarding the AAV-Display are not included in this article.

All these manuscripts are also provided in this thesis in the form of the original publication or submission.

The work on the AAV-Display was presented at the 5th international meeting of the American Society of Gene Therapy and awarded with the “Excellence in Research Award”.
Also the presentation of this results received the 2nd price at the “young Masters Tournament” during the 2002 Congress of the German, Swiss and Austrian Society for Hematology and Oncology.

CHAPTER III

IN VITRO SELECTION OF VIRAL VECTORS WITH MODIFIED TROPISM: THE ADENO-ASSOCIATED VIRUS DISPLAY

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Perabo L., Büning H., Kofler D., Ried M., Girod A., Wendtner C., Enssle J. and Hallek M.

Abstract

Improving the efficiency and specificity of gene vectors is critical for the success of gene therapy. In an effort to generate viral mutants with controlled tropism we produced a library of adeno-associated virus (AAV) clones with randomly modified capsids and used it for the selection of receptor targeting mutants. After several rounds of selection on different cell lines that were resistant to infection by wild-type (wt) AAV, infectious mutants were harvested at high titers. These mutants transduced target cells with an up to 100 fold increased efficiency, in a receptor-specific manner and without interacting with the primary receptor for wt AAV. The results demonstrate for the first time that a combinatorial approach based on a eukaryotic virus library allows to generate efficient, receptor specific targeting vectors with desired tropism.

Introduction

A successful targeting of gene vectors is important to increase the efficiency and safety of gene therapy, since it would allow to restrict the gene transfer into the desired tissue (Kay *et al.*, 2001; Pfeifer and Verma, 2001). Towards this goal, several attempts have been made to modify the capsids or envelopes of viruses in order to enforce the interaction of viral particles with specific cellular receptors (Baranowski *et al.*, 2001; Curiel, 1999; Lavillette *et al.*, 2001; Romanczuk *et al.*, 1999). In the case of AAV, a parvovirus considered as one of the most promising vectors for human gene therapy (Monahan and Samulski, 2000a), it is possible to redirect its tropism by genetic capsid modifications (Baranowski *et al.*, 2001; Girod *et al.*, 1999b; Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Wu *et al.*, 2000). This approach is based on the introduction of sequences conferring the ability to bind particular cellular receptors at an appropriate site of the *cap* gene. In particular, we have demonstrated that the insertion of an integrin binding Arg-Gly-Asp (RGD) motif at amino acid position 587 of the capsid protein VP1 enabled AAV particles to infect cells via $\alpha_V\beta_1$ integrin (Aumailley *et al.*, 1990; Girod *et al.*, 1999b). However, such modifications of the capsid may interfere with the stability of the virion, resulting in impaired function. In addition, when inserted into the architecture of the AAV viral capsid, heterologous receptor binding sequences could lose the biological properties they were chosen for. Moreover this approach requires an optimal three-dimensional fit of each inserted ligand. Therefore, we reasoned that these limitations might be overcome by a combinatorial approach, where a large number of randomly generated capsids is screened in the structural context of the vector and on the desired target cell.

Materials and Methods

Plasmids and Viruses. For the construction of plasmid pWt.oen, the HCMV promoter/enhancer cassette and the GFP open reading frame in the plasmid pEGFPC-1 (Clontech, Palo Alto, California) were substituted with the wt AAV-2 genome encoding fragment of plasmid pUC-AV2 (Girod *et al.*, 1999b). A DNA fragment encoding amino acids AAAstopA and the restriction sites NotI and AscI was inserted between amino acid position 587 and 588 by PCR mutagenesis. To generate a library of AAV plasmids (p587Lib7) a pool of single strand DNA molecules was synthesized as 5'-TTGGCGCGCCGCVNNVNNVNNVNNVNNVNNVNNGGCGGCCGCTTTTTTCCTTGA-3' (bottom strand) and HPLC purified (Metabion GmbH, Martinsried, Germany). For the synthesis of double-stranded molecules a 5'CTCAAGGAAAAAAGC3' primer was used. dsDNA molecules were cloned into the AscI-NotI large fragment of plasmid pWt.oen. p587Lib7was electroporated into E. coli strain DH5α using a Gene Pulser (Biorad, Hercules, California) and amplified DNA was purified. The efficiency of the transformation was controlled by plating sample aliquots. DNA of more than 20 clones was controlled by sequencing with the primer 4066Back (5'-ATGTCCGTCCGTGTGTGG-3'). The screened sequences showed no homology, indicating a complete randomization of the inserted sequence. Plasmids pRC, pXX6 (obtained from J. Samulski, Chapel Hill, North Carolina) and psub/CEP4/EGFP were previously described (Gao *et al.*, 1998; Girod *et al.*, 1999b; Wendtner *et al.*, 2002).

For the production of viruses, 15 150 mm Petri-dishes of 293 cells at 80% confluence were co-transfected with 37.5 µg of DNA. For the production of the AAV library, p587Lib7 and plasmid pXX6 were co-transfected at a molar ratio of 1:1.

The size of the viral library was calculated statistically on the basis of the ligation efficiency (number of bacterial colonies obtained by seeding a small aliquote of the electroporation solution) of the randomized DNA oligos inserted at position 587. The number of viral clones represented by this number of plasmid clones was calculated as (no. of DNA clones x 0.3) to take into account the degeneration of the genetic code. Since the titer of the viral preparation obtained by transfection of this pool of plasmids into 293 cells did not differ from the titer obtained for a control wt virus preparation (approx. $4x10^{11}$/ml, data not shown) performed by transfection of an identical amount of AAV plasmid DNA, we assumed that the diversity of the library was not significantly diminished during the packaging procedure.

It is important to note that only a minimal part of the combinations theoretically generated by randomization of a sequence of 21 nucleotides was represented in the recovered viral library. A further optimization of the cloning process would allow to enhance the diversity of the viral library and therefore the probability to isolate clones with desired biological properties.

For the production of rAAV-wt, the cells were co-transfected with vector plasmid psub/CEP4/EGFP, packaging plasmid pRC and adenoviral plasmid pXX6 at a molar ratio of 1:1:1. For the production of the capsid modified GFP expressing rAAV mutants, pRC plasmids modified to contain the appropriate NotI-AscI retargeting insertion were used. L14-AAV was produced using plasmid pI-587 instead of pRC (Girod *et al.*, 1999b). After 48 hrs cells were collected and pelleted by centrifugation. Cells were resuspended in 150 mM NaCl, 50 mM Tris-HCl (pH 8.5), freeze-thawed several times, and treated with Benzonase (50U/ml) for 30 min at 37°C. Cell debris was removed by centrifugation, supernatant was loaded onto an iodixanol gradient and subjected to 69000 rpm for 1 hr at 18°C as described (Zolotukhin *et al.*, 1999). Virions were then harvested from the 40% iodixanol phase and titrated by DNA dot-blot hybridization with a *rep* or a *gfp* probe (Girod *et al.*, 1999b).

Cell culture. HeLa cells (human cervix epitheloid carcinoma, ATCC CCL 2), M-07e cells, a human megakaryocytic leukemia cell line (obtained from James D. Griffin, Boston, Massachussets), Mec1, a cell line derived from a patient with B-CLL in prolymphocytoid transformation (obtained from Federico Caligaris-Cappio, Torino, Italy), CO-115 cells (human colon carcinoma), and 293 cells (human embryonal kidney) were maintained in Dulbecco's modified Eagle's medium (DMEM) (HeLa and 293), DMEM/NUT.Mix.F-12 medium (CO-115), RPMI medium (M-07e) or Isocove's medium (Mec1) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml), and L-glutamine (2 mM), at 37 °C and 5% CO_2. For M-07e cells, 10 ng/ml interleukin 3 (IL-3) was added to the medium.

Peripheral blood was obtained with informed consent from four patients with an established diagnosis of B-CLL. Mononuclear cells were isolated on a Ficoll/Hypaque (Seromed, Berlin, Germany) density gradient by centrifugation, depleted of monocytes by adherence to plastic tissue culture flasks and cultivated in Isocove's medium supplemented as for Mec1 cells. More than 98% of isolated cells co-expressed CD5 and CD19 as assessed by flow cytometry, therefore non-malignant B cells did not constitute a meaningful fraction of the total cells isolated. Patients were either untreated or had not received cytoreductive

treatment for a period of at least one month before investigation and were clinically stable and free from infectious complications.

Determination of transduction efficiencies. Cells were seeded in 96 or 24 well plates (Nunc, Wiesbaden, Germany) and infected with rAAV-GFP clones, harvested 48 hrs p.i., washed and resuspended in 1 ml PBS. The percentage of GFP expressing cells was determined by flow cytometry with a Coulter Epics XL-MCL (Beckman Coulter, Krefeld, Germany). A minimum of 5000 cells were analyzed for each sample. Infectivity of the retargeted mutants was determined in the presence or absence of various concentrations of GRGDTP or GRGES peptides (Bachem, Bubendorf, Swiss), GENQARS or QNEGSRA peptides (Dr. Arnold, Genzentrum München, Germany) or 5 I.U./µl soluble heparin (Braun, Melsungen, Germany).

Selection of AAV-2 retargeted mutants. 10^7 target cells were co-infected with 1000 genomic library particles/cell and with adenovirus at an MOI of 20 and incubated at 37°C. 2 hrs p.i. cells were pelleted by centrifugation, resuspended in fresh culture medium and incubated at 37°C. 48 hrs p.i., cells were rinsed with 5 ml PBS, resuspended in 5 ml of lysis buffer (150 mM NaCl, 50 mM Tris/HCl, pH 8.5) and lysed through 3 freeze/thaw cycles. Cellular debris was removed by centrifugation and the supernatant was used to infect the next batch of target cells (second round of infection). After each selection round viral DNA was purified from a 100 µl aliquot of the crude lysates by phenol/chloroform extraction and the 587 region was sequenced (primer 4066-back).

Results and discussion

Production and screening of an AAV Library. We generated a library of 4 x 10^6 capsid modified viral particles carrying random insertions of 7 amino acids at the position 587 (Fig. 10; see Methods). The choice of a 7-mer for the insertion was empirical and dictated by the need to insert a sequence long enough to generate an acceptable amount of diversity but without impairing the packaging efficiency of the viral particles. The 587 site was originally identified as retargeting site on the basis of alignment studies with the canine parvovirus capsid protein (Girod *et al.*, 1999b). The recent publication of the three-dimensional structure of AAV showed that the position 587 is located in the proximity of the 3-fold simmetry axis of the capsid, protruding from the external surface of the virion and facing the inside of a

crown-like structure that is believed to constitute the major receptor-binding site (Xie *et al.*, 2002). This confirmed that the 587 site is an appropriate site for the retargeting of AAV.

The library of capsid mutants was subjected to repeated cycles of infection and harvesting of the viral progeny from the target cells (Fig. 1). Virions with impaired ability to enter the cells were removed by changing the culture medium 2 hrs post infection (p.i.). Viral progeny was extracted from the cells 48 hrs p.i. by freeze/thaw cycles and used to infect a new batch of target cells in a new selection round. After each harvest, a small aliquot (100 µl) of the crude lysate was used to extract viral DNA. By titrating this DNA and sequencing the 587 region it was possible to monitor the evolution of the library (Fig. 2). The selective pressure provided by the culture environment drove the selection by means of the ability of the viral clones to accomplish every step in the infection process, namely binding, uptake, uncoating, nuclear translocation, replication and gene expression.

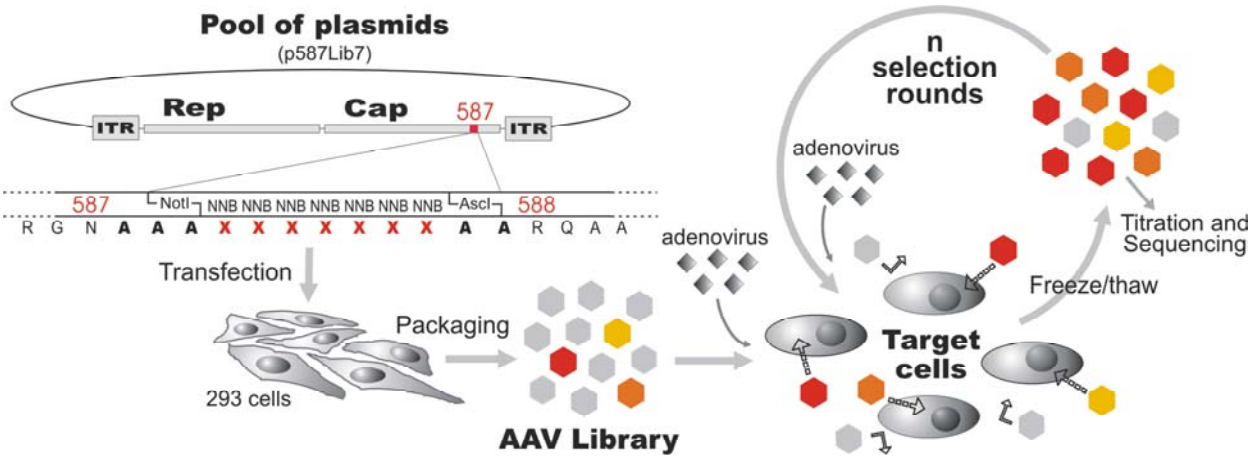

Fig. 1: *Schematic representation of the construction of the library of AAV-2 capsid modified particles and selection protocol for the isolation of retargeted mutants. A pool of randomly generated oligonucleotides was cloned into an AAV-2 genome encoding plasmid at the site corresponding to amino acidic site 587 of capsid protein VP1. Following a standard AAV production protocol (for details see Material and Methods), a library of approximately 4 x 10^6 capsid modified AAV-2 clones was generated. For the selection of retargeted mutants, target cells were co-infected with the pool of AAV-2 mutants and with adenovirus. The viral progeny collected 48 hrs p.i. was used for the next infection round.*

Identification of selected viral mutants. The potential of the AAV display system for the generation of retargeted mutants was tested on two cell lines that are resistant to wt AAV-2 infection. M-07e is a human megakaryocytic cell line (Avanzi *et al.*, 1988). Failure of AAV-2 to infect these cells has justified the use of this cell line as negative control in several reported AAV-2 infection experiments (Bartlett *et al.*, 1999; Ponnazhagan *et al.*, 1997b). Mec1 is a cell line derived from B-cell chronic lymphocytic leukemia (B-CLL) cells in prolymphoid transformation (Stacchini *et al.*, 1999), which is also poorly transduced by vectors based on AAV-2. A typical selection is depicted in Fig. 2. The amount of viral DNA detected in the crude lysates and the analysis of the sequence showed that the number of recovered virions increased after each round, while the heterogeneity of the pool was progressively lost. After 5 rounds only one single clone was present in the viral progeny. Application of $2x10^{10}$ viral particles of the library (corresponding to 5,000 copies of each of the $4x10^{6}$ different clones represented in the library) to M-07e cells led to the selection of a clone carrying an RGDAVGV sequence at the 587 site (Fig. 2).

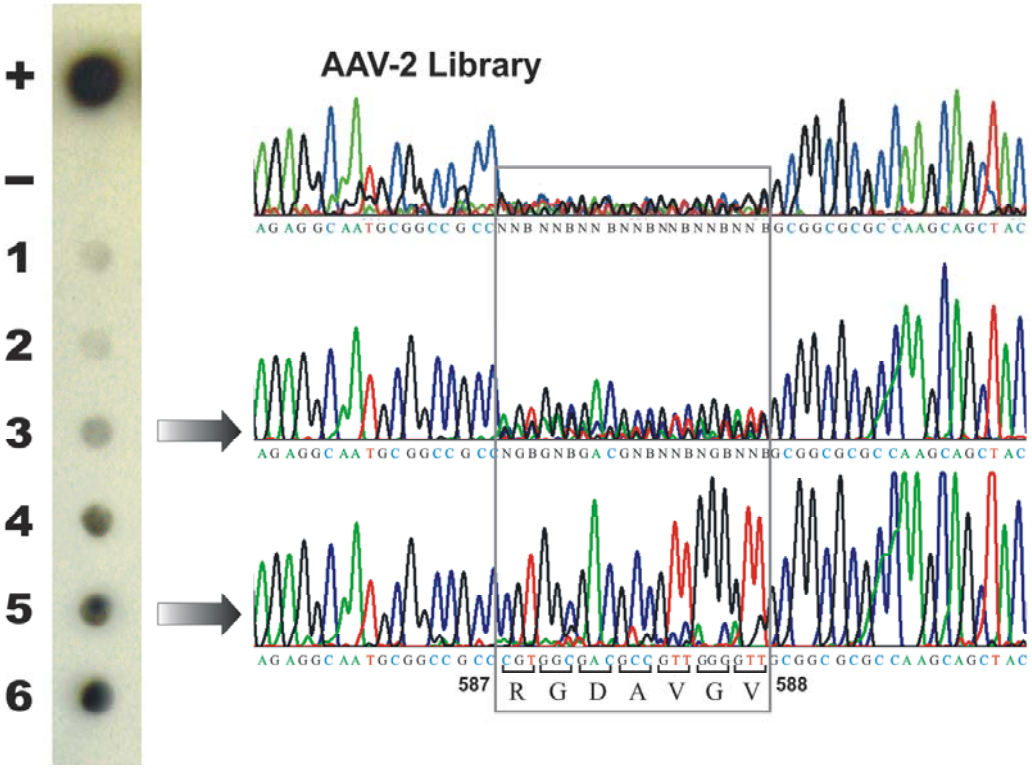

Fig. 2: *Example of evolution of the viral population during 6 selection rounds on M-07e cells. Dot blot assay quantification of viral progeny harvested after each infection cycle and sequencing of the random insertion containing region of the cap gene demonstrated a progressive increase of viral titer and a loss of heterogeneity in the viral population. After 5 rounds a single clone (in the shown example carrying an RGDAVGV inserted sequence) was detected in the viral progeny.*

In a parallel experiment we used $2x10^{12}$ viral particles (500,000 copies of each clone**)** and isolated a clone which carried an RGDTPTS sequence. Interestingly, both clones isolated from M-07e cells led to the selection of an RGD motif, known to bind to several types of cellular integrins (Ruoslahti, 1996). Besides the difference in the sequence of the 4 downstream amino acids, which proved to be irrelevant for the biological behaviour (see below), no significant difference in the selection process could be observed when using different initial amounts of the library. Two analogous experiments performed infecting Mec1 cells with $2x10^{10}$ library particles led to the identification of clones carrying GENQARS and RSNAVVP peptides, respectively.

Characterization of selected viral mutants. The selected DNA sequences were cloned into appropriate plasmids for the production of capsid-modified recombinant AAV (rAAV) vectors encoding the enhanced Green Fluorescent Protein (GFP). Corresponding GFP-expressing retargeted vectors rAAV-M07A (RGDAVGV insertion), rAAV-M07T (RGDTPTS insertion), rAAV-MecA (GENQARS insertion) and rAAV-MecB (RSNAVVP insertion) were produced (see Methods) and genomic titers were determined by dot blot assay. Genomic titers of the selected mutants were comparable or higher than titers of AAV vectors with unmodified capsid (rAAV-wt) (Tab. 1).

Viral Clone	Inserted Sequence	Genomic Particles/ml
rAAV-wt	- - - - -	$5x10^{10}$
rAAV-M07A	RGDAVGV	$5x10^{10}$
rAAV-M07T	RGDTPTS	$5x10^{10}$
rAAV-MecA	GENQARS	10^{11}
rAAV-MecB	RSNAVVP	10^{11}

Tab. 1*: Genomic titers of rAAV-GFP viral preparations as assessed by dot blot assay*

The selected capsid mutants were tested for their ability to transduce M-07e cells (Fig. 3*a*, black bars). At a genomic particle/cell ratio of $2x10^4$, the mutants rAAV-M07A and rAAV-M07T transduced 50 ± 2.5% and 47 ± 2.7% of M-07e cells, respectively, representing a 100 and 94 fold increase in comparison to rAAV-wt transduction efficiency (0.5 ± 0.01%). In contrast, rAAV-MecA and rAAV-MecB transduced M-07e cells with an efficiency of only 8.1 ±1.5% and 16 ± 2%. The vector rAAV-L14, carrying an RGD motif inserted at position 587 (Girod *et al.*, 1999b), was also compared. Interestingly, rAAV-L14 transduced only 10 ± 0.7% of M-07e cells, which was five times less efficient than the selected mutants rAAV-M07A and rAAV-M07T. This highlighted the advantage of the combinatorial approach when compared with the simple insertion of an exogenous sequence.

We then examined whether the transduction of M-07e cells by rAAV-M07A and rAAV-M07T vectors was specifically mediated by the amino acids inserted at position 587. In the capsid of wt AAV, the region around position 587 is involved in the binding to heparan sulfate proteoglycan (HSPG) (Nicklin *et al.*, 2001; Wu *et al.*, 2000), the primary receptor of AAV-2 (Summerford and Samulski, 1998). Pre-incubation with soluble heparin, an HSPG analogue and competitor, inhibited transduction of M-07e cells by rAAV-MecB but not by rAAV-M07A, rAAV-M07T and rAAV-MecA (Fig. 3*a*, white bars). This indicated that the insertion of appropriate heterologous amino acids at this site abolished the requirement of AAV to use HSPG as a primary receptor for transmembrane entry. Mutant MecB, despite carrying the insertion of a peptide (RSNAVVP) that is not known as an heparin binding motif, could be inhibited by soluble heparin from transduction of all tested cell lines (Figs. 3 and 5*a*). Therefore, it is possible that this particular peptide, in the context of site 587 restored the ability to bind HSPG. Such a behaviour of some 587-insertion mutants has been previously reported. Grifman et al. (Grifman *et al.*, 2001) inserted a NGRAHA peptide which is similar to the wt sequence, therefore suggesting that it could mimic it's biological function. Shi et al. (Baranowski *et al.*, 2001) inserted a completely different sequence that however contained a lysine. Even if further investigations are required, these and previous observations (Girod *et al.*, 1999b; Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Wu *et al.*, 2000; Xie *et al.*, 2002) suggest the role of this capsid region (and in particular of the charged amino acids that cluster in that region) in the interaction with HSPG. It is also reasonable to expect that the three-dimensional conformation of the particular insertions might be important for the effect on HSPG binding.

In marked contrast, pre-incubation of M-07e cells with a competing soluble GRGDTP peptide (450 μM) almost completely inhibited transduction of M-07e cells by rAAV-M07A and rAAV-M07T (Fig. 12*a*, gray bars). This effect was concentration-dependent (Fig. 4). Pre-incubation with an inactive (GRGES) peptide (450 μM) had no effect (Fig. 3*a*, checked bars). Taken together, the results demonstrate that rAAV-M07A and rAAV-M07T transduce target cells through the specific interaction of the selected RGD motif presented on the viral capsid with an integrin receptor expressed on the surface of the target cells.

The mutants MecA and MecB showed a reduced but clearly measurable level of transduction of M-07e cells (Fig 3a). In order to identify the ligands inserted at position 587 in the MecA and MecB mutants, we performed an extensive screening in protein sequence databases, but were unable to find any homologous sequence. The selected insertions of mutants MecA and MecB most likely interact with a receptor(s) that is expressed on both cell lines. The cell lines Mec1 and M-O7e probably share identical receptors, since they derive from the same lympho-hematopoietic progenitor cell. Competition experiments with GRGDTP and GENQARS peptides demonstrated that this receptor was different from the targeting sequences on mutants M07A and M07T (Figs. 3*a*, 4 and 5*a*).

We also examined the selected mutants on cells which expressed HSPG and were permissive for wt AAV-2 infection. In human colon carcinoma CO-115 cells (Carrel *et al.*, 1976), the transduction efficiency of the virus mutants rAAV-M07A, rAAV-M07T, rAAV-MecA and rAAV-MecB was reduced by 50, 43, 12 and 31%, respectively, when compared to wt AAV-2 (Fig. 3*b*), while it was similar to wt AAV-2 in HeLa cells (Fig. 3*c*). In both cell lines, transduction by mutants rAAV-M07A and rAAV-M07T was blocked almost completely by the GRGDTP peptide, but not by the GRGES peptide nor by heparin. In contrast, transduction by rAAV-wt and rAAV-MecB was inhibited by heparin but not by the GRGDTP peptide. Moreover, cells which lacked the expression of an integrin receptor were not permissive for transduction by the mutants rAAV-M07A and rAAV-M07T (data not shown). Taken together, these results demonstrate that the integrin receptor recognizing the RGD peptide on rAAV-M07A and rAAV-M07T capsids is also expressed on CO-115 and HeLa cells. Therefore, the tropism of the selected capsid mutants is not restricted to hematopoietic cell lines, but to an integrin receptor, which is probably widely expressed.

Successful retargeting of mutants selected on Mec1 cells is depicted in Fig. 5*a*. While transduction of Mec1 cells by rAAV-wt was not detectable, mutants rAAV-MecA and rAAV-MecB transduced up to 23% of these cells at a genomic particle/cell ratio of $4x10^4$.

Preincubation of target cells with soluble GENQARS peptides abolished transduction of the cells by mutant MecA (which carries the GENQARS sequence at the 587 site) but not by MecB (which carries a different insertion) (Fig. 5*a*), in a concentration dependent manner (Fig 4).

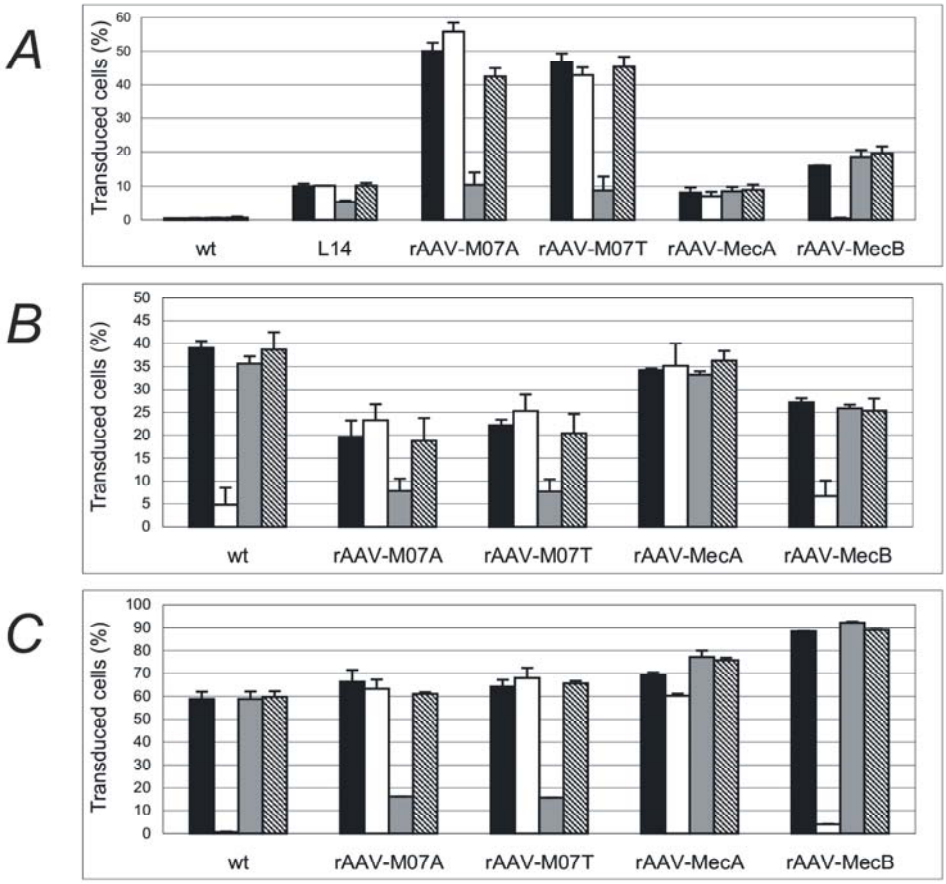

Fig. 3: *Transduction efficiencies ± standard deviation as determined by FACS analysis in duplicate experiments for selected rAAV-GFP mutants (black bars). Transduction rates were also assessed after pre-incubation of viral preparation with soluble heparin (white bars) or pre-incubation of the cells with competing G<u>RGD</u>TP (gray bars) and inactive G<u>RGE</u>S (checked bars).* ***(a)*** *M-07e cells* ***(b)*** *CO-115 cells.* ***(c)*** *HeLa cells.*

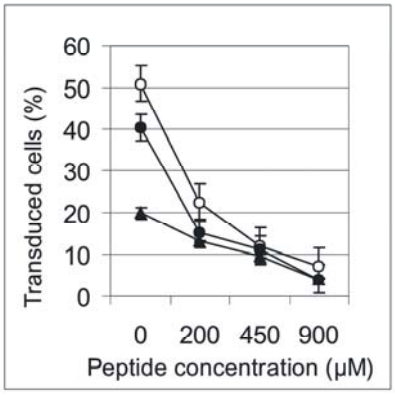

Fig. 4: *Concentration dependence of <u>RGD</u>TP (circles) and GENQARS (triangles) mediated inhibition of M-07e or Mec1 (triangles) cells transduction by rAAV-M07A (white circles), rAAV-M07T (black circles) and rAAV-MecA (black triangles).*

Successful transduction of primary B-CLL cells by the selected MecA mutant. Using rAAV-MecA, we then examined the transduction efficiency in primary leukemia cells in order to explore the potential clinical relevance of the AAV display technology. Primary B-CLL cells are poorly transduced by most currently available viral vector systems including AAV (Cantwell *et al.*, 1996) at least without stimulation with CD40L (Wendtner *et al.*, 2002). In remarkable contrast to vectors with unmodified capsid, rAAV-MecA ($8x10^4$ genomic particles/cell) transduced primary leukemia cells isolated from four B-CLL patients at an efficiency of 54, 49, 23 and 21%, respectively (Fig. 5*b*). In agreement to our observations in Mec1 cells, pre-incubation of the virus mutants soluble heparin did not affect transduction rates (Fig.14*c*). In contrast, rAAV-M07A and rAAV-M07T failed to transduce primary B-CLL cells (data not shown).

These results indicate that such modified vector might be useful for an AAV-based gene therapy of B-CLL (Cantwell *et al.*, 1997; Wierda *et al.*, 2000).

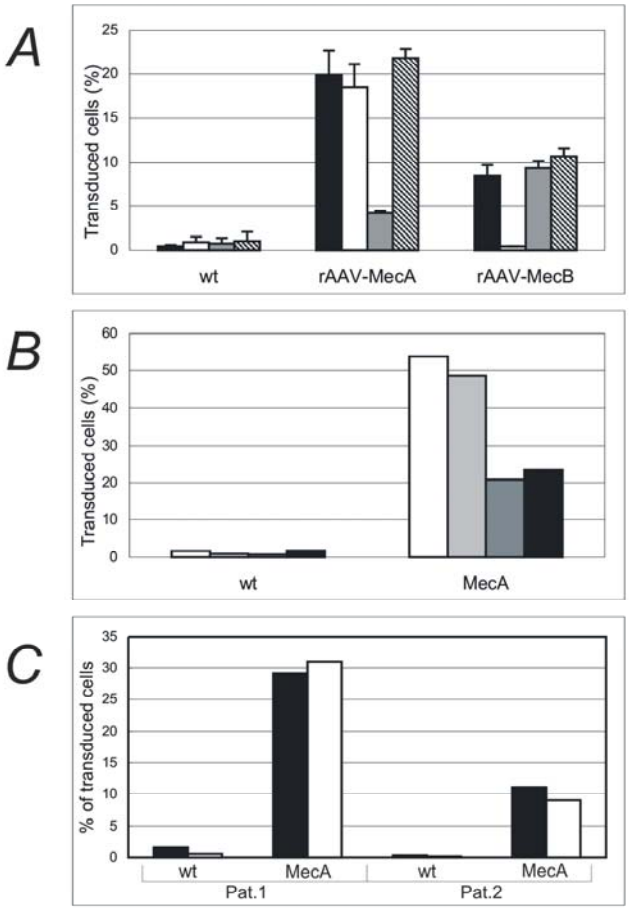

Fig. 5: *B-CLL cells transduction efficiencies of the mutants selected on Mec1 cells (black bars). (**a**) Mec1 cells transduction rates were also assessed after pre-incubation of viral preparation with soluble heparin (white bars) or pre-incubation of the cells with GENQARS (gray bars) and QNEGSRA (checked bars) peptides. (**b**) Primary B-CLL cells obtained from 4 different patients (**c**) Transduction efficiencies on primary B-CLL cells obtained from 2 more patients after (white bars) or without (black bars) pre-incubation of viral preparation with soluble heparin.*

Conclusions. Any successful attempt to molecularly engineer viruses for human somatic gene therapy will depend on our ability to generate retargeting vectors that retain the major functions required for viral entry and appropriate intracellular processing of the virus. Our findings seem highly relevant in this regard. Because of the complexity and our incomplete understanding of the virus-cell interaction, it is highly advantageous to screen appropriate virus mutants from a large library rather than to generate a limited number of virus variants by more or less educated guesses.

The results described in this manuscript provide the proof of principle that surface display approaches can be applied to select eukaryotic virus clones that have a desired tropism. Since no refinement of the selection process was undertaken in this first set of experiments, some limitations remained: the selected capsid mutants showed receptor specificity, but not cell specificity. In particular, in the case of the selected RGD containing mutants, a ligand was selected that is likely to be the more efficient in leading the virions to an infection of the cells. As RGD binding integrins are widely expressed receptors, this resulted in remarkable efficiency, but poor specificity of the obtained vectors. However, we are confident that the goal of producing viral clones with a restriction of the viral tropism to the desired cell type can be achieved by adding negative-selection steps to the screening process, which will deplete those clones able to infect undesirable cell types. For example, normal selection steps on target cells could be preceeded by infection of other types of cells and harvesting of the supernatant after giving the undesired virions enough time to bind or enter the cells. In the case where cell-type specific receptors were known, the library could be subjected to pre-selection in order to select the capsids able to bind to the receptors immobilized on a solid phase. An additional upgrade of this technology might be the generation of an AAV library with randomized insertions in multiple sites of the capsid.

In summary, this report shows for the first time that the AAV display technology holds promise for the generation of retargeting mutants. We believe that the use of this technology will facilitate the generation of efficient and cell specific AAV vectors in the future.

Acknowledgments

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CHAPTER IV

GENETIC MODIFICATIONS OF THE ADENO-ASSOCIATED VIRUS TYPE 2 CAPSID REDUCE THE AFFINITY AND THE NEUTRALIZING EFFECTS OF HUMAN SERUM ANTIBODIES

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Summary

The high prevalence of human serum antibodies against adeno-associated virus type 2 (AAV) vectors represents a potential limitation for *in vivo* applications. Consequently, the development of AAV vectors able to escape antibody binding and neutralization is of importance. To identify capsid domains which contain major immunogenic epitopes, six AAV capsid mutants carrying peptide insertions in surface exposed loops regions (I-261, I-381, I-447, I-534, I-573, I-587) were analyzed. Two of these mutants, I-534 and I-573, showed an up to 70% reduced affinity for AAV antibodies as compared to wild-type AAV in the majority of serum samples. In addition, AAV mutant I-587 but not wild-type AAV efficiently transduced cells despite the presence of neutralizing antisera. Taken together, the results show that major neutralizing effects of human AAV antisera might be overcome by the use of AAV capsid mutants.

Introduction

The human parvovirus adeno-associated virus type 2 (AAV) is a promising vector for human somatic gene therapy. Recombinant AAV vectors (rAAV) have many advantages in comparison to other vector systems, including the ability to transduce both dividing and non-dividing cells, long-term gene expression *in vitro* and *in vivo*, and the apparent lack of pathogenicity. AAV has a broad host range and transduces a wide variety of tissues, including muscle, lung, liver, brain, and hematopoietic cells (Allen *et al.*, 2000; Fisher-Adams *et al.*, 1996; Flotte *et al.*, 1993; Kaplitt *et al.*, 1994; Mandel *et al.*, 1997; Russell and Kay, 1999; Xiao *et al.*, 1996). During the infection process, AAV first interacts with its primary receptor heparan sulfate proteoglycan (HSPG), mediating the attachment of the virions to the host cell membrane (Summerford and Samulski, 1998). Two types of co-receptors, $\alpha_v\beta_5$ integrin and fibroblast growth factor receptor 1 (FGFR), have been implicated in the subsequent internalization process (Qing *et al.*, 1999; Summerford *et al.*, 1999). However, conflicting results were reported about the contribution of these co-receptors (Qiu and Brown, 1999b; Qiu *et al.*, 1999). Following receptor binding, AAV enters the cell via a dynamin-dependent endosomal pathway (Bartlett *et al.*, 2000a; Duan *et al.*, 1999). After acidification of endosomes, viral particles are released into the cytoplasm and rapidly transported to the nucleus, involving microfilaments and microtubules, before they enter the nucleus (Douar *et al.*, 2001; Sanlioglu *et al.*, 2000; Seisenberger *et al.*, 2001).

The capsid of wild-type AAV (wtAAV) harbors a linear, single-stranded DNA genome of 4.7 kb, which contains two open reading frames (ORF), *rep* and *cap*, flanked by the inverted terminal repeats (ITR). The 5' ORF encodes the four nonstructural proteins (Rep78, Rep68, Rep52, and Rep40), which are required for replication, transcriptional control and site-specific integration. The three structural proteins, designated VP1, VP2, and VP3, are encoded in the 3' ORF and transcribed from the same promoter (p40) using alternate splicing and different translational initiation codons. The small icosahedral capsid, which is only 25 nm in diameter, is composed of 60 subunits with a relative stoichiometry of about 1:1:8 for VP1, VP2, and VP3 (Kronenberg *et al.*, 2001). Recently the atomic structure of AAV has been determined (Xie *et al.*, 2002). Each subunit has a β-barrel motif, which is highly conserved among parvoviruses. Between the strands of the β-barrel core large loop insertions are found that constitute the majority of the capsid surface. These loop insertions seem to interact with antibodies and cellular receptors and are highly variable among related parvoviruses. The most prominent features of the capsid are the threefold-proximal peaks, which cluster around the threefold axis (Xie *et al.*, 2002). Positions on the AAV capsid where interaction with HSPG takes place have been determined (Rabinowitz *et al.*, 1999; Wu *et al.*, 2000). They map to a positively charged region on the side of the threefold-proximal peaks (Xie *et al.*, 2002). Moreover, epitopes of monoclonal antibodies (mAb) which interfere with the AAV infection process (A20 and C37-B) have also been mapped to this threefold axis (Wobus *et al.*, 2000a; Xie *et al.*, 2002).

Although knowledge is rising about basic AAV biology, there are still obstacles for the application of AAV as vector system for somatic gene therapy. One major problem is the high prevalence of AAV specific antibodies (Ab) in the human population. 50 to 96% are seropositive for AAV Ab, and 18 to 67.5% of them have neutralizing Ab, depending on age and ethnic group (Chirmule *et al.*, 1999; Erles *et al.*, 1999a; Moskalenko *et al.*, 2000a). Especially these preexisting neutralizing Ab have profound implications for the application of AAV in human gene therapy. Animal experiments have shown that neutralizing Ab greatly reduce or even prevent transgene expression after readministration of the vector (Fisher *et al.*, 1997; Xiao *et al.*, 2000; Xiao *et al.*, 1996). Different mechanisms for neutralization of viral infections have been described (Smith, 2001): (i) aggregation of viral particles, (ii) induction of conformational changes in the capsid, (iii) interference with receptor attachment, and (iv) inhibition of uncoating due to virion stabilization. In related parvoviruses immunogenic sites are formed by highly variable and accessible domains on the capsid surface, which can be generally found in the threefold spike region (Fig. 1): on the tip and the shoulder of the spike,

and between the twofold dimple and the spike (Chapman and Rossmann, 1993). In canine parvovirus (CPV) two dominant neutralizing epitopes, which are both conformational epitopes, are found on the shoulder of the threefold spike (epitope A: residues 93, 222, 224, 426 and epitope B: residues 299, 300, 302 of VP2) (Strassheim *et al.*, 1994). Similarly, the epitopes of two monoclonal neutralizing Ab of B19 parvovirus have also been mapped to the threefold spike region (Chipman *et al.*, 1996). Therefore it is possible that immunogenic sites of AAV are also located in variable regions of the threefold spike region.

In a previous study we had generated six AAV capsid mutants bearing an integrin specific peptide ligand (L14) insertion at position 261, 381, 447, 534, 573, or 587 of the AAV VP1 protein (Girod *et al.*, 1999a). These positions were originally selected based on structural alignments with the related parvoviruses CPV, B19 and feline panleukopenia virus (FPV), with regard to flexible, highly variable, and immunogenic domains of these viruses (see Fig. 1; Girod et al., 1999). Indeed, we and others could show that the insertion sites are displayed on the capsid surface (Girod *et al.*, 1999a; Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Wu *et al.*, 2000) and the recently published atomic structure of AAV confirmed the localization of the selected positions in surface exposed loop regions (Xie *et al.*, 2002). Moreover, binding studies with the neutralizing mAb A20 and C37-B demonstrated that the mutations impaired their affinities for the AAV capsid. This indicated that immunogenic domains had been affected by the capsid mutations (see Fig. 2a; Wobus et al., 2000). In this paper we analyzed these six AAV capsid mutants with polyclonal human serum samples in binding and neutralization assays. This allowed us to characterize immunogenic and neutralizing regions on the AAV capsid.

Results

AAV antibodies from human serum samples have a reduced affinity for AAV insertion mutants

To determine major antigenic domains involved in the humoral immune response of humans against the capsid of AAV, we analyzed the ability of human antisera to recognize AAV capsid mutants carrying a 14 amino acid (aa) peptide (L14, QAGTFALRGDNPQG) of the laminin fragment P1 inserted at positions 261, 381, 447, 534, 573, or 587 (Girod *et al.*, 1999a).

In a first step, the capsid morphology of the six different VP3 mutants was characterized by electron microscopy (EM). All viral preparations showed an EM morphology similar to wtAAV and contained predominantly intact particles (data not shown).

Table1. *Titers of AAV stocks*

Virus Stocks	Physical particles / ml [1]	Infectious particles / ml [2]
wtAAV	**4.9×10^{12}**	**7×10^{9}**
I-261	**1.0×10^{12}**	**1×10^{4}**
I-381	**1.0×10^{12}**	**$<1 \times 10^{2}$**
I-447	**2.2×10^{12}**	**1×10^{6}**
I-534	**3.5×10^{12}**	**$<1 \times 10^{2}$**
I-573	**3.1×10^{12}**	**$<1 \times 10^{2}$**
I-587	**2.5×10^{12}**	**6×10^{6}**
rAAV	**2.5×10^{12}**	**3×10^{9}**
rAAV-587/L14	**3.0×10^{11}**	**4×10^{6}**
rAAV-587/MecA	**1.3×10^{12}**	**2×10^{8}**

[1] Physical particle titers were determined by EM for *rep/cap*-containing particles and by dot-blot analysis for recombinant vectors

[2] Determined on HeLa cells by immunofluorescence for preparations containing a *rep* gene, and by FACS analysis for GFP encoding particles

The presence of antibodies against wtAAV was then analyzed in 65 human serum samples. In an ELISA, 43 out of 65 serum samples (66%) were positive for AAV antibodies. Out of these 43 seropositive samples, 29 sera with a high titer of AAV specific antibodies (OD>0.6 after subtraction of background) were selected for further analysis. The binding affinity of these 29 serum samples to the six AAV insertion mutants (I-261, I-381, I-447, I-534, I-573, and I-587) was analyzed in an ELISA and compared to wtAAV. Identical numbers of particles of the respective insertion mutants and of wtAAV, as determined by EM (Table 1) and confirmed by Western blotting, were coated on the ELISA plates. Binding of serum Ab to wtAAV was set as 100%, and the change in serum binding to the AAV mutants was determined. Several patterns of interaction of human sera with AAV mutants could be distinguished (Table 2). One group of serum samples, designated *class A*, reacted with all six AAV mutants in a way similar to wtAAV (e.g. serum P17, Fig. 2b). This was the case for 10 out of 29 sera (34%). A second group of serum samples (12 out of 29, 42%), designated *class B,* displayed a reduced binding affinity only to mutants I-534 and I-573 (e.g. serum P37, Fig. 2b). The average reduction for both, I-534 and I-573, was 31% as compared with wtAAV. A third and smaller group of serum samples (7 out of 29, 21%), *class C*, additionally showed a reduced affinity for the other 4 capsid mutants (e.g. serum P26, Fig. 2b). On average, binding to these 4 mutants was decreased by 7% (I-381) to 26% (I-447) (Table 2). Nevertheless, insertions at positions 534 and 573 reduced binding of serum antibodies to the capsid more than insertions at other positions, i.e. by 51% and 45%, respectively (Table 2). Interestingly, binding of *class C* sera to these two capsid mutants was more affected than binding of *class B* serum samples. The observed differences in binding affinity did not significantly change when using various serum dilutions.

Strikingly, mutations at positions 534 or 573 had an effect on the affinity of human antisera in 19 of 29 cases. In some cases, serum binding was reduced up to 70%. In 7 serum samples, mutations at position 261, 381, 447, or 587 also resulted in a reduced binding affinity, albeit to a smaller extent. Based on these findings, we conclude that insertions in positions 534 or 573 affected major antigenic determinants of the humoral immune response against the AAV capsid.

Table 2.

Binding of seropositive human sera to AAV insertion mutants compared to wtAAV

Class [1]	Serum	I-261	I-381	I-447	I-534	I-573	I-587
A	P1	–	–	–	–	–	–
	P2	–	–	–	–	–	–
	P6	–	–	–	–	–	–
	P14	–	–	–	–	–	–
	P17	–	–	–	–	–	–
	P19	–	–	–	–	–	–
	P24	–	–	–	–	–	–
	P31	–	–	–	–	–	–
	P47	–	–	–	–	–	–
	P60	–	–	–	–	–	–
	Reduction [2]	0.3% ± 6.6%	0.2% ± 4.7%	7.5% ± 5.5%	0.0% ± 7.4%	3.9% ± 8.3%	- 2.3% ± 5.4%
B	P5	–	–	–	↓	↓↓	–
	P7	–	–	–	↓↓↓	↓↓	–
	P16	–	–	–	↓↓	↓	–
	P27	–	–	–	↓↓↓	↓↓	–
	P29	–	–	–	↓	↓	–
	P32	–	–	–	–	↓	–
	P33	–	–	–	↓	↓	–
	P37	–	–	–	↓↓	↓↓	–
	P48	–	–	–	↓↓	↓↓	–
	P51	–	–	–	↓	↓↓	–
	P53	–	–	–	↓	↓↓	–
	P59	–	–	–	↓	↓	–
	Reduction	3.4% ± 5.8%	1.8% ± 8.9%	9.2% ± 5.5%	30.9% ± 13.5%	30.7% ± 7.2%	4.6% ± 3.5%
C	P3	↓	–	↓↓	↓↓↓	↓↓↓	–
	P26	↓	–	↓	↓↓↓	↓↓↓	↓
	P35	–	–	↓↓	↓↓	↓↓	–
	P40	–	↓↓	–	↓↓	↓↓	–
	P54	–	–	↓	↓↓↓	↓↓	–
	P57	↓	↓	↓↓	↓↓	↓↓	↓
	P65	↓	–	↓	↓↓↓	↓↓↓	↓
	Reduction	12.5% ± 10.6%	6.8% ± 9.9%	25.8% ±11.7%	51.3% ± 10.9%	44.8% ± 5.0%	8.8% ± 9.1%
mAb	A20	↓↓↓↓↓	↓↓↓↓↓	–	↓↓↓	↓↓↓	–
	C37-B	↓↓↓↓↓	–	↓↓↓	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓↓

The symbols –, ↓, ↓↓, ↓↓↓ and ↓↓↓↓↓ illustrate a reduction in affinity in comparison to wtAAV of 0-14%, ≥15%, ≥25%, ≥50% and 100%, respectively.

[1] Classification of the serum samples: A, affinity to insertions mutants like to wtAAV; B, reduced affinity to I-534 and I-573; C, reduced binding with I-534, I-573 and other capsid mutants

[2] The mean reduction and standard deviation are given

Transduction of HeLa cells by rAAV-587/L14 is not inhibited by preexisting neutralizing antibodies in human serum samples

A detailed understanding of major immunogenic domains on the AAV capsid is not only important with regard to the binding of serum antibodies to the virus and its subsequent neutralization by the immune system, but also with regard to the existence of neutralizing antibodies that directly inhibit infection of the target cells by AAV vectors. To analyze the interference of different human antisera with AAV transduction, we used a recombinant AAV vector carrying the L14 ligand at position 587 (rAAV-587/L14) to determine whether this modification would block the neutralizing ability of human antisera. Unfortunately, only rAAV-587/L14 could be purified to sufficiently high titers to perform these studies, therefore the other insertion mutants were not further tested (Table 1).

First, we determined the presence of neutralizing Ab in human serum samples. The 43 positive serum samples were tested in a neutralization assay with an AAV vector coding for GFP, which carried the wild-type capsid (rAAV). rAAV was incubated with serial dilutions of serum samples prior to transduction of HeLa cells. Thereafter, the number of GFP expressing cells was assessed by FACS analysis. Neutralizing titers were defined as the serum dilution where transduction was reduced by 50% (N_{50}). Serum samples were considered as neutralizing when the N_{50} was 1:320 or higher. 31 of these 43 serum samples (72%) contained neutralizing Ab against AAV, in agreement with previously published data (Erles *et al.*, 1999a).

15 of these 31 serum samples, equally distributed over the above mentioned three classes (5 *class A*, 6 *class B*, and 4 *class C*), were selected for further analysis. The effect of these serum samples on the transduction of HeLa cells by rAAV-587/L14 as compared with rAAV was determined (Fig. 3a). In addition, the neutralizing mAb C37-B and an anti-L14 serum (see Materials and Methods) were tested. For these experiments identical transducing particle numbers of rAAV-587/L14 and rAAV were used. Both vectors were incubated with serial dilutions of neutralizing serum samples prior to transduction of HeLa cells. For all serum samples tested, transduction by rAAV-587/L14 was 8 up to 64 fold less reduced than transduction by rAAV (mean 15 fold). In 13 out of 15 serum samples, transduction by rAAV-587/L14 was only slightly impaired, with neutralizing titers of 1:80 or lower, demonstrating the ability of rAAV-587/L14 to escape the effects of neutralizing Ab (Fig. 3a). Strikingly, rAAV-587/L14 was able to escape the neutralizing Ab in serum P47 at any dilution tested, and serum samples P17, P31 and P37 reduced transduction only at a dilution of 1:20, where unspecific interactions could not be excluded. Figures 3b and 3c show one

representative experiment with serum P35, which completely inhibited transduction by rAAV at a 1:80 dilution (Fig. 3b). In marked contrast, transduction by rAAV-587/L14 was not affected (Fig. 3c). Only two serum samples (P16 and P48) were able to neutralize rAAV-587/L14 transduction efficiently, with a N_{50} of 1:320. We assume that this was due to the high neutralizing Ab content in these serum samples, because transduction by rAAV-587/L14 still remained less affected than transduction by rAAV. As an additional control, the mAb C37-B was tested. C37-B is a neutralizing Ab that inhibits binding of AAV to the host cell (Wobus *et al.*, 2000a). It failed to bind I-587 in an ELISA, therefore it should not interfere with rAAV-587/L14 transduction. As expected, rAAV-587/L14 transduction was not neutralized by C37-B, while rAAV transduction could be totally inhibited by this antibody (data not shown). In marked contrast, anti-L14 serum, which was generated against the L14 ligand, neutralized rAAV-587/L14 transduction completely at a 1:160 dilution, while rAAV transduction remained unaffected (Fig. 3a). To rule out the possibility that these observations were due to different numbers of physical particles used for rAAV and rAAV-587/L14, we performed additional control experiments, where neutralization assays were performed with identical numbers of physical particles for both AAV vectors. These experiments yielded identical results (data not shown). Taken together, these results demonstrate that the mutant rAAV-587/L14 is able to escape preexisting neutralizing Ab in human serum samples.

Neutralizing sera do not interfere with the L14 mediated tropism of rAAV-587/L14 on B16F10 cells

Insertion of the integrin specific L14 peptide in 587 expands the tropism of AAV to non-permissive B16F10 cells (Girod *et al.*, 1999a). To determine if rAAV-587/L14 was able to retain its ability to infect the target cell line B16F10 via the inserted ligand L14 in the presence of neutralizing antisera, we performed additional experiments with selected serum samples. rAAV-587/L14 was incubated with serial dilutions of P35 serum before transduction of irradiated B16F10 cells. After 72 hours GFP expression was measured. rAAV-587/L14 efficiently transduced B16F10 cells despite incubation with P35 at a 1:80 dilution, whereas anti-L14 serum completely inhibited transduction at this dilution (Fig. 4b and c). When testing P37 and P26, the same neutralizing titers as determined on HeLa cells were obtained (data not shown). These findings showed that the AAV L14 targeting vector could escape neutralizing antibodies in human sera while retaining its retargeting ability.

The ability of rAAV-587 to escape neutralizing sera does not depend on the inserted L14 ligand

To exclude that the escape from neutralizing antisera was caused by a specific ligand, we tested another insertion mutant, rAAV-587/MecA that carries a 7 aa ligand (GENQARS) at position 587. This mutant has been selected by AAV-display on Mec1 cells and efficiently transduces Mec1 cells and primary B-cells from chronic lymphocytic leukemia patients in a receptor specific manner (Perabo *et al.*). rAAV-587/MecA and rAAV were incubated with the serum P35 before Mec1 cells were infected. Transduction of Mec1 cells by rAAV-587/MecA was not affected by the neutralizing Ab of serum P35 (1:80 dilution). In contrast, rAAV transduction was almost completely inhibited by this serum (Fig. 5). Experiments with other neutralizing serum samples provided identical results (data not shown). In additional control experiments the neutralizing Ab A20 was able to inhibit transduction by rAAV-587/MecA, while C37-B had no effect (data not shown).

Taken together, the results demonstrate that the insertion of different heterologous ligands at position 587 allows escape from preexisting neutralizing antibodies. Targeting properties of these vectors are retained in these capsid mutants, even in the presence of neutralizing antisera.

Discussion

Because of the high prevalence of antibodies against AAV in the population, it is essential in gene therapy approaches to understand the immunogenic determinants of the AAV capsid and to develop strategies to circumvent antibody binding and neutralization of AAV vectors. In this study, we analyzed six AAV capsid mutants (I-261, I-381, I-447, I-534, I-573, and I-587) with a 14 aa peptide ligand inserted into the VP3 part of the capsid protein to identify immunogenic domains on the AAV capsid. We showed that peptide insertions at position 534 or 573 reduced binding of human antisera in 66% of the analyzed samples, indicating that these regions might be preferentially recognized by human AAV antibodies. In addition, we analyzed AAV vectors modified at position 587 to study the potential of AAV capsid mutants to escape the neutralizing effects of human antisera with regard to the transduction efficiency. We demonstrated that these modified vectors were able to escape neutralizing Ab in human antisera without losing their ability to infect cells via the targeted receptors. In marked contrast, transduction of AAV carrying the unmodified capsid was significantly reduced or

inhibited. These findings suggest that modifications at site 587 reduce the ability of AAV antibodies in human blood to neutralize the transduction by rAAV vectors.

The atomic structures of related parvoviruses like CPV, FPV and B19 have been resolved during the past decade and antigenic sites have been determined (see Fig. 1) (Agbandje *et al.*, 1994; Chang *et al.*, 1992; Chapman and Rossmann, 1993; Chipman *et al.*, 1996; Strassheim *et al.*, 1994 ; Tsao *et al.*, 1991). At the beginning of our studies the capsid structure of AAV was still unknown. Alignments of these related parvoviruses with AAV led to hypothetical models of the AAV capsid, and systematic mutagenesis helped to map functional sites on the capsid (Girod *et al.*, 1999a; Rabinowitz *et al.*, 1999; Wu *et al.*, 2000). Based on our structural alignments, six sites on the AAV capsid, selected with regard to flexible, highly variable loops and immunogenic domains of related parvoviruses (Fig. 1), were identified to accept the insertion of an integrin specific RGD ligand (L14, QAGTFALRGDNPQG) (Girod *et al.*, 1999a). Immunological analysis demonstrated the surface localization of the inserted L14 peptide (Girod *et al.*, 1999a). Characterization of other AAV serotypes revealed that the selected positions are also within highly variable regions amongst these serotypes (Chiorini *et al.*, 1999). The recent unveiling of the atomic structure of AAV (Xie *et al.*, 2002) broadly confirmed the flexible loop regions predicted by our initial structural model (Girod *et al.*, 1999a). When mapping the six insertion sites used in this report on the three-dimensional structure of AAV, they can all be found on the capsid surface within the threefold spike region (Fig. 6).

The high prevalence of AAV specific Ab causes substantial problems for human gene therapy. Different approaches have been pursued to map epitopes on the AAV capsid. Moskalenko et al. (2000) used small overlapping peptides (15aa) spanning the VP1 protein and human antisera to screen the AAV capsid protein by peptide scan. They identified several linear epitopes presented on the capsid surface, amongst them sites mapping to I-261 and I-447. However, some of the identified peptides might block Ab binding unspecifically or might not be displayed on the capsid surface (Wobus *et al.*, 2000a; Xie *et al.*, 2002). Moreover, conformational epitopes cannot be identified by this method. Wobus et al. (2000) used murine mAb, which recognize conformational epitopes (D3, C37-B, C24-B, and A20), to identify epitopes on the AAV capsid. Immunological analysis of these mAb with our six AAV insertion mutants helped mapping the epitopes of these antibodies on the AAV capsid and provided information about regions involved in receptor attachment. However, murine mAb cannot mimic the polyclonal Ab repertoire after an infection in humans. We therefore analyzed the ability of human antisera to recognize the six AAV insertion mutants in order to

determine major antigenic domains of AAV involved in the humoral immune response. By using an ELISA we could demonstrate that the majority of the serum samples had a reduced affinity towards two insertion mutants, I-534 and I-573. Although at 39 aa distance from each other on the primary sequence, these sites are found in close proximity in the assembled capsid, on the side of the peaks at the threefold rotation axis (Xie *et al.*, 2002). These data indicate the importance of the threefold-proximal peak region in the recognition by the humoral immune response, as it has been already shown for B19 or CPV (Chapman and Rossmann, 1993; Chipman *et al.*, 1996; Strassheim *et al.*, 1994). It remains to be elucidated whether these mutations interfere with Ab binding directly or indirectly due to structural changes in adjacent regions.

Insertions at sites 261, 381, 447, and 587 affected binding of serum Ab only in a minority of serum samples. This was surprising, because mutations at position 261, 381 and 587 abolished binding of murine mAb A20 and C37-B, respectively (Fig. 2a). Moreover, amino acids corresponding to positions 261, 381, and 447 are part of major antigenic determinants in CPV (Strassheim *et al.*, 1994). Different explanations are conceivable for this minor effect of these mutations on human Ab binding. (i) It is possible that epitopes, especially linear epitopes, which are adjacent to these insertion sites, have not been affected and that they are responsible for the remaining reactivity of serum Ab towards these mutants. (ii) In contrast to CPV, AAV residues 261 and 381 are located in the valley between two peaks of the threefold symmetry axis and this region might be less accessible for Ab binding, or less relevant for inducing a humoral immune response. (iii) Serum samples consist of a polyclonal Ab population. Thus, epitopes which only induce a weak Ab response might not have been detected in this binding assay, although they were affected by the mutations. For the same reason, we also did not expect a complete inhibition of binding of the polyclonal Ab, as observed with the monoclonal Ab A20 with I-261, I-381 and C37-B with I-534, I-573, and I-587. (iv) In addition, the specificity of Ab for a given antigen is dependent on the B-cell repertoire, T-cell repertoire and the major histocompatibility complex (MHC), and is therefore different at the species and individual level. Murine Ab generated against viral antigens may differ in their targeted sequences to those generated in humans. All six insertions are directly at or close to the spike region. It is likely that in individuals the positions of the major antigenic determinants of AAV are different. In this case differences in human serum Ab binding were only seen if the major antigenic determinants were close to the spike region. This might also explain why serum samples of *class A* displayed no reduced affinity for any of the six insertion mutants, but this does not exclude that different mutants

would impair binding of these sera. Consequently, it is reasonable to assume that other immunogenic determinants exist which have not been identified so far.

For the *in vivo* application of AAV, epitopes which interact with neutralizing antibodies are of particular importance. We therefore investigated the ability of AAV vectors with insertion of different peptide ligands at 587 to escape the preexisting neutralizing Ab in human antisera. Such vectors have previously been shown to efficiently retarget infection to wtAAV resistant cells (Girod *et al.*, 1999a; Nicklin *et al.*, 2001). Moreover, Wu and colleagues (2000) demonstrated that this 587 region is involved in binding to the primary attachment receptor HSPG. 15 neutralizing serum samples were analyzed for their ability to neutralize rAAV-587/L14 transduction in comparison to rAAV. Strikingly, rAAV-587/L14 could escape the neutralizing effects exerted by 13 of the 15 neutralizing serum samples and efficiently transduced various cell lines. The targeting properties of rAAV-587/L14 were not affected, and escape could also be observed with a second ligand (MecA) that differed in size and sequence, demonstrating that the escape did not depend on the particular L14 insertion. Unfortunately, infectious titers of the other insertion mutants were not sufficient to perform neutralization assays. Therefore, our results do by no means allow the conclusion that insertions at position 587 are the only site to generate mutants, which are able to escape the neutralizing effects of human antisera.

It seems contradictory that I-587 only slightly impaired binding of serum samples in the ELISA, while it had such strong effects on the neutralizing capacity of human antisera. At least two explanations are possible: Human sera consist of a polyclonal Ab population directed against various epitopes on the AAV surface, but only a small amount of these Ab are capable of neutralizing AAV transduction. Therefore, in an ELISA these neutralizing Ab may not have a noticeable effect on the overall Ab binding, whereas they inhibit virus transduction in a neutralization assay. The three dimensional structure of AAV shows that the sites for interaction with the viral receptor HSPG are located within the peaks of the threefold axis, proximate to residue 587 (Wu *et al.*, 2000; Xie *et al.*, 2002). Furthermore, the neutralizing Ab C37-B, which inhibits binding of wtAAV to the host cell, has its epitope adjacent to this site in the assembled capsid. Therefore this region seems to be critical for receptor binding. It is very likely that neutralizing Ab are preferentially directed against this region and explains why the action of these Ab is affected by insertions at this region (Wobus *et al.*, 2000a; Xie *et al.*, 2002). Another explanation is that neutralizing Ab, which have been generated against the wtAAV capsid, only block the wt capsid mediated transduction. After insertion of a targeting ligand at position 587 the virus mutant can use a different uptake route

than wtAAV, which does no longer depend on HSPG binding (rAAV-587/L14 via the integrin receptor) (Girod *et al.*, 1999a; Nicklin *et al.*, 2001). Therefore it is supposable that these neutralizing Ab cannot block the interaction of these AAV mutants with alternative cell surface receptors. Of course, these explanations are not mutually exclusive, and due to the complexity and various mechanisms of the neutralization process other explanations are also possible. However, it is reasonable to assume that capsid modifications at position 587 might not only allow to alter the tropism of AAV but also to generate immune escape variants. We are fully aware that animal experiments are now needed to corroborate the utility of this concept.

Taken together the results indicate that the threefold-proximal peaks on the AAV capsid are major antigenic determinants for antibody binding as well as for neutralization of AAV transduction. Moreover, our results demonstrate that modifications at site 587 could allow to generate AAV vectors with the ability to escape neutralization by human antisera. Importantly, these modified vectors retain their ability to transduce specific target cells. These findings might be useful for the production of AAV vectors suitable for repeated administration in human gene therapy.

Materials and Methods

Cell culture. HeLa, 293 and B16F10 cells were grown in Dulbecco´s modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM). Mec1 cells were cultivated in Iscove's modified DMEM supplemented with 10% FCS, penicillin-streptomycin and L-glutamine. Cells were maintained at 37°C in a 5% CO_2 humidified incubator.

Antibodies and human serum samples. B1, A20 and C37-B were generated by immunization of mice with purified AAV capsid proteins and synthetic peptides, respectively, followed injection with AAV empty particles (Wistuba *et al.*, 1995; Wobus *et al.*, 2000a). 76/3 was generated by immunization with purified Rep protein as described (Wistuba *et al.*, 1995). Serum against the L14 ligand was obtained after immunization of a rabbit with L14 peptide (Girod *et al.*, 1999a). Serum samples from human patients were kindly provided by the Klinikum Großhadern in Munich, Germany.

Plasmids. The plasmid pUC-AV2 contains the full-length AAV2 genome and was constructed as described in Girod et al. (1999). The plasmids pI-261, pI-381, pI-447, pI-534,

pI-573, pI-587 are derived from pUC-AV2, with the L14-encoding sequence inserted in the *cap* gene. The AAV2-based helper plasmids pRC, pRC(I-587) and pRC(587/MecA) contain the AAV2 Rep and Cap encoding regions but lack the viral ITRs (Girod *et al.*, 1999a; Perabo *et al.*). pRC(587/MecA) contains a DNA fragment coding for the MecA ligand inserted between amino acid position 587 and 588. The pGFP plasmid is an AAV2-based vector plasmid in which the AAV ITR sequences flank the hygromycin selectable marker gene controlled by the thymidine kinase promoter and the enhanced GFP gene regulated by the cytomegalovirus promoter (Ried *et al.*, 2002). The adenovirus helper plasmid pXX6 (Xiao *et al.*, 1998) was kindly provided by R. J. Samulski.

Preparation of virus stocks. The AAV stocks were generated as described previously (Ried *et al.*, 2002) with the following modifications. 293 cells seeded at 80% confluence in plates with 15mm of diameter were transfected with a total of 37.5 µg of vector plasmid (pGFP) and packaging plasmid (pRC for wt capsid, pRC(I-587) for L14 carrying capsid, and pRC(587/MecA) for MecA carrying capsid) and adenoviral plasmid (pXX6) at a 1:1:1 molar ratio. For viruses containing an AAV *rep* and *cap* gene, the pUC-AV2 plasmid or mutated plasmids were transfected with pXX6 in a 2:1 molar ratio. After 48 h cells were collected and pelleted by centrifugation. Cells were resuspended in 150 mM NaCl, 50 mM Tris-HCl (pH 8.5), lysed by repeated freeze-thaw cycles, and treated with Benzonase (50 U/ml) for 30 min at 37°C. Cell debris was removed by centrifugation and the supernatant was loaded onto an iodixanol gradient as described (Zolotukhin *et al.*, 1999). After centrifugation at 69,000 rpm for 1 h at 18°C the AAV containing iodixanol phase was harvested.

Evaluation of AAV titers. Particle titers of virus stocks containing *rep/cap* were determined by electron microscopy and confirmed by Western blotting. Electron microscopy was performed at the DKFZ (Heidelberg). Iodixanol gradient purified viral particles were adsorbed onto Formvar-carbon-coated copper grids and negatively stained with uranyl acetate. Titers were calculated in comparison to a known viral standard (Grimm *et al.*, 1999).Western blot analysis was performed to confirm the titers obtained by electron microscopy. Equal numbers of AAV particles were separated on a 10% SDS-PAGE and blotted on nitrocellulose membrane using standard protocols. Capsid proteins were detected by B1 hybridoma supernatant, followed by incubation with a peroxidase-coupled secondary antibody (Sigma) and visualized by enhanced chemiluminescence (Pierce). For recombinant vectors encoding the GFP protein genomic titers were quantified by dot-blot analysis as described (Girod *et al.*, 1999a). Briefly, serial dilutions of the AAV preparations were first

incubated in 2 M NaOH, then blotted onto a nylon membrane, and finally hybridized with a random-primer *gfp* probe by standard methods. Infectious particle titers of the GFP encoding virus stocks were determined by infecting irradiated HeLa cells (70 Gy from a ^{137}Cs gamma irradiation source) with serial dilutions of the AAV preparation in a 12-well plate. After 48 h cells were harvested and assayed for GFP expression by fluorescence-activated cell sorting (FACS). Infectious titers on B16F10 and Mec1 cells were performed accordingly by co-infection with adenovirus 5 (AdV). Titers of AAV stocks carrying the *rep* and *cap* gene were determined by infection of HeLa cells after AdV co-infection and detection of the viral Rep proteins with Cy3-labeled 76/3 monoclonal antibody (Cy3 mono-Reactive Dye Pack, Amersham, according to the manufacturer's protocol).

ELISA. Identical particle amounts (5×10^8 per well) as determined by electron microscopy of wtAAV und AAV insertion mutants were coated onto microtiter plates (MaxiSorp; Nunc Nalgene International) in PBS overnight at 4°C. After blocking with 3% BSA / 5% sucrose in washing buffer (PBS/0,05% Tween 20) wells were incubated with A20- or C37-B-hybridoma supernatant or human serum diluted 1:50 to 1:400 in blocking buffer for 1h at room temperature. After washing wells were incubated with a biotin-conjugated anti-human or anti-mouse secondary antibody (Dianova) diluted in washing buffer for one hour. Detection and quantification was performed as described previously (Girod *et al.*, 1999a). Serum samples were considered as seropositive for AAV antibodies when the measured OD was 0.2 or higher at a 1:300 dilution after subtraction of background.

Neutralization Assay: HeLa cells or B16F10 cells were seeded in 96-well plates (5×10^3 cells per well) and infected with AdV (MOI 5) or irradiated 2 h prior to infection with AAV, respectively. Identical transducing particle numbers (MOI 5) of rAAV (wt capsid) and rAAV-587/L14 were incubated with serial dilutions (1:10 to 1:1200) of human serum in PBS for 2 h at 4°C in a total volume of 30 µl. Before addition of the AAV/serum-mixture medium was replaced by 100 µl of fresh medium. 48 h (HeLa) or 72 h (B16F10) after infection GFP-positive cells were detected by FACS analysis and fluorescence microscopy. Similarly, Mec1 cells were seeded at 5×10^4 cells per well, infected with AdV followed by infection with rAAV or rAAV-587/MecA, which had been incubated with serial dilutions of human serum as described above. The neutralizing titers are expressed as the dilution at which transduction was 50% reduced compared to the positive control (N_{50}). Serum samples were considered as neutralizing when the N_{50} was 1:320 or higher.

Acknowledgments

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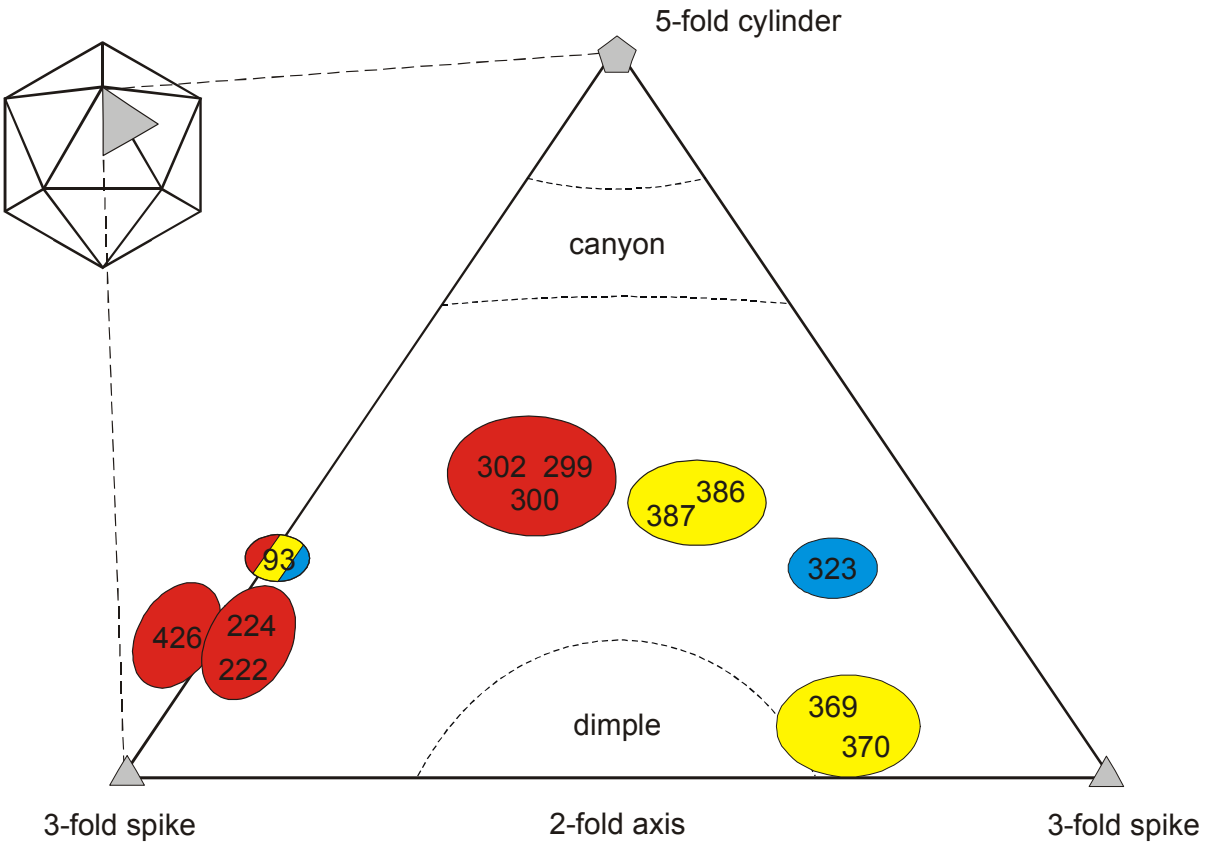

Figure 1. *Map of major antigenic regions in CPV, FPV and B19. The triangle represents one asymmetric subunit of the CPV major capsid protein. The positions of the 5-fold, 3-fold and 2-fold symmetry axis are indicated. The amino acid positions of major antigenic determinants in CPV (red), B19 (yellow) and FPV (blue) are given (aligned to the CPV capsid protein) (Chapman and Rossmann, 1993).*

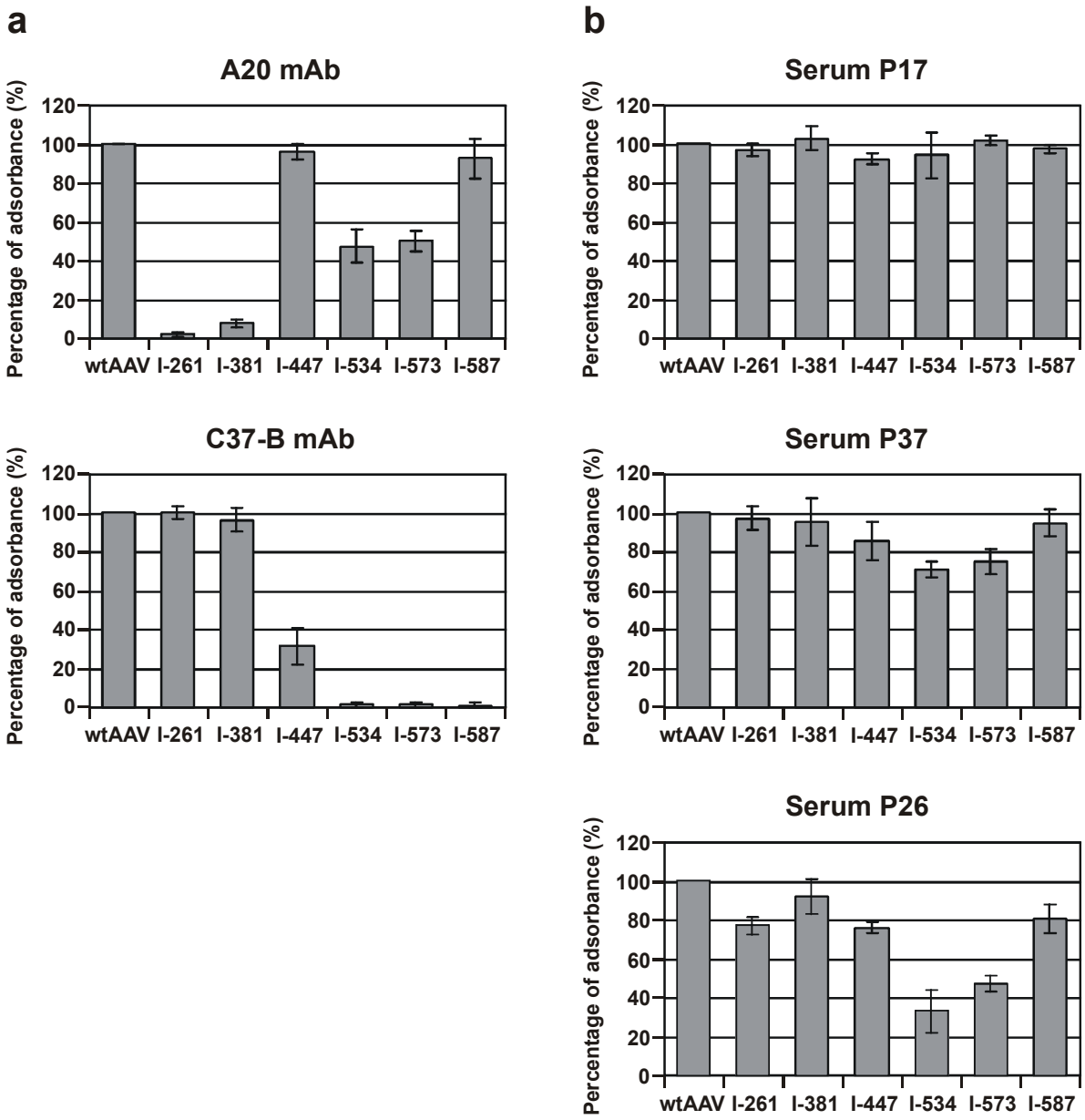

Figure 2. *Binding of A20 and C37-B monoclonal antibody (a) and human serum samples (b) to wtAAV and AAV capsid mutants as determined by ELISA. Microtiter plates were coated with identical particle amounts of wtAAV and AAV insertion mutants and incubated with hybridoma supernatants of either A20 or C37-B mAb or with serum samples of human patients as described in Materials and Methods. Binding of antibodies to wtAAV was set at 100% (y axis). Each experiment was repeated independently at least three times; the figure shows the mean values and standard deviations (indicated by the error bars).*

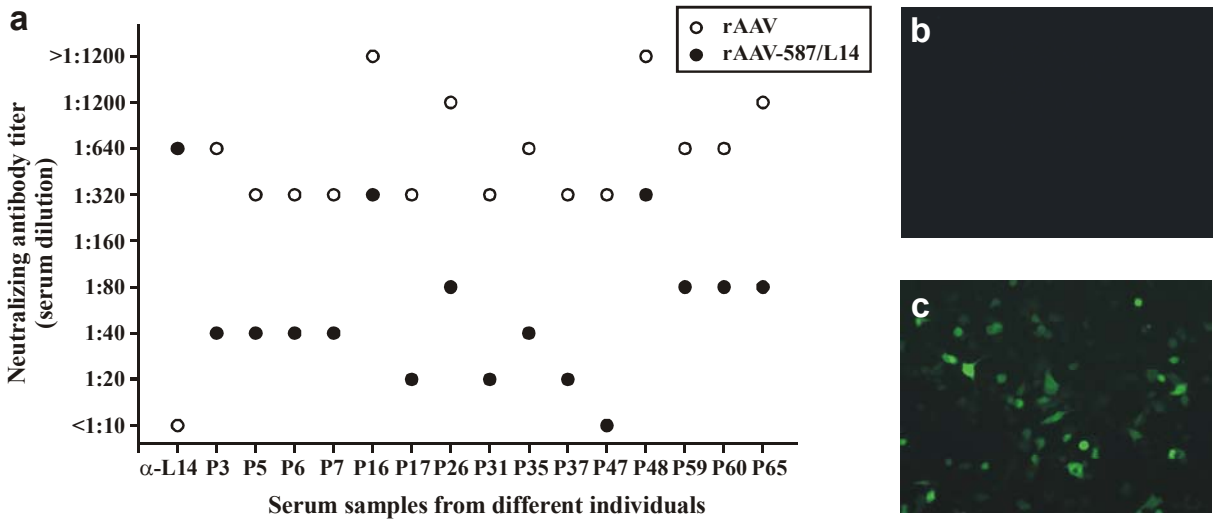

Figure 3. *Neutralization assay on HeLa cells. (a) Neutralizing antibody titers against rAAV and rAAV-587/L14. Serial dilutions (1:10 – 1:1200) of 15 neutralizing human serum samples were analyzed on HeLa cells. As control, rabbit serum directed against the inserted L14-ligand (α-L14) was tested. The neutralizing titers (N_{50}) are expressed as the dilution at which transduction was 50% reduced compared to the positive control. rAAV (b) and rAAV-587/L14 (c) were incubated with serum P35 (1:80) prior infection of HeLa cells. GFP expression was monitored 48 hours post infection.*

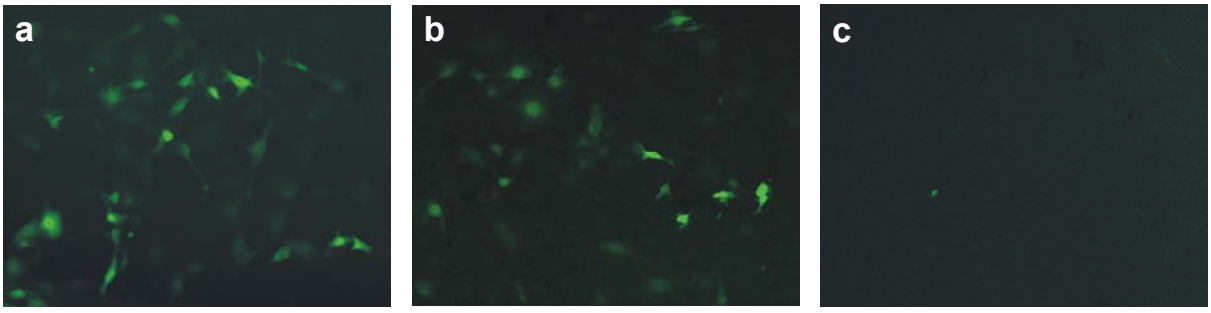

Figure 4. *Neutralization assay on B16F10 cells. Infection of irradiated B16F10 cells with rAAV-587/L14 alone (a) or after co-incubation with P35 serum (b) or anti-L14 serum (c) at a 1:80 serum dilution. Cells were analyzed for GFP expression after 72 hours.*

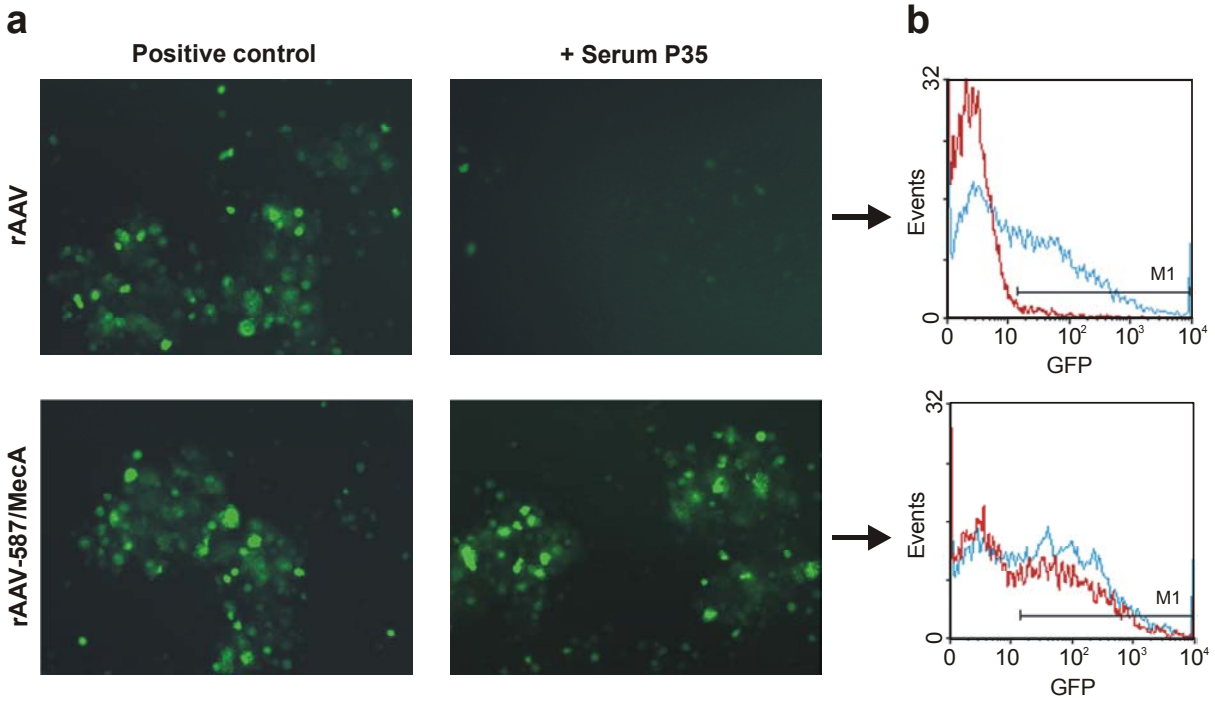

Figure 5. *Effect of neutralizing antisera on rAAV-587/MecA transduction. (a) After infection with adenovirus, Mec1 cells were infected with rAAV (top row) and rAAV-587/MecA (bottom row) alone (positive control) or after co-incubation with serum P35 at a 1:80 dilution (+ serum P35). Note that more physical particles were used for rAAV to achieve similar transduction. (b) FACS analysis of rAAV (top row) and rAAV-587/MecA (bottom row) incubated with serum P35 (red line) in comparison to their positive controls (blue line). GFP expression was determined 48 hours post infection.*

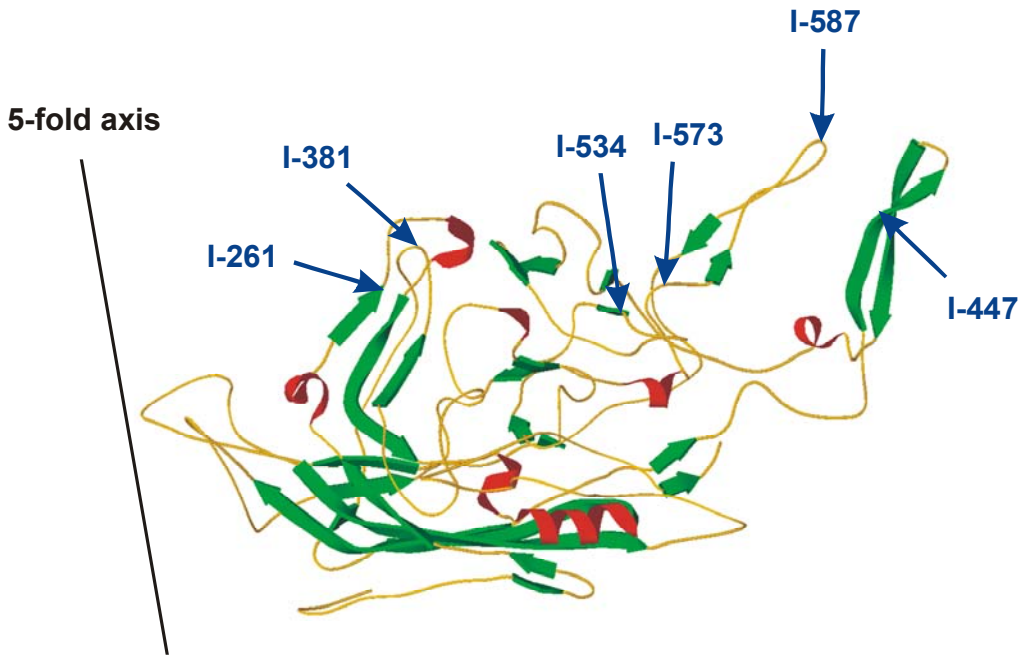

Figure 6. *Model of the AAV major capsid protein according to the atomic structure by Xie et al. (2002) (taken from The Protein Data Bank). The sites of insertion are marked with arrows.*

CHAPTER V

EFFICIENT GENE TRANSFER OF CD40 LIGAND INTO PRIMARY B-CLL CELLS USING RECOMBINANT ADENO-ASSOCIATED VIRUS (rAAV) VECTORS

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Abstract

B cells of chronic lymphocytic leukemia (B-CLL) are resistant to transduction with most currently available vector systems. Using an optimized adenovirus-free packaging system, recombinant adeno-associated virus (rAAV) vectors coding for the enhanced green fluorescent protein (AAV/EGFP) and CD40 ligand (AAV/CD40L) were packaged and highly purified resulting in genomic titers up to $3x10^{11}$ per ml. Cells obtained from 24 patients with B-CLL were infected with AAV/EGFP and/or AAV/CD40L at a multiplicity of infection (MOI) of 100 resulting in transgene expression in up to 97% of cells as detected by flow cytometry 48 hours after infection. Viral transduction could be specifically blocked by heparin. Transduction with AAV/CD40L resulted in upregulation of the costimulatory molecule CD80 not only on infected CLL cells but also on noninfected bystander leukemia B cells, while this effect induced specific proliferation of HLA-matched allogeneic T cells. Vaccination strategies for B-CLL patients using leukemia cells infected *ex vivo* by rAAV vectors seem now possible in the near future.

Introduction

B-CLL cells are inefficient antigen-presenting cells because they lack costimulatory molecules for efficient T cell activation.[1] Moreover, CD40 ligand (CD40L), a critical molecule for T cell activation, is downregulated on T-cells in CLL patients.[2] This defect can be partially corrected by gene transfer of CD40L into B-CLL cells. This strategy was shown to induce an autologous immune recognition of B-CLL cells *in vitro* and in patients using recombinant adenoviral vectors.[3,4] However, transduction by recombinant adenovirus requires a high concentration of viral particles per cell (MOI up to 2000) because B-CLL cells lack the fiber receptor essential for adenoviral attachment.[5] Furthermore, the use of adenovirus has raised the question whether adenoviral fiber structures mediate an unspecific immune stimulation.[6] In contrast, AAV, a non-enveloped human parvovirus, has gained attention for human gene therapy, since it does not seem to be pathogenic. AAV vectors have been proven to be efficient gene transfer vehicles, in particular for solid tumors.[7-10] However, sufficient gene transfer into primary B-CLL cells with AAV vectors has not been achieved so far. Previous efforts to transduce primary B-CLL cells with rAAV were inefficient with transgene expression levels below 3%.[11] A recent improvement in the AAV packaging process was the introduction of a plasmid that encodes the required adenoviral gene products for the helper virus.[12,13] In addition, concentration of viral particles by density-gradient steps using

iodixanol, a nonionic dimeric X-ray contrast solution, instead of cesium chloride was shown to yield higher titers.[14] By using these improvements, we could prepare high-titer rAAV stocks free of adenovirus in order to reassess the susceptibility of primary B-CLL cells for rAAV vectors.

Materials and Methods

Patients, cells and cell culture

After informed consent, peripheral blood was obtained from patients satisfying diagnostic criteria for B-CLL.[15] Mononuclear cells were isolated on a Ficoll/Hypaque (Seromed, Berlin, Germany) density gradient by centrifugation and depleted from monocytes by adherence to plastic tissue culture flasks. More than 98% of isolated cells coexpressed CD5 and CD19, as assessed by flow cytometry, therefore nonmalignant B cells did not constitute a meaningful fraction of the total cells isolated. Patients were either untreated or had not received cytoreductive treatment for a period of at least one month before investigation. All patients were clinically stable and free from infectious complications when blood samples were collected. Staging was perfomed according to the Binet classification. Clinical characteristics of the patients studied are summarized in **Table 1**.

HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) whereas the 293 cells were a gift from M. Lohse, Max-Planck-Institute of Biochemistry (Martinsried, Germany). Cells were cultured at 37^0C in 5% CO_2 in air, in culture medium consisting of DMEM (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom), 2mM L-glutamine (Biochrom), 100 U/ml penicillin (Biochrom) and 100µg/ml streptomycin (Biochrom).

HeLa/SF cells transfected with human CD40L cDNA were produced as previously described.[16] Cells were γ-irradiated at 200 Gy, plated at $3x10^4$ cells/well in 96-well plates in media and incubated overnight at 37^0C in a 5% CO_2 humidified atmosphere. Before addition of B-CLL cells, the feeder layers were washed twice with phosphate-buffered saline, and tumor cells were cultured at $2x10^6$ cells/ml in Iscove´s medium (GibcoBRL) supplemented with 20% heat-inactivated fetal calf serum (FCS), 2mM/l L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin. For functional assays, CLL cells were harvested, purified by ficoll density gradient centrifugation, washed and analyzed by flow cytometry.

Antibodies and reagents

Immunophenotyping was performed with the following monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or phycoerythrin cyanine 5 (PE-Cy5): CD5, CD19, CD80, anti-κ, anti-λ (Beckman Coulter, Krefeld, Germany). Fluorescein-conjugated mAbs specific for murine CD40L was purchased from BDPharMingen (Heidelberg, Germany) and expression was controlled by an isotype hamster IgG_3 mAb (BDPharMingen). Heparin (10,000 U/ml; Braun, Melsungen, Germany) was used for blocking experiments with infectious AAV.

Plasmids

The adenoviral pXX6 plasmid was a friendly gift of R. Samulski and described previously.[17] The gene for enhanced green fluorescent protein (EGFP) was obtained by excising the Asp718-NotI fragment of pEGFP-N1 (Clontech, Heidelberg, Germany) and inserted into the Asp718-NotI site of psub/CEP4(Sal invers). psub/CEP4(Sal invers) is a derivative of psub201(+), which was digested with XbaI, blunt ended and ligated to the blunt ended 3923bp SalI-NruI-fragment of pCEP4(Sal invers).[18] pCEP4(Sal invers) differs from pCEP4 (Invitrogen, Groningen, Netherlands) by inversion of the SalI(8)-SalI(3-1316)-fragment. The construct pAAV/mCD40L contains the murine CD40L encoding gene driven by the CMV promoter. To generate pAAV/mCD40L, the 0.8 kb open reading frame (orf) was released by BamHI digestion from pCEP4/mCD40L and ligated into an Asp718/NotI-digested psubCEP4(Sal invers).

rAAV vector production and purification

293 cells were seeded at 80% confluency and cotransfected by calcium phosphate with a total of 37.5µg of vector plasmid (pAAV/EGFP or pAAV/mCD40L), packaging plasmid pRC and adenoviral plasmid pXX6 at a 1:1:1 molar ratio.[19] 48 hours after transfection cells were harvested and pelleted by low speed centrifugation. Cells were resuspended in 150mM NaCl, 50mM Tris-HCl (pH 8.5), freeze-thawed several times, and treated with Benzonase (50U/ml) for 30 min at 37^0C.[20] Cell debris was spun down at 3700g for 20 min at 4^0C. Supernatant was purified by ammoniumsulfate precipitation. The pellet was resuspended in PBS-MK buffer (1x phosphate buffered saline (PBS), 1 mM $MgCl_2$ and 2.5 mM KCl) and loaded onto an iodixanol gradient and purified as previously described.[20] The iodixanol fraction was further purified by heparin affinity column chromatography and the virus dialysed against PBS-MK before being used.[21]

Viral vector titering assays

The vector particle titer for AAV/EGFP and AAV/CD40L was determined by treating vector dilutions with DNase I (end concentration 0.5µg/µl; Boehringer Mannheim GmbH, Mannheim, Germany) for 60 min at 25^0C to remove putatively free viral genomes that could be subsequently hybridized with the probe. The viral preparations were then blotted in a serial two-fold dilution and finally hybridized with a random-primed transgene specific probe by standard methods. Particle titers were determined by comparing the intensity of the hybridization signals with that obtained for the vector plasmid standard of known concentration blotted on the same membrane. The transducing titer was determined as follows: $7x10^4$ HeLa cells per well were seeded in a 12-well-plate ($4cm^2$), 24 hours later the cells were irradiated with 100 Gy and serial dilutions of the recombinant virus were added to the cells. After 48 hours, the number of transgene expressing cells was quantified by flow cytometry.

AAV transduction

$5x10^5$ primary CLL cells per well (96-well plate) were incubated in a total of 50µl IMDM medium supplemented with 20% FCS and infectious AAV was added resulting in an MOI between 1 and 500. Cells were incubated for 2 hours at 37^oC in 5% CO_2 in air. Thereafter infected cells were transferred on a γ-irradiated feeder layer expressing CD40L (HeLa/SF) as outlined above and 150µl Iscove´s medium was added.

Flow cytometry

48 hours after AAV transduction, CLL cells were harvested, purified by Ficoll density gradient centrifugation and washed. Specific, directly conjugated antibodies were applied to cells for 30 min in PBS, 4% FCS, 0.1% sodium azide, 20mM HEPES, and 5mM EDTA pH 7.3 on ice and washed. Nonspecific binding was controlled by incubation with isotypic controls (murine isotype IgG_1 mAb and hamster isotype IgG_3 mAb, BDPharMingen, Heidelberg, Germany). Fluorescence was measured with a Coulter Epics XL-MCL (Beckman Coulter, Krefeld, Germany). A minimum of 5,000 cells were analyzed for each sample. The percentage of positive cells is defined as the fraction beyond the region of 99% of the control-stained cells. Data were analyzed with the use of WinMDI2.8 FACS software. We calculated the mean fluorescence intensity ratio (MFIR) to compare the relative staining intensities of two or more stained cell populations. The MFIR is the mean fluorescence intensity (MFI) of

cells stained with a fluorochrome-conjugated antigen-specific mAb divided by the MFI of cells stained with a fluorochrome-conjugated isotype control mAb.[3]

Transactivation Assay

CD40L-transduced or mock-infected B-CLL cells were prelabeled with a green fluorescent dye (CellTracker^{TM} Green CMFDA, Molecular Probes) at a concentration of 1μM for 15 min at 37^{0}C. After extensive washing labeled stimulator cells were cocultured with noninfected, nonstained CLL cells from the same patient at 37^{0}C for another 48 hours. Expression of CD80 on noninfected, nonlabeled naive CLL cells was assessed by PE-conjugated anti-CD80 mAb (Beckman Coulter).

Mixed lymphocyte reaction (MLR)

T cells derived from HLA.A2 positive healthy donors were isolated to >95% purity by depleting CD19, CD14, CD56 and CD16 positive cells from blood mononuclear cells using the Pan T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and magnetic separation columns (Miltenyi Biotec). CLL cells isolated from HLA.A2 positive patients were transduced with AAV (AAV/EGFP, AAV/CD40L) and after separation from HeLa/SF feeder cells coincubated for 72 hours with untransduced CLL cells (ratio 1:10) derived from the same patient. Irradiated (200Gy) CLL cells were used as stimulators, cocultured at 1x10^4 cells/well in a final volume of 200μl with allogeneic T cells at 1x10^5 cells/well in 96-well round-bottom plates, and incubated for 4 days in IMDM medium (Biochrom) supplemented with IL-2 (20 IU/ml; Panbiotech, Aidenbach, Germany) at 37^{0}C in a 5% CO_2 humidified atmosphere. During the last 6 hours of the 96-hour culture period, cells were pulsed with 0.5μCi [^{3}H] thymidine (Amersham, Braunschweig, Germany). Cells were harvested onto glass fiber filters and dried, and the [^{3}H] thymidine incorporation was measured by scintillation spectrophotometry in a Wallac Microbeta Plus 1450 scintillation counter (Turku, Finland).

Statistics

Statistical associations between dependent subgroups were analysed by the *t test* for paired samples; statistical associations between independent subgroups were carried out using the *median two-sample test*. In the case of multiple comparisons *p*-values were adjusted by the *Bonferroni method*. A statistical significance was accepted when the *p* value was less than 0.05. The calculations were determined by the statistical software package SAS, version 8.2.

Results

Efficient packaging of high-titer, purified rAAV vectors coding for EGFP and CD40L with an improved packaging system

Using a new helpervirus-free packaging protocol, efficient production of rAAV virions coding for EGFP and CD40L was achieved. The peak fraction of rAAV preparations contained a concentration of 3.8 x 10^{11} (SEM: 0.6 x 10^{11}) viral particles per ml for AAV/EGFP and 2.4 x 10^{11} (SEM: 0.8 x 10^{11}) for AAV/CD40L, respectively. In order to exclude the possibility that contaminating DNA accounted for hybridization signals, viral stocks were preincubated with DNAse. No significant decrease in titer was seen suggesting that the majority of viral particles detected was intact (Fig. 1). In several viral preparations infectious titers of 3.7 x 10^9 (SEM: 0.3 x 10^9) per ml for AAV/EGFP and 4.7 x 10^9 (SEM: 0.3 x 10^9) per ml for AAV/CD40L were achieved. This corresponds to an infectious to particle titer ratio of about 1:100 for AAV/EGFP and 1:50 for AAV/CD40L, respectively.

High expression of EGFP and CD40L on primary B-CLL after AAV transduction

Freshly isolated CD5/CD19 positive cells from patients with established diagnosis of B-CLL were infected with AAV/EGFP and AAV/CD40L at an MOI of 100 and assessed for transgene expression by flow cytometry after 48 hours. In Fig. 2**,** results with cells double-stained for the lineage marker CD19 and the transgene are shown for four representative patients (No. 4, 5, 21, 22 see Tab. 1). While green fluorescence was detected in only 1% of untransduced cells, expression increased to 42.4% (MFIR 5.3), 44.9% (MFIR 6.6), 50.3% (MFIR 5.4) and 63.0% (MFIR 15.2), respectively, after transduction with AAV/EGFP. Leukemic cells of the same patients were transduced with AAV/CD40L, and CD40L expression showed a marked shift to 77.8% (MFIR 209.0), 97.7% (MFIR 312.2), 78.9% (MFIR 80.2) and 81.5% (MFIR 164.9), respectively.

Cumulative data for 24 different patients with B-CLL studied for AAV infection are given in Tab. 1. The mean age of the study population was 62.3 years (SEM: 1.9 years) with a median of 62 years. Ten patients presented with early disease (Binet stage A) and 14 with advanced disease (Binet stage B: n=4, Binet stage C: n=10). Eighteen patients were studied for EGFP transduction, the mean percentage of EGFP transduced cells was 30.3% (SEM: 3.6%). For CD40L-transduced samples (n=15), 44.0% (mean) of CLL cells could be infected (SEM: 7.4%). Using the *median two-sample test*, there was no correlation seen between transduction efficiency with AAV/EGFP or AAV/CD40L, respectively, and clinical stage of

disease (p = 0.36 and p = 0.48), gender (p = 0.61 and p = 0.35) or age (p = 0.36 and p = 0.20). Furthermore, the association between the clinical stage of disease and susceptibility to AAV/EGFP or AAV/CD40L infection, respectively, was examined by the *median two-sample test* using the *Bonferroni* method with adjustment for sex (male, female) and age (≤ median age, > median age). Therefore, p values less than 0.0125 (0.05 divided by 4, due to 4 subgroups) would indicate a significant association at the 0.05 level. For all four subgroups (male, female, age ≤62 years and >62 years), p values were greater than 0.0125 demonstrating no association between clinical stage and susceptibility to AAV/EGFP and AAV/CD40L infection, respectively.

Studying the patients´ samples which were both transduced for EGFP and CD40L (n=9), values for transduction efficiency were not statistically different (t test for paired samples: p=0.17): a mean percentage of 37.3% (SEM: 5.3%) of infected cells could be transduced by AAV/EGFP and 50.6% of CLL cells by AAV/CD40L (SEM: 11.3%). Transduction efficiencies of both viral vectors correlated in these patients with each other (Spearman correlation coefficient test: p = 0.01). Using the Bonferroni method for adjustment with respect to clinical stage and age of patients (adjustment for sex was not performed because of only one female patient studied both for EGFP and CD40L transduction), a significant p value in the Spearman correlation coefficient test ($p<0.0001$) was only achieved for samples derived from patients with advanced clinical stage (Binet B or C).

Concentration and time kinetics of AAV transduction in CLL cells

Primary B-CLL cells were infected with AAV/EGFP at different MOIs ranging from 10 to 500. 48 hours after infection cells were analyzed for expression of EGFP by flow cytometry. At an MOI of 10, an average of 8.3% of cells (SEM: 3.9%) were positive for transgene expression. At an MOI of 100, a significant increase of the fraction of transgene positive cells was observed (t test for paired samples: p = 0.02): an average of 32.2% of cells (SEM: 4.8%) showed an EGFP expression. At higher MOI of 500, only a slight, but insignificant (t test: p = 0.31) increase in the fraction of EGPF positive cells was detectable (38.3%; SEM 3.5%). (Fig.3)

Recently, membrane-associated heparan sulfate proteoglycans (HSPG) were identified as the primary receptors for AAV virions thus mediating both AAV attachment to and infection of target cells.[21] Inhibition of AAV attachment and infection was shown to be achieved in competition experiments using heparin, a molecule chemically very similar to heparan sulfate glycosaminoglycan thus functioning as a soluble receptor analog. Therefore,

viral supernatants coding for EGFP (AAV/EGFP) were mixed with heparin and incubated with B-CLL cells. As seen in Fig. 3**,** viral transduction of EGFP at an high MOI of 500 was significantly decreased by heparin (t test: $p = 0.007$) resulting in background level of EGFP expression: a mean percentage of only 0.05% of cells (SEM: 0.05%) were EGFP positive.

Transgene expression was assessed over time using an MOI of 100. As seen in Fig. 4, 12 hours after EGFP infection only small amounts of EGFP positive cells were detected by flow cytometry (mean: 0.4%, SEM: 0.1%). 24 hours after transduction a mean of 4.6% of cells were positive (SEM: 1.3%). A significant (t test: $p = 0.005$) shift in the fraction of EGFP expressing cells was seen after 48 hours (mean: 32.2%; SEM: 4.8%). A further although insignificant (t test: $p = 0.11$) increase was detectable another 48 hours later (i.e., 96 hours after infection) with an average EGFP positive cell population of 53.0% (SEM: 8.6%). The mean percentage of EGFP positive cells was decreased one week after infection at significant levels in comparison to transduction data obtained 48 hours after infection (20.9%; SEM: 9.1%) (t test: $p = 0.04$).

Upregulation of CD80 on B-CLL cells after AAV/CD40L transduction

In order to prove that CLL cells transduced with CD40L became highly proficient antigen presenting cells, expression of the costimulatory molecule CD80 was assessed before and 8 days after infection with AAV/CD40L. In CLL samples derived from three different patients, CD80 expression could be induced from 6.9% (MFIR 1.6) to 19.4% (MFIR 2.3) (patient 3), from 1.7% (MFIR 1.2) to 10% (MFIR 2.8) (patient 6) and from 4% (MFIR 0.8) to 27% (MFIR 2.4) (patient 7), respectively. CD80 expression on CD40L-transduced CLL cells (mean: 18.8%, SEM: 4.9%) was significantly higher in comparison to uninfected control CLL cells (mean: 4.2%, SEM: 1.5%) (median two-sample test: $p = 0.03$). Incubation with an anti-murine CD40L mAb could significantly inhibit CD80 upregulation (mean: 4.8%, SEM: 0.9%) in these three patients with an expression of 5.4% (MFIR 0.7), 3% (MFIR 1.8) and 6% (MFIR 1.9), respectively (median two-sample test: $p = 0.03$) (Fig. 5). Primary CLL cells infected with AAV/EGFP did not result in any upregulation of CD80 in comparison to uninfected controls (data not shown).

Transactivation of non-infected bystander leukemia cells by CD40L-transduced CLL cells

The transactivation capacity of AAV/CD40L infected CLL cells was assessed after they were labeled with a green fluorescent dye (CellTracker^{TM}) and used as stimulator cells for equal numbers of nonlabeled CLL B cells from the same patient. Uninfected nonlabeled bystander CLL cells of patient 16 (see Tab. 1) were induced to express CD80 when cocultured with CD40L-infected CLL cells (29.4%/MFIR 4.0), but not after coincubation with uninfected control CLL cells (1.3%/MFIR 1.0). Similar results were obtained when naive CLL cells were stained and coincubated with CD40L-transduced nonlabeled leukemia cells: CD80 was upregulated on naive bystander cells (29.7%; MFIR 4.2), incubation with uninfected control CLL cells resulted in very low CD80 expression (0.3%; MFIR 0.9). This stimulatory effect on bystander CLL cells could be abrogated by coincubation of stimulator cells with an anti-mCD40L mAb (3.2%/MFIR 1.5). Furthermore, stimulation of naive CLL cells by mock-infected (wild-type AAV) CLL cells did not effect CD80 expression levels (0.7%; MFIR 0.8). Specific upregulation of CD80 on bystander CLL cells was also observed in two other patients´ samples studied (No. 12, 15) (Fig. 6). CD80 expression on bystander cells after coincubation with CD40L-transduced CLL cells (mean: 18.4%, SEM: 6.1%) was significantly higher for all samples studied in comparison to data obtained after coincubation with wild type AAV-infected (mean: 1.4%, SEM: 0.5%) or uninfected CLL cells (mean: 1.3%, SEM: 0.8%) (median two-sample test: $p = 0.03$).

CD40L-transduced CLL cells induce a proliferative T-cell response

We examined whether AAV/CD40L-infected CLL cells could stimulate allogeneic T cells in an HLA.A2-matched mixed lymphocyte reaction (MLR). For this purpose, we used highly purified allogeneic T cells from HLA.A2 positive healthy donors and incubated them with γ-irradiated CLL cells from HLA.A2 positive CLL patients. In order to minimize unspecific T cell stimulatory effects by HeLa/SF-feeder cocultivation of infected CLL cells, transduced CLL cells were mixed 1:10 with naive bystander cells derived from the same CLL patient and used as mixture in allogeneic T cell proliferation assays. Upon co-culture with AAV/CD40L-infected CLL cells, allogeneic T cells were induced to undergo proliferation at rates significantly greater (mean: 13274 cpms, SEM: 3493 cpms) than that observed with CLL cells infected with AAV/EGFP (mean: 2467 cpms, SEM: 142 cpms) (median two-sample test: $p = 0.03$) (Fig. 7). The experiments shown are representative for different allogeneic T-cell donors and different CLL samples examined.

Discussion

By use of a novel adenovirus-free packaging method we were able to generate very high-titer and pure viral preparations of rAAV. The infectious to particle titer achieved is quite favorable in comparison to other packaging procedures for AAV because eradication of contaminating helpervirus particles is unnecessary.[7] Using this purified helpervirus-free rAAV, we were able to transduce primary B-CLL cells at low MOIs with high efficiency. In comparison to adenoviral transgene vectors, rAAV vectors were efficient to transduce CLL cells at much lower MOIs.[3] We have shown that the transduction potency of AAV vectors is independent of the transgene used. Differences observed between individual patients are independent of clinical stage of disease, age or gender. We were able to provide evidence that the transduction of CLL cells by rAAV was specific and not due to a pseusotransduction since viral transduction could be significantly abrogated by heparin. Since membrane-associated heparan sulfate proteoglycans (HSPG) were identified as the primary receptors for AAV virions mediating both AAV attachment and infection, the blockade of infection by heparin, a chemical analog of heparan sulfate glycosaminoglycan, is thought to be specific for this type of vector transduction and has been demonstrated by others.[21,24] After attachment of the virus it is assumed that AAV is internalized by clathrin-mediated endocytosis promoted by the interaction of the virus with the β_5-subunit of $\alpha_v\beta_5$-integrins.[25] The role of $\alpha_v\beta_5$ as functional receptor for AAV is not totally clarified since neither $\alpha_v\beta_5$, RGD peptides nor functional blocking mAb were able to block AAV-2 transduction as has been shown by others.[26] Besides integrins, fibroblast growth factor receptor 1 (FGFR1) has been implicated as co-receptor of AAV entry into target cells. Cells which do not express either HSPG or FGFR1 fail to bind AAV and are resistant to AAV infection.[27]

Besides expression of specific receptors on target cells, transduction efficiency of AAV vectors correlates with the phosphorylation state of cellular proteins, especially the single-stranded D-sequence-binding-protein (ssD-BP). This protein interacts with D(-) sequences within the inverted terminal repeat (ITR) of AAV.[28,29] Efficient transgene expression requires dephosporylation of ssD-BP which is usually phosphorylated at tyrosine residues by the protein kinase activity of the cellular epidermal growth factor receptor (EGFR). Inhibition of the EGFR protein tyrosine kinase by the specific inhibitor tyrphostin results in dephosphorylation of ssD-BP and augmentation of AAV-mediated transgene expression.[30,31]

During AAV transduction primary B-CLL cells were incubated on a feeder layer expressing CD40L itself. By this CD40 activation, malignant B cells are shifted in a more

proliferative status as has been shown previously.[32] Based on cell cycle profile assessment using propidium iodide staining and flow cytometric analysis, Granziero et al. demonstrated an increase in the proliferative pool with 0.5 to 5.3% of CLL cells in S phase after 3 days of CD40 stimulation by human soluble CD40L.[33] In own preliminary experiments we could show an increase in the fraction of CLL cells in S phase (<5%) after cultivating them on CD40L-expressing feeder cells (data not shown). Therefore, besides an improved packaging protocol the preactivation of B cells by CD40 cross-linking remains a prerequisite for efficient transgene transduction by AAV. For adenoviral infection of primary CLL cells this CD40 stimulus was shown to improve transduction efficiency.[3] A future goal for AAV-mediated transduction of CLL cells will be to replace this time-consuming cellular feeder system: besides AAV-2 other serotypes of AAV will be studied for their B cell tropism. Serotypes 1, 3, 4 and 5 have been shown to transduce muscle cells more efficiently than recombinant AAV-2 particles.[34] Furthermore, packaging of self-complementary rAAVs (scAAVs) could enable a feeder-independent transduction by rAAV. These are AAV vectors with about half of the size of wild type AAV and are therefore preferentially packaged to dimeric molecules, i.e. scAAVs contain two complementary sequences of the genome in an inverted repeat configuration. Thus the limiting step of conversion of a single-stranded AAV vector to a double-stranded transgene sequence in the target cell is no more necessary. In a murine model scAAV particles coding for erythropoietin showed a faster and higher transgene expression in comparison to monomeric AAV particles after intramuscular injection.[35] Finally, retargeting of AAV vectors by modification of their capsid proteins could enable an improved transduction of malignant B cells. For recombinant AAV-2 vectors a region I-587 was recently defined at the capsid which allowed insertion of a peptide ligand thus allowing efficient retargeting of AAV vectors to cells with specific integrin receptors.[19] Using an immunoglobulin binding domain at this insertion site, B cell specific antibodies could redirect via their F_c region AAV particles to B-CLL cells.

By infection with viral particles encoding murine CD40L, we modified primary human B-CLL cells to express a functional ligand for CD40. We used murine CD40 ligand since it was shown that recombinant soluble murine CD40L is able to bind human CD40 on B cells and induces a T cell activation.[36] Furthermore, this heterologous ligand could be distinguished from endogenously expressed human CD40L on infected human cells since no cross reactivity of murine and human Ab was observed. Therefore it can be excluded that putatively unspecific stimulation of endogenous human CD40L during AAV infection or cytophilic soluble human CD40L made by the HeLa/SF feeder cells account for false positive

transduction signals. In a clinical phase I trial using murine CD40L no unspecific immune reactions were observed in treated patients. CD40L transduction of B-CLL cells resulted not only in expression of the immune accessory molecule CD80 on infected cells, but also in activation of noninfected bystander leukemia B cells. Similar results with an upregulation of CD54 and CD86 on leukemic bystander cells were observed previously using an adenoviral transfer systems.[3] We could show that this bystander effect was specific since incubation with an antibody directed against murine CD40L abrogated the induction of costimulatory molecules. Furthermore, coincubation of bystander cells with mock-infected or untransduced CLL cells did not affect the expression of CD80. Infection with AAV/CD40L significantly improves the functional antigen presenting capacity of CLL cells *in vitro* since allogeneic T cells can be induced to proliferate at higher levels in comparison to mock-infected CLL cells. Similar results have also been shown for CLL cells transduced by adenoviral vectors coding for CD40L.[3,4] In our experimental setting this T cell proliferative response is mainly mediated by activated CLL bystander cells which were coincubated with CD40L-transduced CLL cells thus resembling an *in vivo* vaccination situation where the number of resident nontransduced leukemia cells would greatly outnumber the infused CD40L-infected CLL cells.[4] Having a non-immunogenic vector transfer system like AAV in hands, it is now possible in future experiments to exclude any unspecific T cell induction and define CLL specific cytolytic T cell responses using putative CLL-associated antigens.[37,38]

In conclusion, in the present study we have shown that an efficient transduction of primary B-CLL cells with the costimulatory molecule CD40L can be achieved by high-titer, adenovirus-free rAAV vectors. CD40L gene transfer resulted in specific upregulation of the costimulatory molecule CD80 on infected B-CLL cells and on leukemic bystander cells and induced an allogeneic T cell response. Therefore transduction of immunostimulatory molecules into CLL cells is now possible with these biologically active preparations of rAAV and enables vaccination strategies with this safe vector system in the near future.

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References

1. Ranheim EA, Kipps TJ. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J Exp Med. 1993;177:925-935.

2. Cantwell MJ, Hua T, Pappas J, Kipps TJ. Acquired CD40-ligand deficiency in chronic lymphocytic leukemia. Nat Med. 1997;3:984-989.

3. Kato K, Cantwell MJ, Sharma S, Kipps TJ. Gene transfer of CD40-ligand induces autologous immune recognition of chronic lymphocytic leukemia B cells. J Clin Invest. 1998;101:1131-1141.

4. Wierda G, Cantwell MJ, Woods SJ, Rassenti LZ, Prussak CE, Kipps TJ. CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. Blood. 2000;96:2917-2924.

5. Cantwell MJ, Sharma S, Friedmann T, Kipps TJ. Adenovirus vector infection of chronic lymphocytic leukemia B cells. Blood. 1996;88:4676-4683.

6. Yang R, Nunes FA, Berencsi K. Cellular immunity to vital antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci USA. 1994;91:4407-4411.

7. Chiorini JA, Wendtner CM, Urcelay E, Safer B, Hallek M, Kotin RM. High-efficiency transfer of the T cell co-stimulatory molecule B7-2 to lymphoid cells using high-titer recombinant adeno-associated virus vectors. Hum Gene Ther. 1995;6:1531-1541.

8. Wendtner C-M, Nolte A, Mangold E et al. Gene transfer of the costimulatory molecules B7-1 and B7-2 into human multiple myeloma cells by recombinant adeno-associated virus enhances the cytolytic T cell response. Gene Ther. 1997;4:726-735.

9. Maass G, Bogedain C, Scheer U et al. Recombinant adeno-associated virus for the generation of autologous, gene-modified tumor vaccines: evidence for a high transduction efficiency into primary epithelial cancer cells. Hum Gene Ther. 1998;9:1049-1059.

10. Hanazono Y, Brown KE, Handa A et al. In vivo marking of rhesus monkey lymphocytes by adeno-associated viral vectors: direct comparison with retroviral vectors. Blood. 1999;94:2263-2270.

11. Rohr UP, Kronenwetter R, Haas, R. Transduction efficiencies of primary normal and malignant cells using a recombinant AAV-2 vector depend on cell type and cell cycle [abstract]. Blood. 1999;94:181a.

12. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J Virol. 1998;72:2224-2232.

13. Grimm D, Kern A, Rittner K, Kleinschmidt JA. Novel tools for production and purification of recombinant adeno-associated virus vectors. Hum Gene Ther. 1998;9:2745-2760.

14. Hermens WTJMC, Brake OT, Dijkhuizen PA et al. Purification of recombinant adeno-associated virus by iodixonal gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. Hum Gene Ther. 1999;10:1885-1891.

15. Cheson B, Bennett JM, Grever M et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood. 1996;87:4990-4997.

16. Buhmann R, Nolte A, Westhaus D, Emmerich B, Hallek M. CD40-activated B-cell chronic lymphocytic leukemia cells for tumor immunotherapy: stimulation of allogeneic versus autologous T cells generates different types of effector cells. Blood. 1999;93:1992-2002.

17. Nathwani AC, Hanawana H, Vandergriff J, Kelly P, Vanin EF, Nienhuis AW. Efficient gene transfer into human cord blood CD34+ cells and the CD34+CD38- subset using highly purified recombinant adeno-associated viral vector preparations that are free of helper virus and wild-type AAV. Gene Ther. 2000;7:183-195.

18. Samulski RJ, Chang LS, Shenk T. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J Virol 1989;61:3096-3101.

19. Girod A, Ried M, Wobus C et al. Genetic capsid modifications allow efficient retargeting of adeno-associated virus type 2. Nat Med. 1999;5:1052-1056.

20. Zolotukhin S, Byrne BJ, Mason E et al. Utilization of AAV receptor affinity to develop an efficient and novel purification protocol for vector production. Gene Ther. 1999;6:973-985.

21. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol. 1998;72:1438-1445.

22. Alexander IE, Russell DW, Miller AD. DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. J Virol 1994;68:8282

23. Russell DW, Alexander IE, Miller AD. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. Proc Natl Acad Sci USA 1995;92:5719

24. Hacker UT, Gerner FM, Büning H et al. Standard heparin, low molecular weight heparin, low molecular weight heparinoid, and recombinant hirudin differ in their ability to inhibit transduction by recombinant adeno-associated virus type 2 vectors. Gene Ther 2001;8:966-968.

25. Summerford C, Bartlett JS, Samulski RJ. $\alpha_v\beta_5$ integrin: a co-receptor for adeno-associated virus type 2 infection. Nat Med 1999;5:78-82.

26. Qiu J, Brown KE. Integrin alphaVbeta5 is not involved in adeno-associated virus type 2 (AAV2) infection. Virology 1999;264:436-440.

27. Qing KY, Mah C, Hansen J, Zhou S, Dwarki V, Srivastava A. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat Med. 1999;5:71-77.

28. Qing KY et al. Role of tyrosine phosphorylation of a cellular protein in adeno-associated virus 2-mediated transgene expression. Proc Natl Acad Sci USA 1997;94:10879-10884.

29. Qing KY et al. Adeno-associated virus type 2-mediated gene transfer: correlation of tyrosine phosphorylation of the cellular single-stranded D sequence-binding protein with transgene expression in human cells *in vitro* and murine tissues *in vivo*. J Virol 1998;72:1593-1599.

30. Mah C et al. Adeno-associated virus 2-mediated gene transfer: role of epidermal growth factor receptor protein tyrosine kinase in transgene expression. J Virol 1998;72:9835-9843.

31. Hansen J, Qing KY, Kwon H-J, Mah C, Srivastava A. Impaired intracellular trafficking of adeno-associated virus 2 vectors limits efficient transduction of murine fibroblasts [abstract]. Blood. 1999;94:410b.

32. Teoh G, Tai Y-T, Urashima M et al. CD40 activation mediates p53-dependent cell cycle regulation in human multiple myeloma cell lines. Blood. 2000;95:1039-1046.

33. Granziero L, Ghia P, Circosta P et al. Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. Blood. 2001;97:2777-2783.

34. Chao HC, Liu Y, Rabinowitz J, Li C, Samulski RJ. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol Ther 2000;2:619-623.

35. McCarty DM, Monahan PE, Samulski RJ. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. Gene Therapy 2001;8:1248-1254.

36. Lane P, Brocker T, Hubele S, Padovan E, Lanzavecchia A, McConnell F. Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell-dependent activation. J Exp Med 1993;177:1209-1213.

37. Trojan A, Schultze JL, Witzens M, Vonderheide RH, Ladetto M, Donovan JW, Gribben JG. Immunoglobulin framework-derived peptides function as cytotoxic T-cell epitopes commonly expressed in B-cell malignancies. Nature Med 2000;6:667-672.

38. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 1999;10:673-679.

Table 1: *Clinical features of patients with B-CLL and transduction efficiency into primary CLL cells by AAV/EGFP and AAV/CD40L.*

Patient	Stage (Binet)	Sex/ Age	EGFP (%)	EGFP (MFIR)	CD40L (%)	CD40L (MFIR)
1	C	M/53	13.5	6.0	n.d.	n.d.
2	A	F/56	15.5	3.6	n.d.	n.d.
3	C	M/83	13.0	4.3	n.d.	n.d.
4	A	M/61	42.4	5.3	77.8	209.0
5	A	M/73	44.9	6.6	97.7	312.2
6	A	M/64	41.0	8.4	15.0	2.5
7	A	F/59	36.5	6.0	15.0	3.7
8	A	F/65	24.5	3.4	n.d.	n.d.
9	C	M/62	14.0	2.0	n.d.	n.d.
10	C	M/64	n.d.	n.d.	13.4	3.0
11	C	M/70	13.4	2.7	10.0	1.3
12	A	M/53	16.0	2.6	48.0	19.3
13	B	M/75	n.d.	n.d.	34.4	3.0
14	C	M/57	28.0	4.7	31.8	3.5
15	C	M/73	n.d.	n.d.	23.3	2.7
16	B	M/53	n.d.	n.d.	45.5	11.4
17	A	M/72	34.7	3.6	n.d.	n.d.
18	B	F/63	41.4	5.2	n.d.	n.d.
19	A	F/60	18.6	2.1	n.d.	n.d.
20	A	M/46	34.0	3.7	n.d.	n.d.
21	C	M/62	50.3	5.4	78.9	80.2
22	B	M/70	63.0	15.2	81.5	164.9
23	C	M/60	n.d.	n.d.	30.6	6.6
24	C	M/41	n.d.	n.d.	56.6	14.0

(Abbreviations: M=male, F=female; MFIR=mean fluorescence intensity ratio; n.d.= not determined)

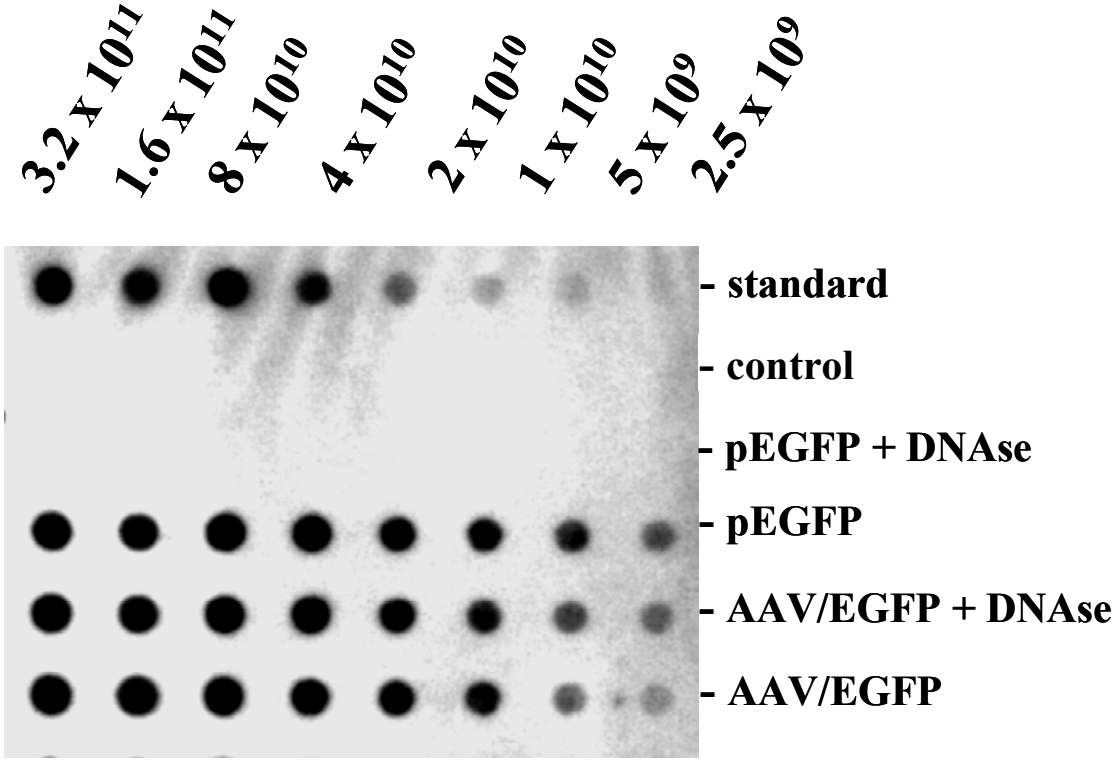

Figure 1: *Genomic titers of rAAV. AAV/EGFP preparations and the vector plasmid pEGFP were blotted in a serial two-fold dilution and hybridized with a random-primed transgene specific probe by standard methods. Particle titers were determined by comparing the intensity of the hybridization signals with that obtained for the vector plasmid standard of known concentration blotted on the same membrane. A DNA fragment encoding resistance for Neomycin^R served as negative control in the hybridization reaction. DNAse treatment (0.5µg/µl DNAse I, Boehringer Mannheim, Mannheim, Germany; 60 min at 25°C) was performed to remove free, unpackaged viral genomes.*

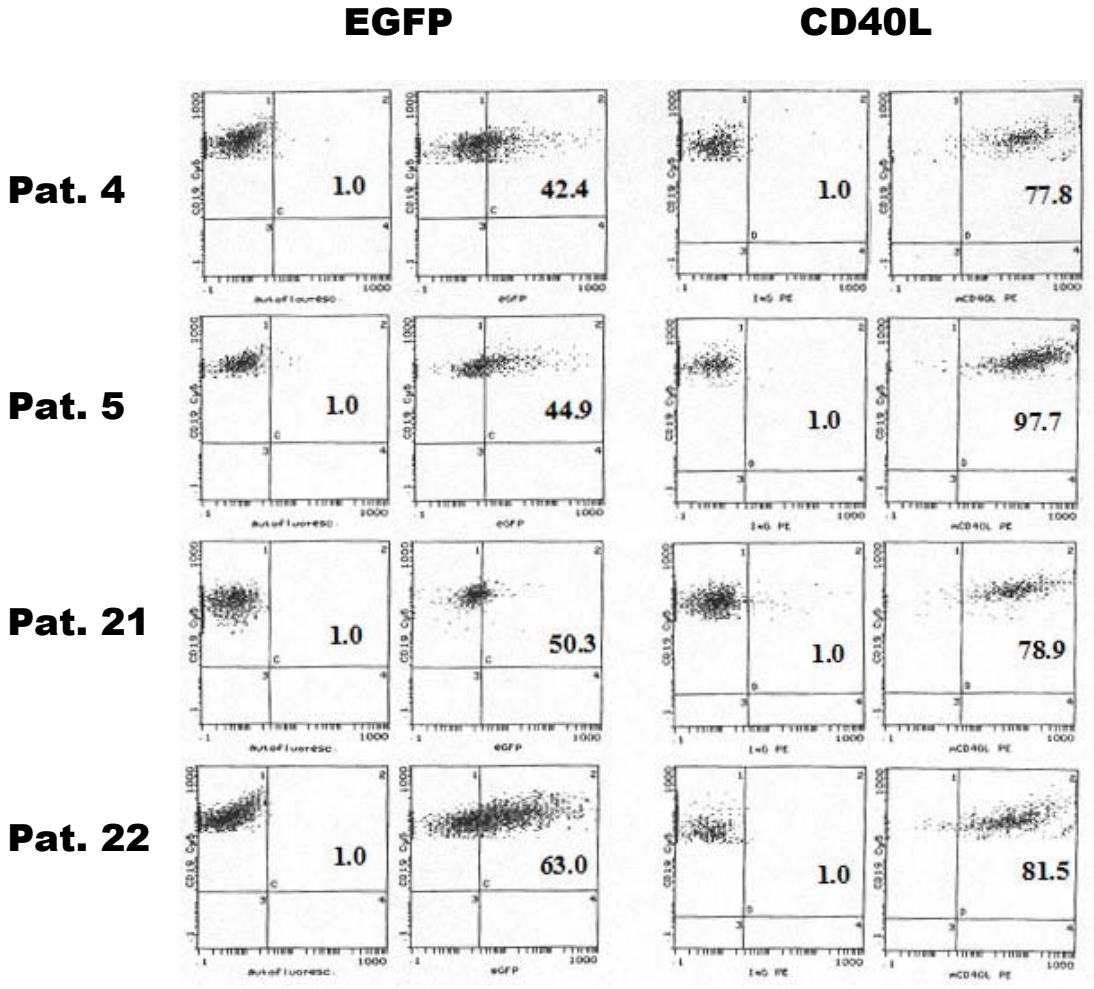

Figure 2: *EGFP and mCD40L expression after rAAV transduction (MOI 100). Primary B-CLL cells derived from patient 4, 5, 21 and 22 (see Tab.1) were transduced at an MOI of 100 with rAAV vectors encoding EGFP and CD40L and analyzed for transgene by flow cytometry 48 hours after later. The panels on the left (control) represent the autofluorescence (EGFP) and isotype (CD40L) controls, respectively, and the panels on the right show the cells transduced with rAAV vectors (AAV/EGFP, AAV/CD40L). Dot-plot analysis of the double staining for the B-cell lineage marker CD19 (Cy5-conjugated mAb) and transgene expression (EGFP and PE-conjugated anti-murine-CD40L) is shown.*

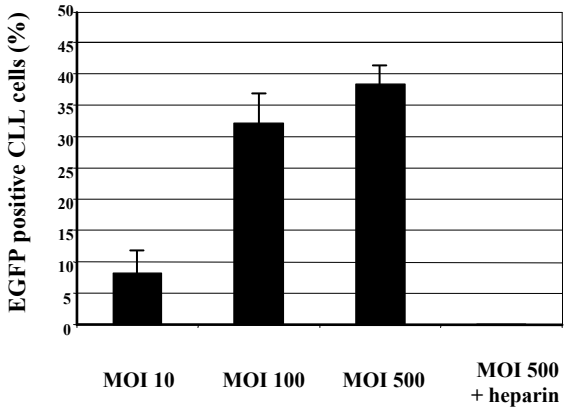

Figure 3: *Concentration kinetics of AAV/EGFP. Primary CLL cells of four patients (17-20, see Tab.1) were infected at an MOI of 10, 100 or 500 with rAAV vectors encoding EGFP. The cells were analyzed for expression of EGFP by flow cytometry 48 hours after infection. Shown is the mean percentage of transduced cells with standard error of mean (error bars). Infected cells (MOI 500) were incubated with 500IU heparin and EGFP expression detected 48 hours later.*

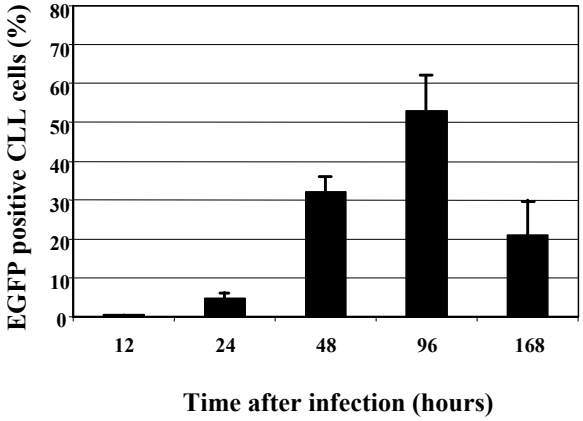

Figure 4: *Time kinetics of AAV/EGFP. CLL samples (patients 17-20, Tab. 1) were infected with AAV/EGFP (MOI 100) and EGFP expression detected 12, 24, 48, 96 and 168 hours later. Shown is the mean percentage of the fraction of EGFP positive cells with corresponding standard error of mean (error bars).*

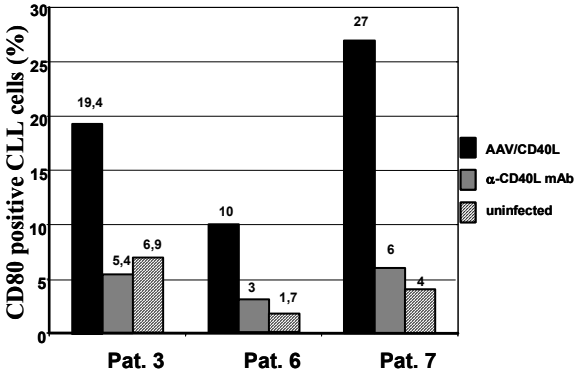

Figure 5: *CD80 expression on B-CLL cells after CD40L transduction. CD80 expression was assessed in samples derived from three patients (3, 6, 7) after infection with AAV/CD40L (black bars) in comparison to uninfected control CLL cells derived from the same patient (striped bars). Expression of CD80 was also detected after coincubation with specific anti-murine-CD40L mAb (grey bars).*

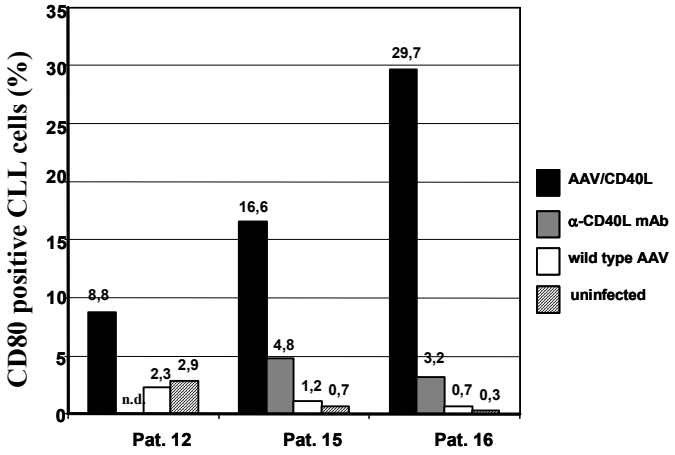

Figure 6: *Transactivation of bystander CLL cells after coincubation with CD40L-transduced CLL cells. CLL cells ($1x10^5$) transduced with AAV/CD40L were coincubated with equal amounts of naive CLL cells labeled with a fluorescence dye (CellTracker™). CD80 expression on naive bystander leukemic cells was detected 72 hours later by flow cytometry (black bars). In control assays anti-murine CD40L mAb was added to the mixture of transduced and bystander CLL cells before CD80 expression was detected 72 hours later (grey bars). As further controls uninfected (striped bars) or wild type AAV-infected cells (open bars) were used for stimulation of bystander CLL cells.*

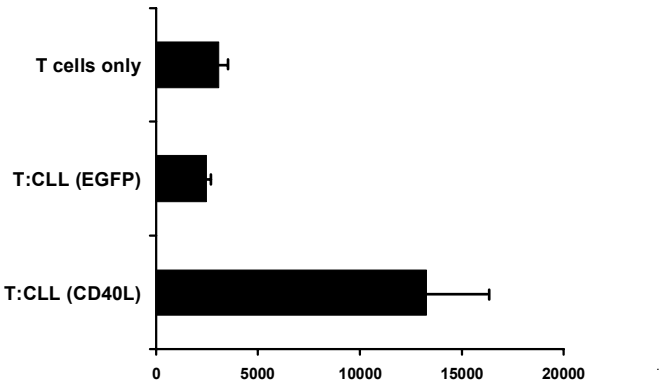

Figure 7: *Allogeneic, HLA-matched mixed lymphocyte reaction. HLA.A2 positive CLL cells were either infected with AAV/CD40L or AAV/EGFP and incubated for 72 hours with naive CLL cells (1:10) derived from the same patient. 1x10^4 CLL cells irradiated with 200 Gy were co-cultured with 1x10^5 purified normal donor T cells (HLA.A2 positive) for another 96 hours, pulsed with [^{3}H]-thymidine and T cell proliferation determined 6 hours later (cpms). The data from one representative patient are depicted. Error bars represent standard error of mean of triplicate measurements.*

CHAPTER VI

RECEPTOR TARGETING OF ADENO-ASSOCIATED VIRUS VECTORS

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Summary

Adeno-associated virus (AAV) is a promising vector for human somatic gene therapy. However, its broad host range is a disadvantage for *in vivo* gene therapy, because it does not allow the selective tissue- or organ-restricted transduction required to increase the safety and efficiency of the gene transfer. Therefore, increasing efforts are being made to target AAV-2 based vectors to specific receptors. The studies summarized in this review show that it is possible to target AAV-2 to a specific cell or organ. So far, the most promising approach is the genetic modification of the viral capsid. However, the currently available AAV-2 targeting vectors need to be improved with regard to the elimination of the wild type AAV-2 tropism and the improvement of infectious titers. The creation of highly efficient AAV-2 targeting vectors will also require a better understanding of the transmembrane and intracellular processing of this virus.

Introduction

The development of safe and efficient gene transfer vehicles is critical for the success of gene therapy. One of the most promising viral vectors is based on adeno-associated virus type 2 (AAV-2), a member of the parvovirus family. AAV-2 was discovered as a co-infecting agent during an adenovirus outbreak, without any apparent pathogenicity contributed by AAV-2.(Blacklow, 1988; Blacklow *et al.*, 1968a; Blacklow *et al.*, 1971) Until now, no human disease caused by AAV-2 has been detected. Moreover, AAV-2 seems to be protective against bovine papillomavirus and adenovirus mediated cellular transformation.(Hermonat, 1989; Khleif *et al.*, 1991; Mayor *et al.*, 1973) AAV-2 does not induce cytotoxic effects and does not elicit a cellular immune response as commonly seen with other viral vectors. (Carter and Samulski, 2000) Finally, AAV-2 has the unique potential to integrate site specifically into the q-arm of human chromosome 19.(Kotin *et al.*, 1990; Samulski *et al.*, 1991)

AAV-2 has a broad tissue tropism infecting diverse organs such as brain, liver, muscle, lung, retina and heart muscle. This makes AAV-2 attractive for *in vitro* gene transfer into various tissues.(Carter and Samulski, 2000) AAV-2 vectors are now successfully used for *in vivo* gene transfer.(Monahan and Samulski, 2000a; Tal, 2000) However, the studies reported so far clearly demonstrate that clinically relevant gene expression can be reached only in the liver, unless vectors are administered directly into the target tissue or organ. These results emphasize the need for a targeting of AAV vectors in order to overcome the apparent limitations of a broad tissue tropism. In addition, the targeting of AAV vectors would also enhance the safety and efficiency of AAV-mediated gene transfer *in vivo*. Therefore,

increasing efforts are being made to retarget AAV-2 based vectors to specific receptors and to generate selective, tissue- or organ-restricted vectors. The studies summarized in this review show that it might become possible to target AAV-2 to a specific cell type or organ. However, the targeting vectors still need to be optimized by a further reduction of the wild type AAV-2 tropism, or with an increase in infectious titer. With the rapidly increasing knowledge about the functional domains on the AAV-2 capsid involved in receptor binding and subsequent steps of transmembrane and intracellular processing of the virion, we feel justified to predict that the creation of highly efficient AAV-2 targeting vectors will become possible in the near future.

Organization of the AAV-2 genome

AAV-2 is a single stranded, replication deficient non-enveloped DNA virus(Rivadeneira *et al.*, 1998) composed of an icosahedral protein capsid and a viral genome of 4680 nucleotides. The AAV-2 genome encodes the two large open reading frames *rep* and *cap*. It is flanked at both ends by the 145 bp inverted terminal repeat sequences (ITR). The ITRs are required for encapsidation of the viral genome and seem to have enhancer and/or weak promoter activity. They are besides the viral Rep proteins necessary for the site specific integration of wild type AAV-2 and for the rescue of proviruses. The 5' open reading frame *rep* encodes four overlapping, multifunctional proteins (Rep78, Rep68, Rep52 and Rep40) controlled by two different promoters.(Balague *et al.*, 1997) The large Rep proteins (Rep78 and its splice variant Rep68) are controlled by the p5 promoter and are necessary for viral DNA replication, transcriptional control and site-specific integration. Rep52 and its splice variant Rep40 are known as small Rep proteins. They are transcribed from the p19 promoter and play an essential role in the accumulation of single-stranded progeny genomes used for packaging. The 3' ORF *cap* accommodates the three capsid proteins VP1 (90 kDa), VP2 (72 kDa) and VP3 (60 kDa), which form the 60 subunits of the AAV-2 viral capsid at a 1:1:20 ratio.(Rabinowitz and Samulski, 2000) They are controlled by the p40 promoter, share the same stop codon, but differ due to alternative splicing and different initiation codons resulting in progressively shorter proteins from VP1 to VP3. All three capsid proteins are necessary for the generation of infectious particles, although capsids are formed in the absence of VP1.(Hermonat *et al.*, 1984; Smuda and Carter, 1991; Tratschin *et al.*, 1984) The capsid assembly itself occurs in the nucleus.(Wistuba *et al.*, 1997a; Wistuba *et al.*, 1995) The N-terminus of VP2 contains a nuclear localization sequence by which it transports VP3 into the nucleus.(Hoque *et al.*, 1999; Ruffing *et al.*, 1992) The encapsidation of the AAV-2 genome

probably takes place in the nucleoplasm and Rep-tagged DNA seems to initiate packaging by interaction with capsid proteins.(Dubielzig *et al.*, 1999)

If *rep* and *cap* are provided *in trans* on a helper plasmid, 96% of the wild type AAV genome can be removed and replaced by a transgene, because the ITRs are the only *cis* elements necessary for the generation of recombinant AAV (rAAV).(Carter and Samulski, 2000) The protocols to generate high-titer and highly purified viral preparations have undergone continuous improvements.(Hermens *et al.*, 1999; Summerford and Samulski, 1999) Until now, rAAV is commonly produced by transfection of a vector plasmid (containing the ITR flanked transgene) and a helper plasmid (encoding *rep* and *cap*) into HeLa or 293 cells, followed by superinfection with adenovirus type 5. Alternatively, a triple transfection of vector-, helper- and an adenovirus helper plasmid can be used.(Ferrari *et al.*, 1997; Girod *et al.*, 1999b; Grimm *et al.*, 1999; Xiao *et al.*, 1998) After harvesting, AAV is purified using iodixanol or CsCl gradient ultracentrifugation and/or chromatography.(Chiorini *et al.*, 1995; Hermens *et al.*, 1999; Summerford and Samulski, 1999; Zolotukhin *et al.*, 1999) After purification, *infectious* particle titers of AAV-2 of $>10^9$/ml are easily reached, which is sufficient for most *in vitro* and *in vivo* experiments, at least in smaller rodents. However, when it comes to larger animals or human beings in clinical applications, it is strongly desirable to enhance the target specificity of AAV vectors by receptor retargeting in order to reduce the amount of vector particles to be administered.

Three-dimensional structure of AAV-2

Recently, Xie et al. were able to determine the atomic structure of AAV-2 to a 3-Å resolution by x-ray crystallography.(Xie *et al.*, 2002) Like Kronenberg et al., who investigated empty capsids by electron cryo-microscopy and icosahedral image reconstruction(Kronenberg *et al.*, 2001), Xie et al. observed substantial differences in the surface topology between AAV-2 and other parvoviruses.(Xie *et al.*, 2002) The inner surface of the AAV-2 capsid is composed of a jelly-roll β-barrel motif, comprising two antiparallel β-sheets. This motif is common in virus capsids and has also been described for other parvoviruses like canine parvovirus (CPV),(Tsao *et al.*, 1991) feline panleukopenia virus,(Agbandje *et al.*, 1993) minute virus of mice,(Agbandje-McKenna *et al.*, 1998) or the human parvovirus B19.(Agbandje *et al.*, 1994) However, the interstrand loops located between the strands of the core β-barrel have quite different structures in the different parvoviruses and are the regions responsible for interactions with antibodies and cellular receptors. The most prominent features of the AAV-2 surface topology are the “threefold-proximal” peaks. These peaks cluster around each

icosahedral three-fold rotation axis. Unique for AAV-2 is that neighbouring subunits interact intimately at this threefold axis. Additional, but more modest interactions were also observed for residues in the HI, BC and EF loop of neighbouring subunits. The threefold proximal peaks are mainly formed by a so called GH loop. This loop is missing in densoviruses (insect parvoviruses) and is structurally different in CPV. This loop is, as expected, mainly involved in binding to the primary receptor of AAV-2 (see below) and contains the epitope recognized by the neutralizing antibody C37-B. Moreover, the most promising position for the insertion of receptor specific peptides (amino acid position 587) is also located in this loop.

Before the characterization of the three-dimensional structure of the AAV-2 capsid potential insertion sites as well as the determination of antibody and receptor binding regions were identified by epitope mapping, mutagenesis studies of capsid proteins, as well as sequence alignments of AAV-2 and related parvovirus including other serotypes. Although it was possible by these approaches to determine functional relevant regions of the AAV-2 capsid, the now solved three dimensional structure will accelerate this process by using a more rational, structure-based approach.

Infectious pathway of wild type AAV-2

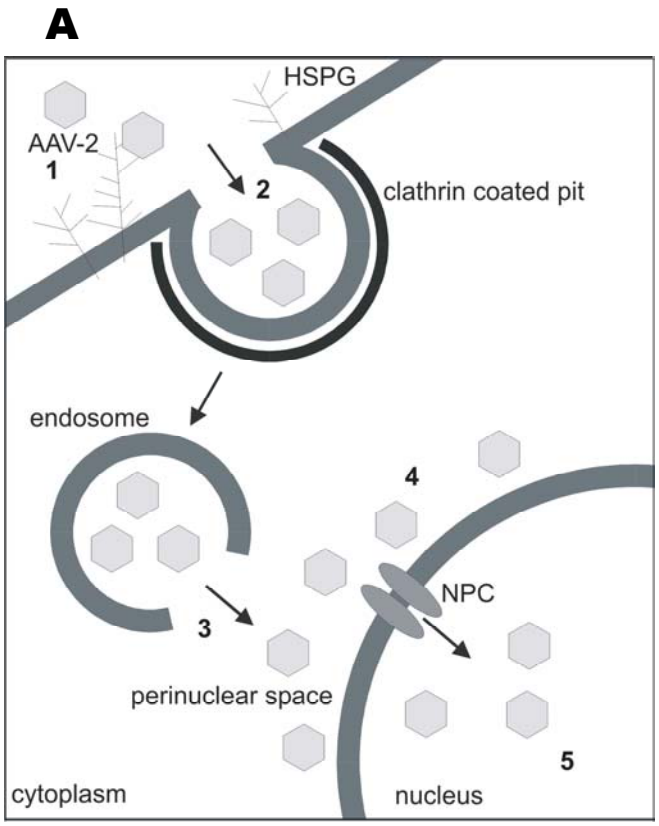
A successful viral infection is a multistep process starting with the attachment of the virus to the cell surface, followed by viral uptake, intracellular trafficking and - in most of the cases - nuclear transport and deposition or replication of the viral genome in the cell nucleus. In the current model of infection of permissive cells by AAV-2 (Fig. 1A), AAV-2 first binds to heparan sulfate proteoglycans (HSPG), which act as primary or attachment receptors.(Summerford and Samulski, 1998) Since all adherent cells express glycosaminoglycans on their surface, this offers a simple explanation for the broad tropism of AAV-2. No distinct heparin binding motif was identified so far in the AAV-2 capsid. However, Wu et al. mapped two regions involved in HSPG binding by alanine substitution and insertion of the hemagglutinin (HA) epitope YPVDVPDYA.(Wu *et al.*, 2000) These regions encompass amino acids 509 to 522 and 561 to 591 (in VP3) and are cluster around the threefold-proximal peaks.(Xie *et al.*, 2002) The alanine substitution mutant 585-RGNR-588 and the HA insertion mutant 591 for example are located on the side of the threefold-proximal peak facing the valley, which separates this peak from its neighbour, whereas the alanine insertion mutant 509 is on the floor of the valley. The other two mutants generated by Wu et al. were mapped at the base of (alanine substitution at 561-565) and underneath (HA insertion

at 522) the peak facing the twofold axis. Xie et al. assume that mutations in the regions underneath the peak (insertion at 522(Wu *et al.*, 2000) and 519(Rabinowitz *et al.*, 1999)) are not directly affecting the HSPG binding of AAV-2.

Two types of AAV-2 co-receptors have been identified, $\alpha_v\beta_5$ integrin and fibroblast growth factor receptor 1 (FGFR1).(Bartlett *et al.*, 2000a; Qing *et al.*, 1999; Summerford *et al.*, 1999) It is postulated that FGFR1 enhances the attachment process.(Bartlett *et al.*, 2000a; Qing *et al.*, 1999) Antibodies against $\alpha_v\beta_5$ integrin do not interfere with cell binding but inhibit endocytosis. Therefore, $\alpha_v\beta_5$ integrins seem to be required for endocytosis of AAV-2,(Sanlioglu *et al.*, 2000) which is mediated mainly by clathrin-coated pits.(Bartlett *et al.*, 2000a; Duan *et al.*, 1999) This endocytotic process and the subsequent steps are still poorly understood. However, it is possible to assume that like for adenovirus α_v integrin clustering facilitates the localization of virus particles to coated pits.(Wang *et al.*, 1998) This could then activate the endocytosis in which Dynamin, a 100 kDa cytosolic GTPase, is involved. Although the precise function of Dynamin in vesicle formation remains controversial,(Marks *et al.*, 2001; Sever *et al.*, 2000) it is known that it is essential for scission of newly formed vesicles from the plasma membrane.(Marks *et al.*, 2001) For AAV-2 it was shown that the introduction of a dynamin mutant results in the decrease of AAV mediated transduction(Bartlett *et al.*, 2000a; Duan *et al.*, 1999), although it was not possible to abolish the AAV-2 infection. However, Sangioglu et al. assumed that the binding to the integrin could have an additional effect, which is the activation of Rac1.(Sanlioglu *et al.*, 2000) They propose that Rac1 activation results in the stimulation of phosphoinositol-3 kinase (PI3K) which facilitates the rearrangements of microfilaments and microtubuli. These rearrangements are necessary to support the initiation of the intracellular movements of AAV-2 to the nucleus after endocytosis. The release of AAV-2 from the endosome at the early-to-late endosomal transition requires a low endosomal pH.(Bartlett *et al.*, 2000a; Douar *et al.*, 2001) As with other viruses,(Marsh and Helenius, 1989) the low pH is likely to induce conformational changes of key viral proteins necessary for a successful endosomal release or nuclear entry. Interestingly, the unique region of VP1 contains a potential phospholipase A2 (PLA2) domain,(Girod *et al.*, 2002) which might be involved in this process. PLA2 inactivating point mutations do not influence the capsid assembly, packaging, cell binding or entry of AAV-2, but delay the onset and reduce the amount of early gene expression. Thus, the PLA2 activity in the N-terminus of VP1 may be required for the exit of AAV-2 from the endosome and the transfer of the viral genome to the nucleus. A PLA2 domain with similar function has been found in porcine parvovirus.(Zadori *et al.*, 2001) The destiny of AAV-2 after this endosomal

release is mostly unclear. Some studies have observed a perinuclear accumulation of AAV-2 particles,(Bartlett *et al.*, 2000a) before the virus slowly enters the nucleus, probably via nuclear pore complexes. However, the perinuclear accumulation of AAV-2 observed by conventional fluorescent and confocal laser microscopy may suffer from some methodological shortcomings such as interference effects and the cellular virus overload required for these conventional imaging studies (see next paragraph). These results need to be confirmed by independent methods.

The development of a novel technique, called *Single Virus Tracing* (SVT), which allows the visualization of an individual virus in a living cell with high spatial and temporal resolution, may permit a more detailed analysis of specific steps of the cellular infection(Seisenberger *et al.*, 2001) (Fig. 1B). Using this technique Seisenberger et al. observed AAV-2 movements towards the cell surface, which were followed by repetitive touching and short diffusion paths in the vicinity of the cell surface. The touching events were clearly visible as short periods of immobility at the cell surface, with a mean touching time of t_t = 62 ms. Inside the cell, three different kinds of AAV-2 movements were observed in the cytoplasm and the nuclear area, namely directed motion, anomalous diffusion and normal diffusion. In agreement with the current model of AAV-2 infection, most virions followed a normal diffusion in endo- or lysosomal particles. However, in marked contrast to the above findings with conventional microscopic techniques, neither a nuclear accumulation nor a slow penetration of the nuclear membrane was observed by SVT. Interestingly, the total time measured for membrane penetration, trafficking through the cytoplasm and entry into the nuclear area was much shorter than determined by other methods.(Seisenberger *et al.*, 2001) By this new method it was for example possible to detect at least one Cy5- labeled AAV-2 in the nucleus of 50% of the cells 15 minutes after adding virus to the cells.



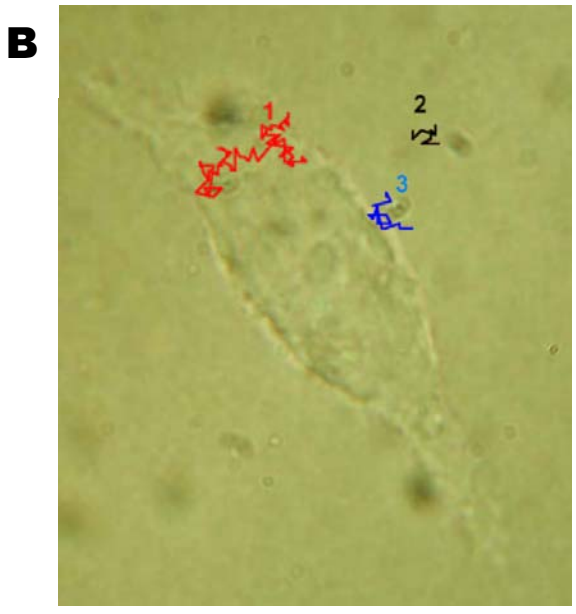


***Fig. 1**. Infectious pathway of wild type AAV-2. **(A)** Schematic representation of AAV entry and endocytic trafficking in HeLa cells as seen by fluorescent and confocal laser microscopy.(Bartlett et al., 2000a) Following binding to heparan sulfate proteoglycans (HSPG) on the cell surface (1), AAV is rapidly internalized via clathrin-coated pits (2) through a process involving $\alpha_v\beta_5$ integrin, Dynamin and Rac1.(Bartlett et al., 2000a; Duan et al., 1999; Summerford et al., 1999). Following endocytosis, the activation of phosphatidyinositol-3 kinase seems to support the initiation of the intracellular movement.(Sanlioglu et al., 2000) After acidification AAV is released from the endosome into the cytoplasm (3), where it is found in a perinuclear localization (4). Then AAV slowly enters the nucleus (5) probably via nuclear pore complexes (NPC). **(B)** Trajectories of single AAV-Cy5 particles analysed by Single Virus Tracing.(Seisenberger et al., 2001) The traces show single diffusion virus particles at different times. In this figure three examples of the various stages of the AAV-2 infection are visible (diffusion in the cytoplasm after cell entry (**1**), diffusion in solution (**2**) and touching at the cell membrane (**3**)). Normal diffusion with a diffusion coefficient of $D = 7.5\ \mu m^2/s$ could be measured for AAV-2 outside the cell (**2**). A deceleration of AAV-2 near the cell could be observed. When approaching the cell, a repetitive touching of the cell membrane by AAV-2 occured, which was interrupted by short diffusion path in the vicinity of the cell surface (**3**). Inside the cell AAV movements using direct motion, anomalous and normal diffusion were observed (**1**). This method also allowed to distinguish between free viruses and AAV inside endosomes by calculating the diffusion constants of the particles traced by SVT.*

AAV serotypes other than AAV-2

Most AAV vectors are based on the AAV-2 serotype, as it was the first serotype from which an infectious clone was available.(Samulski *et al.*, 1982) 50 to 96% of the population is seropositive for AAV-2. Five additional primate AAV serotypes (AAV-1, -3, -4, -5, and 6) have been characterized at the nucleotide level.(Chiorini *et al.*, 1999; Chiorini *et al.*, 1997; Muramatsu *et al.*, 1996; Rutledge *et al.*, 1998) With the exception of AAV-6, which has a >99% amino acid homology with AAV-1, all serotypes show a significantly different amino acid sequence of the capsid proteins, which is most prominent in VP3(Rabinowitz and Samulski, 2000) and most obvious for AAV-4 and -5. It remains to be determined how these differences influence the binding of neutralizing antibodies, the viral tropism and the intracellular processing.

An investigation of the humoral immunity against AAV performed with a cohort of 85 human volunteers revealed that none of the sera contained neutralizing antibodies against AAV-5, although neutralizing antibodies against AAV-1 and AAV-2 were detected in 19 and 25% of the sera, respectively.(Hildinger *et al.*, 2001) Furthermore, neutralizing antibodies against AAV-4 or AAV-5 do not cross-react.(Rabinowitz and Samulski, 2000) Serum from mice immunized with AAV-2 vectors did not neutralize AAV-6 infection in tissue culture, neutralized AAV-3 only partially, but inhibits AAV-2 almost completely.(Halbert *et al.*, 2000) Similar results were obtained with AAV-3 used for immunization. Serum from AAV-6 immunized animals did not cross-react with AAV-2 or AAV-3 and neutralized the infection by AAV-6 only weakly.

In AAV-2, VP3 is responsible for receptor binding and therefore mainly determines the viral tropism. Differences in this region should result in a different receptor usage and viral tropism. Therefore it was not unexpected that AAV-4 and AAV-5, which show the lowest similarity to AAV-2, use α2-3 O-linked (AAV-4) and N-linked (AAV-5) sialic acid for cell binding(Kaludov *et al.*, 2001) instead of HSPG. AAV-6, which has a homology of about 60% to AAV-4 and –5,(Bantel-Schaal *et al.*, 1999) also binds to sialic acid,(Seiler *et al.*, 2002) whereas AAV-3 binds to HSPG.

The various AAV serotypes show a different tissue or cell tropism.(Halbert *et al.*, 2000; Hildinger *et al.*, 2001; Rabinowitz *et al.*, 2002) AAV-1 is more efficient than AAV-2 for the transduction of skeletal muscle.(Xiao *et al.*, 1999) AAV-3 is superior for the transduction of megakaryocytes.(Handa *et al.*, 2000) Compared to AAV-2, AAV-5 and AAV-

6 infect apical airway cells more efficiently.(Halbert *et al.*, 2001; Zabner *et al.*, 2000) AAV-2, -4 and –5 transduce cells of the central nervous system, but differences in the distribution and the target cell types (Davidson *et al.*, 2000) exist. It can be anticipated that further work on AAV serotypes will result in the identification of all domains involved in receptor binding and uptake. This knowledge will be very useful for the creation of AAV retargeting vectors.

Receptor Targeting of AAV

In principle, at least two different strategies are possible to achieve a receptor targeting of AAV:(Cosset and Russell, 1996)

1. *Indirect targeting*: In contrast to wild type (Fig. 2A) the interaction between the viral vector and the target cell is mediated by an associated molecule (e.g. a glycoside molecule or a bispecific antibody) which is bound to the viral surface and interacts with a specific cell surface molecule(Miller, 1996) (Fig. 2B).
2. *Direct targeting*: The cell specific targeting of the vector is mediated by a ligand which is directly inserted into the viral capsid(Walter and Stein, 1996)(Fig. 2C).

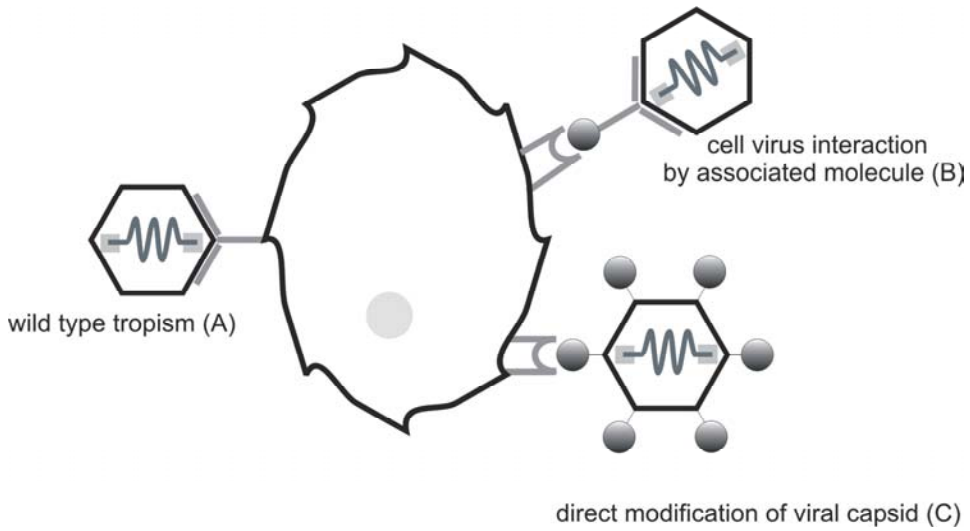


Fig. 2: *Possibilities of targeting viral vectors.Viral vectors with wild type tropism (**A**) show a direct binding of structural capsid components to the cell surface receptor. In targeting vectors, the virus-cell interaction is mediated by a molecule associated with the capsid (indirect targeting, **B**) or by a ligand directly inserted into the capsid (direct targeting, **C**).*

For indirect targeting (Fig. 2B) it is not necessary to know the three-dimensional structure of the viral surface if high affinity viral surface binding molecules such as monoclonal antibodies are available. For this strategy, the stability of the interaction of the virus with the intermediate molecule and the efficiency by which the complex is generated are rate limiting. In addition, the intermediate molecules must bind to cell-specific receptors which allow the uptake and correct intracellular processing of the virus.

A combination of two important parameters is required for the successful generation of a targeting vector by direct modifications of the capsid (Fig. 2C). The first parameter is a good choice of the insertion site to ensure that packaging of the mutant remains efficient and the inserted ligand is exposed on the virus surface. Until now two alternative strategies have been used for AAV-2 to identify candidate positions for insertion of heterologous ligand: a) sequence alignment between AAV-2 and other parvoviruses for which the X-ray crystal structure is known;(Girod *et al.*, 1999b; Grifman *et al.*, 2001) b) a systematic, insertional mutagenesis of the whole AAV-2 capsid.(Rabinowitz *et al.*, 1999; Shi *et al.*, 2001; Wu *et al.*, 2000) The second important parameter is the choice of the targeting peptide. It is difficult to predict the secondary structure of the ligand inserted into the AAV capsid. Therefore, the ligand should be structure-independent and not too large to avoid the destabilization of the entire capsid. Moreover, the ligand should be cell type specific. Finally, the ligand-receptor complex should be internalized in a way that allows an efficient transport of the virus and the release of the viral DNA in the cell nucleus.

Both approaches and a combination thereof have been used to retarget AAV-2 (Bartlett *et al.*, 1999; Girod *et al.*, 1999b; Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Ried *et al.*, 2002; Shi *et al.*, 2001; Wu *et al.*, 2000; Yang *et al.*, 1998) and will be described in the following paragraphs.

Targeting by bispecific antibodies

The feasibility to target AAV-2 using a bispecific antibody which mediates the interaction between virus and target cell (Fig. 2B) was first shown by Bartlett et al. (1998). The antibody used was generated by a chemical cross-link of the Fab arms of monoclonal antibodies against the $\alpha_{IIb}\beta_3$ integrin (AP-2 antibody) and the intact AAV-2 capsid (A20 antibody(Wistuba *et al.*, 1995)).(Bartlett *et al.*, 1999) The major ligand for $\alpha_{IIb}\beta_3$ is fibrinogen, which becomes internalized via endocytosis. Therefore, AAV-2 targeted to this integrin was expected to become internalized via receptor-mediated endocytosis, similar to wild type virus. This

targeting vector transduced MO7e and DAMI cells, which are not permissive for wild type AAV-2 infection (70-fold above background). In contrast, a 90% reduction in AAV-2 transduction was seen on cells negative for the targeting receptor. It remains to be determined whether this reduction was due to steric hindrance or some other mechanism. Another issue that remains to be resolved is the stability of the virus-bispecific antibody complexes *in vivo*.

Targeting by insertion of single chain antibodies or receptor specific ligands at the N-terminus of VP proteins

The first attempt to alter the tropism of AAV-2 was described by Yang et al. (1998). They inserted a single-chain antibody against human CD34, a cell surface molecule expressed on hematopoietic progenitor cells, at the 5'-ends of VP1, VP2 and VP3. Using a transcription and translation assay, they could express all three different single-chain fragment variable region (scFv)-AAV-2 capsid fusion proteins. However, they failed to produce detectable rAAV-2 particles when using either all three scFv-VP fusion proteins or one scFv-VP fusion with two other unmodified capsid proteins. Therefore they had to use all three wild type AAV-2 capsid proteins for the packaging process in addition to one of the three single scFv-VP fusion proteins. Using this procedure, intact viral particles could be generated which were able to infect HeLa cells and showed an increased transduction of CD34 positive KG-1 cells. Although this approach provided the first demonstration that targeting of AAV-2 by direct modification of the capsid is possible, the virus titers ($1.9x10^2$ transducing units/ml on KG-1) were extremely low. Moreover, very heterogeneous viral preparations consisting of an unknown mixture of chimeric, targeting and wild type AAV-2 particles were produced.

Wu et al. (2000) inserted the hemagglutinin (HA) epitope YPVDVPDYA into the N-terminal regions of VP1, VP2 and VP3 and the C-terminus of the *cap* ORF. They observed that the insertion of this and other epitopes at the N termini of VP1 (VPN1) and VP3 (VPN3) and at the C-terminus of the *cap* ORF (VPC) resulted in either no detectable particles (VPN3 and VPC), or in a 2-3 log decrease of infectious and physical particle titers. In agreement with Yang et al. (1998), only the insertion at the N-terminus of VP2 (amino acid position 138) was tolerated.(Wu *et al.*, 2000) Moreover, exchanging the HA epitope by the serpin receptor ligand KFNKPFVFLI(Ziady *et al.*, 1997) resulted in a 15-fold higher infection of the lung epithelial cell line IB3 than by wild type AAV-2. The fact that the N-terminal insertion of different peptides is tolerated in VP2 and allows targeting, albeit at low efficiency, probably reflects the exposure of the N-terminus of VP2 at the viral surface analogous to

CPV.(Chapman and Rossmann, 1993; Weichert *et al.*, 1998) This assumption was further confirmed by the results of Shi et al. (2001), who inserted a 6 amino acid peptide (TPFYLK) from bovine papillomavirus (BPV) at position 139 and were able to detect this epitope on the capsid surface by monoclonal antibodies against BPV. In addition, the insertion of a 10 amino acid peptide (HCSTCYYHKS) derived from the human luteinizing hormone (LH) increased the infection efficiency of a LH-receptor positive human ovarian adenocarcinoma cell line, OVCAR-3.

Targeting of rAAV-2 vectors by insertion of ligand coding sequences into the capsid genes

The first successful demonstration that a genetic capsid modification (direct targeting) can be used to retarget AAV-2 was described by Girod et al. (1999). A sequence alignment of AAV-2 and CPV identified 6 sites (amino acid positions 261, 381, 447, 534, 573, 587) that were expected to be exposed on the surface of the virus capsid and to accept the insertion of a ligand without disrupting functions essential for the viral life cycle (Fig. 3A).

At these positions the sequence for the 14 amino acid peptide L14 (QAGTFALRGDNPQG) was inserted into the capsid gene. The L14 peptide contains the RGD motif of the laminin fragment P1,(Aumailley *et al.*, 1990) is the target for several cellular integrin receptors, and can also serve as a viral receptor.(Aumailley *et al.*, 1990; White, 1993) In addition no specific secondary structure is required for the recognition of the receptor.(Aumailley *et al.*, 1990) All six mutants could be packaged with an efficiency similar to wild type AAV-2 and showed an intact capsid structure in electron microscopy images.(Grimm *et al.*, 1999; Wistuba *et al.*, 1997a) Using an ELISA with an anti-L14 polyclonal antibody it was demonstrated that the L14 epitope was exposed at the viral surface when inserted at amino acid positions 261, 381, 447, 573 and 587. In a cell binding assay insertion mutants I-447 and I-587 were able to bind B16F10 (mouse melanoma) and RN22 (rat swannoma) cell lines, which did not bind to and were not infected by wild type AAV-2. An efficient transduction of B16F10 cells was observed using the AAV insertion mutant I-587 expressing Rep or β-galactosidase.

The same site, 587, was also successfully used for the insertion of an endothelial specific peptide isolated by phage display, and allowed the generation of an AAV-2 mutant able to infect endothelial cells such as human umbilical vein endothelial cells (HUVEC) and human saphenous vein endothelial cells (HSVEC).(Nicklin *et al.*, 2001) In contrast to wild type

AAV-2 infection, the infection of the endothelial specific cells by the mutant was not blocked by heparin, showing that the infection did not depend on HSPG. Moreover, heparin binding studies showed that the mutant was not retained in a heparin column, in contrast to wild type AAV-2. The specificity of the binding was shown by infection studies using different non-endothelial specific cell lines such as HepG2. Furthermore, the mutant seemed to follow an intracellular route different from wild type AAV-2 since compounds such as bafilomycin A2 (an inhibitor of endosomal acidification) did not inhibit transduction. Taken together, all studies underline the potential value of the 587 site of the AAV-2 capsid, which is positioned at the tip the GH loop(Xie *et al.*, 2002) for the generation of cell-specific AAV-2 vectors by the direct targeting approach (Fig. 2C).(Girod *et al.*, 1999b; Nicklin *et al.*, 2001; Ried *et al.*, 2002)

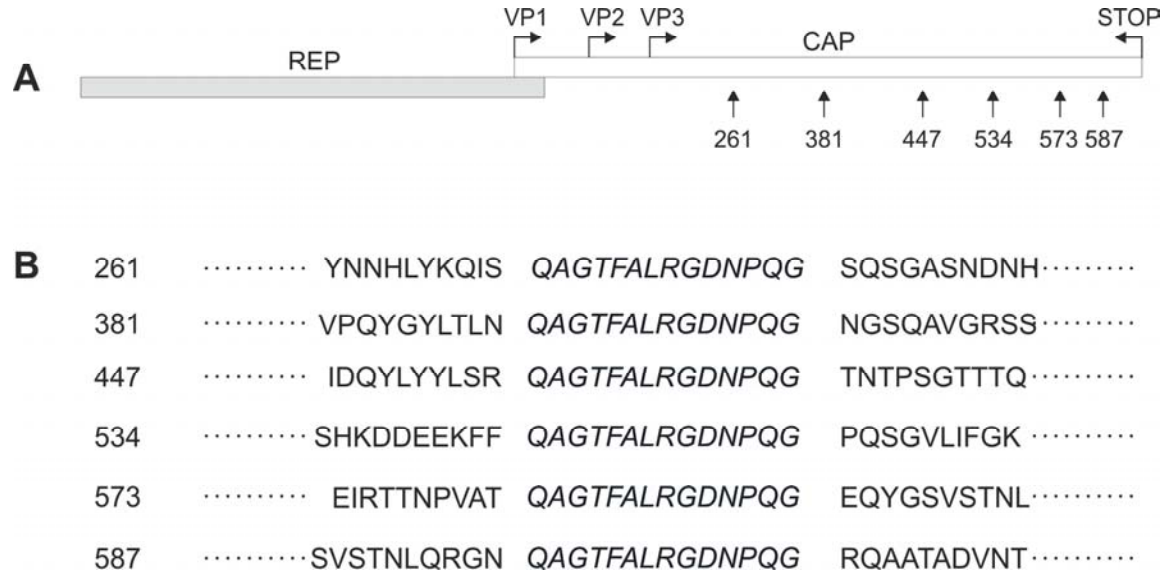


Fig. 3. *L14 peptide insertion sites in the AAV2 capsid.* ***(A)*** *Schematic diagram of the two open reading frames rep and cap. Cap encodes the three capsid protein VP1, VP2 and VP3. Sites of the L14 insertion described by Girod et al. (1999) are marked by arrows (numbers are the amino acid positions N-terminal of the insertion).* ***(B)*** *Sequence of L14 and flanking amino acids at the different insertion sites tested. The insertion mutant I-587 displayed the L14 peptide on the surface and was able to retarget the mutant to the mouse melanoma cell line B16F10 (Girod et al., 1999b) (numbering starts at the start codon of VP1).*

This strategy was successfully repeated by Grifman et al. (2001). They also used a sequence alignment approach to identify potential targeting sites of the AAV-2 capsid by expanding their comparisons to parvoviruses other than CPV and to the other AAV serotypes (AAV-1, -3, -4 and -5). They identified identical regions to Girod et al. (1999), and finally used sites 448 and 587 for their studies. Grifman et al. (2001) inserted the Myc epitope and a CD13 (NGR receptor expressed on angiogenic vasculature and in many tumor cell lines) specific peptide with the sequence NGRAHA, identified by phage display. The insertion of NGRAHA at 587 allowed a cell-specific targeting to different cell lines (KS1767 (Kaposi sarcoma) and RD (rhabdomyosarcoma)). Interestingly, deletion of the 6 amino acids (GNRQAA) at position 586-591 resulted in the loss of heparin binding, whereas the insertion of the targeting peptide (NGRAHA) restored the heparin binding ability. Taking into account that HSPG has a negative charge, the R at position 588 might have an essential role for HSPG binding.

For a systematic characterization of functional domains of the AAV capsid proteins, Wu et al. (2000) constructed 93 mutants at 59 different positions on the AAV-2 capsid by site directed mutagenesis. They identified several putative regions, which were involved in HSPG binding and/or exposed on the capsid surface, with the potential to tolerate the insertion of a ligand. These positions were 34 (in VP1), the N-terminus of VP2 (138), as well as 266, 328, 447, 522, 553, 591, 664 (in VP3). Although all VP3 insertion mutants were precipitated by an antibody against the inserted HA epitope, only 266, 447, 591 and 664 were still infectious. For insertion mutant 522, this could be explained by the loss of the HSPG binding ability.(Rabinowitz *et al.*, 1999; Wu *et al.*, 2000) For the other mutants, a simple explanation is lacking. Wu et al. (2000) tested only the position 34 in the VP1 sequence (FVFLI substitution) and the N-terminus of VP2 (KFNKPFVFLI insertion) for targeting of AAV-2 to IB3 cells via serpin receptor. It was shown that targeting and infection was possible. In this approach the N-terminal VP-2 insertion mutant was 15-fold and the VP1 mutant approximately 62-fold more infectious than the wild type. In both cases, the insertions were placed outside the potential HSPG binding regions. Therefore it was not surprising, that the transduction of the target cells by these mutants was blocked by heparin, suggesting that the serpin-tagged mutants continued to use HSPG as primary receptor and used the serpin receptor as alternative (co-)receptor.

Using insertional mutagenesis, Shi et al. (2001) also tried to identify positions in the AAV-2 capsid which might tolerate the insertion of heterologous peptide ligands. In addition to the VP2 N-terminal insertion mutants mentioned above, mutants with insertions into the capsid sequences were generated. These mutants contained either an insertion of two amino acids (TG or AG) or of longer epitopes derived from the bovine papillomavirus (BPV; TPFYLK), from the human luteinizing hormone (LH; HCSTCYYHKS) or a cyclic RGD-containing peptide specific for α_v integrins (4C-RGD, CDCRGDCFC). None of these mutants were tested for retargeting, but three important observations were made:

1) Five different capsid regions were identified which allowed the surface display of the BPV peptide ligand. These were 139 (N-terminus of VP2), 161 (in the VP2 region), 459, 584 and 587 (in the VP3 region). The positions 139 and 587 confirmed earlier results.(Girod *et al.*, 1999b; Wu *et al.*, 2000) The other sites remain to be tested for functional targeting, because the surface display of a ligand alone is a prerequisite but not sufficient for a ligand dependent infection by the virus mutant.(Girod *et al.*, 1999b)

2) The scaffold sequences flanking the heterologous ligand are important for epitope display, HSGP binding ability and titers. Using for example the amino acids ALS to flank the BPV ligand inserted at 584 resulted in mutants that showed surface display of the epitope, HSPG binding and the production of infectious particles. In contrast, LLA and GLS used as scaffold sequences did not allow the production of infectious particles and reduced the surface display of the BPV ligand. In contrast, the GLS scaffold sequence was better tolerated than LLA or ALS at position 587.

3) Not every ligand, even if comparable in length, is tolerated at a specific insertion site. Shi et al. inserted either the BPV or the LH ligand both flanked by the GLS scaffold sequences at site 459 and observed that the BPV ligand (a 6 amino acid insertion) generated fully infectious particles, whereas the insertion of the 10 amino acid LH peptide created non-infectious virus particles. Our laboratory made similar observation when trying to insert multimers of the L14 sequence at position 587: larger insertions at position 587 resulted in a decrease of the packaging efficiency although the insertion of a 34 amino acid containing the Z34C protein A domain of *Staphylococcus aureus* (see below) was well tolerated. These results show that the maximal length of

the peptide tolerated at this position depends on the sequence itself. The precise determinants of this phenomenon are unknown.

Generation of universal targeting vectors by combining two principles of vector targeting

Inspired by an earlier attempt for Sindbis virus,(Ohno *et al.*, 1997) we tried to use a general targeting vector using a truncated 34 amino acid peptide, Z34C, from protein A of *Staphylococcus aureus*.(Ried *et al.*, 2002) Protein A recognizes and binds the Fc part of immunoglobulins (Ig), but not the variable Ig domain, which therefore remains free to bind the antigen. Z34C is derived from the protein A subunit B, which encompasses 56 amino acids and binds the Fc portion with a dissociation constant of about 10-50 nM.(Sinha *et al.*, 1999) A 38 residue truncation of this domain, selected by phage display, was further truncated and stabilized by insertion of disulfide bonds and showed thereafter a dissociation constant of 20 nM. The insertion of Z34C at position 587 in the AAV-2 capsid (587Z34C) resulted in a 10-fold decrease of packaging efficiency in comparison to wild type AAV-2. In contrast, the combination of the insertion with a 9 amino acid deletion (587Δ9Z34C) resulted in a packaging efficiency similar to wild type AAV-2. Electron microscopy and A20-ELISA revealed a wild type capsid morphology for both mutants, although empty capsids were observed 3-fold more frequently. Interestingly, the wild type tropism of the Z34C insertion mutants decreased by 4 orders of magnitude, in agreement with the results of Nicklin et al. (2001). The insertion of Z34C at position 587 allowed a functional expression of the IgG binding domain, as shown by binding studies using various antibodies. Interestingly, the capsid mutant 587Z34C bound antibodies more efficiently than 587Δ9Z34C, maybe because the binding domain was less accessible with the 9 amino acid deletion. In agreement, Grifman et al. (2001) showed that a substitution at 587 was less efficient than an insertion. Coupling 587Z34C virus with antibodies against CD29 (β1-integrin), CD117 (c-kit-receptor) or CXCR4 resulted in a specific, antibody mediated transduction of hematopoietic cell lines. No transduction could be detected without antibody, whereas the targeted infection was blocked with soluble protein A or with IgG molecules. In addition, no inhibition of transduction by the targeting vector was observed with heparin, demonstrating that the interaction of the 587Z34C mutants with the natural AAV-2 receptor HSPG was not essential for infection or transduction. Taken together, this targeting approach shows that a universal AAV targeting vector can be generated and loaded with different targeting molecules to transduce the desired

cells via specific receptors. However, this approach leaves room for improvement, since the titers obtained with these vectors were relatively low.

Future prospects: understand the infectious biology of AAV

To efficiently retarget AAV vectors a better understanding of the infectious biology of AAV will be required. This includes the virus-cell surface interactions, mechanisms of uptake, endosomal processing and release, nuclear transport and mechanisms leading to gene expression. The structure determination of the AAV-2 capsid (Xie *et al.*, 2002) will tremendously enhance our knowledge of the location and function of different capsid domains.

The identification of HSPG as primary attachment receptor for AAV-2 was an important achievement. However, no distinct binding motif within the capsid has been identified so far, despite some useful information presented in the work of Rabinowitz et al. (1999) and Wu et al. (2000). Such knowledge will be required to specifically modify the natural viral tropism of AAV-2.

A better understanding of the intracellular processing of AAV targeting vectors will be essential, because AAV targeting vectors may be transferred into a cellular compartment from which they will never be released, or in which they will be processed in ways preventing nuclear processing or gene expression. Therefore, the success of creating AAV targeting vectors will ultimately depend on our ability to unveil the detailed mechanisms of AAV transport and processing. Some pieces of the puzzle are already known,(Bartlett *et al.*, 2000a; Sanlioglu *et al.*, 2000; Seisenberger *et al.*, 2001) but the picture is not complete. With this regard, Single Virus Tracing (Seisenberger *et al.*, 2001) will be a very important tool to understand which receptors and cellular compartments need to be used to efficiently re-target AAV. On the other hand, the technique of AAV vector targeting will help to uncover some important, basic functions of AAV capsid proteins, as well as mechanisms of the infectious biology of AAV.

The third important issue is the identification of the optimal ligand or targeting receptor. For the genetic modification strategy chosen by our group, the length and sequence of the ligand are critical, as the insertion of a peptide may result in profound alterations of the three-dimensional capsid structure. One possibility to overcome this problem is the combination of the insertion with one or more deletions. Another possibility is the insertion of a sequence

which is able to form its own secondary structure, for example a loop closed by a cystein bridge.

These difficulties are overcome when using an antibody or another bridging molecule to mediate the interaction between the viral surface and the target cell. However, this approach will encounter other problems such as the stability of the virus-ligand complex, limitations to scale up the vector production, and steric hindrance of the virus uptake by large virus-ligand complexes. To identify new ligands, phage display has been proven to be a valid approach. However, the ligand sequences are selected in an architectural context that is different from that of the final vector. This means that once inserted in the context of AAV, they could destabilize the capsid structure (resulting in low packaging efficiency) or lose their biological properties (resulting in low infectious titers). To overcome these difficulties the screening for new "retargeting" peptides to be inserted might be done more efficiently in the context of the AAV capsid itself (vector display), where a pool of randomized peptide sequences is inserted into the capsid sequence and the viral pool is then screened directly on the target cells (Perabo *et al.*). The exciting results obtained by this approach together with the rapid advance of our knowledge of the structure and biology of AAV raise the expectation of dramatic improvement of the AAV vector technology in near future.

The ultimate goal of all these attempts will be the generation of a recombinant AAV vector, which allows gene delivery exclusively to the desired cells or tissue, thereby widening the therapeutic window of this vector for the clinical application.

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CHAPTER VII

CONCLUSIONS AND OUTLOOK

The potential of AAV as vector for gene therapy should benefit dramatically from further improvement of retargeting procedures.

On one hand, the vector is required to infect and transduce target cells at high rates (*efficiency*). At the same time, it is desired that the vector can avoid transduction of other cell types (*selectivity*). The AAV-Display technology established in this work seems to hold enormous potential in both these regards.

The selections performed on target cells allowed the identification of AAV capsid mutants with remarkable efficiency of infection of AAV-2 non-permissive cell lines. The combinatorial approach makes this possible even in the complete absence of any information regarding the receptors expressed on the surface of the target cells and their ligands.

Moreover, the screening of a large number of different peptides inserted at the re-targeting site provides an efficient optimization of the sequences with regard to all the characteristics that contribute to the functionality of the new capsid in every step that constitutes a productive infection (binding, intrnalization, release from the endosome, nuclear translocation, gene expression, replication and packaging of the viral progeny). The relevance of this advantage is demonstrated in our data by comparison between the selected mutants and the L14 mutant, obtained by simple insertion of a previously known sequence at the 587 site.

At the same time, insertional mutagenesis at the site exploited in this work (amino acid position 587) is likely to abolish the ability of the virus to interact with its primary receptor (HSPG) (Girod *et al.*, 1999b; Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Shi *et al.*, 2001; Wu *et al.*, 2000). Heparan sulfate proteoglycan is a very common molecule expressed on the surface of eucaryotic cells. Therefore, the result of the mutagenesis is that the ability of the capsid to bind to a widely expressed receptor is substituted by the ability to bind another receptor. If this alternative receptor happens to be less widely expressed, the result will be an increased specificity of the mutants.

For the mutants identified in the here reported experiments, we could detect an only limited restriction of the wild type tropism. The mutants failed to show target cells specificity and retained (with some exceptions) the ability to efficiently infect several AAV-2-permissive cell lines. Two simple explanations may account for this behaviour.

First, the screening procedure selected sequences that most likely target widely expressed receptors that are not hematopoietic cells-specific. While this is obvious for the RGD-carrying mutants, it could not be demonstrated for the mutants selected on Mec1, for which the receptors remain unknown.

Cell type specific receptors could be targeted by the introduction of negative selection steps in the AAV-Display screening protocol (Fig. 10). In this case, the pool will be screened for mutants with ability to infect particular cell-types by viral progeny harvest 48 hrs after infection of target cells and for mutants that fail to infect cell types chosen as "noise" cells, for which the infection is undesirable. This latter goal could be pursued by infecting the noise cells and collecting the culture supernatant 2 hrs. p.i. in order to rescue the viral mutants that were not able to bind to their expressed cellular receptors.

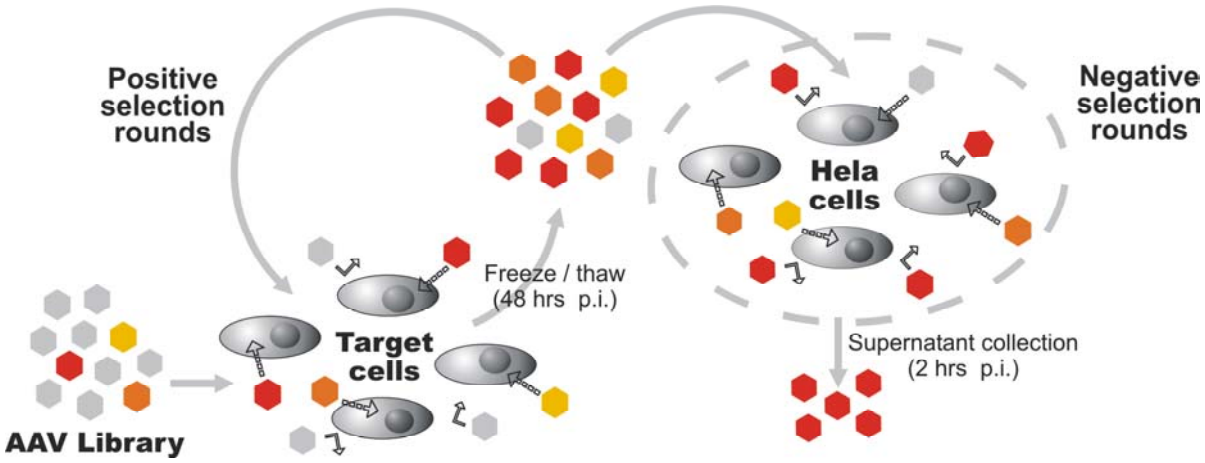

Fig. 10**:** *Example of protocol for screening the AAV library through positive selection rounds on target cells and negative selection rounds on noise cells.*

Another solution is the establishment of in vivo biopanning protocols, were the library of AAV is applied intravenously to an animal, and the viral progeny is collected from a desired tissue (for example tumoral cells previously introduced in the animal). Reiteration of injection/harvesting rounds should select viral mutants that display high efficiency in the infection of target tissues while minimizing their ability to infect other cell types.

The other explanation for the lack of selectivity of the identified mutants is that alhough the 587 site mutated capsids are prone to lack the ability to bind to HSPG, they may at least partially retain wild type tropism characteristics conferred by the nature of the capsid protein in other capsid regions; these structures could drive their ability to bind to other widely expressed molecules (e.g. cellular integrins). In particular, very little is known about the regions of the capsid that bind to the several described secondary receptors for AAV. 587 insertional mutants may retain the wild type capsid ability to bind to these molecules, explaining the still unsatisfactory specificity of all re-targeting mutants described so far. The recent publication of the 3D structure of the AAV-2 capsid should boost the efforts to shed light on the functionality of the capsid and help the identification of relevant sites (Xie *et al.*,

2002). More help could be provided by a further improvement of the AAV-Display technology where the mutations are extended to other regions of the capsid protein. This should be achieved amplifying the previous version of the AAV library by PCR with a modified polymerase that introduces a desired rate of point mutations in the region comprised between the primers (Fig. 11). The outcome would be a library of viral particles with random sequences of aminoacids inserted at the 587 site and point mutations introduced in several random sites of the capsid. Performance of positive/negative selection rounds on target and noise cells should result in the identification of clones with target efficiency provided by the selected insertion and selectivity provided by the natural tropism-abolishing mutations.

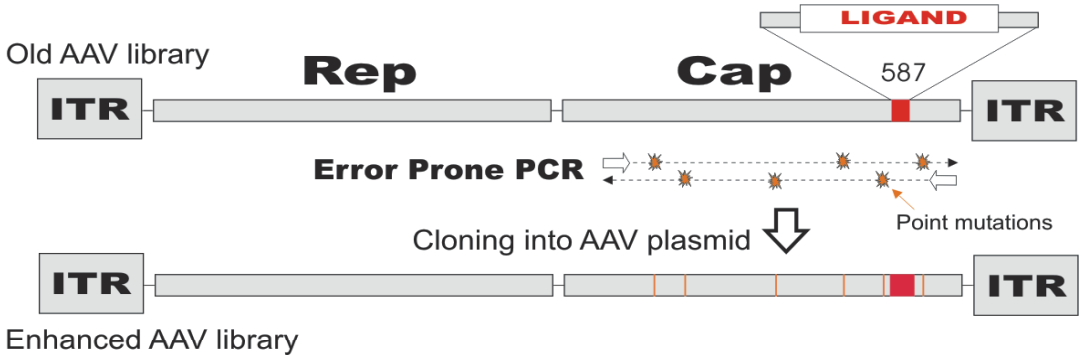

Fig.11: *Generation of a new library of AAV-2 viral particles with modified capsids by error prone PCR.*

Finally, the AAV-display could find application for the identification of mutants that escape neutralization by human antibodies. Previous studies estimated that 30-96% (depending on the geographical and ethnical differences) of all humans are AAV seropositive, while 18-67.5% have neutralizing antibodies (Blacklow *et al.*, 1968b; Chirmule *et al.*, 1999; Erles *et al.*, 1999b; Moskalenko *et al.*, 2000b), making a clinical application for this individuals difficult if not impossible. Our results (Huttner et al., submitted) demonstrated that the insertion of appropriate sequences in the 587 position diminishes the ability of policlonal human serum antybodies to neutralize AAV infection. In this study, one of the mutants that showed this ability was selected for retargeting purposes by AAV-Display. Pre-incubation with wt AAV neutralizing sera failed to inhibit transduction of target cells. A possible explanation is that modifications of the capsid external structure may provide viral particles with a novel shape that can no longer be recognized by preexisting antibodies. The AAV-Display technology could be useful to screen for viral mutants that are able to escape the humoral immune system of the patient. In particular, the AAV library may be subjected to

infection rounds on target cells in the presence of AAV neutralizing human sera in order to provide the selective pressure required to isolate clones that infect target cells despite the presence of antibodies against wt AAV-2.

BIBLIOGRAPHY

Agbandje, M., Kajigaya, S., McKenna, R., Young, N.S. and Rossmann, M.G. (**1994**). The structure of human parvovirus B19 at 8 A resolution. *Virology* 203(1), 106-15.

Agbandje, M., McKenna, R., Rossmann, M.G., Strassheim, M.L. and Parrish, C.R. (**1993**). Structure determination of feline panleukopenia virus empty particles. *Proteins* 16(2), 155-71.

Agbandje-McKenna, M., Llamas-Saiz, A.L., Wang, F., Tattersall, P. and Rossmann, M.G. (**1998**). Functional implications of the structure of the murine parvovirus, minute virus of mice. *Structure* 6(11), 1369-81.

Allen, J.M., Halbert, C.L. and Miller, A.D. (**2000**). Improved adeno-associated virus vector production with transfection of a single helper adenovirus gene, E4orf6. *Mol Ther* 1(1), 88-95.

Anderson, R., Macdonald, I., Corbett, T., Whiteway, A. and Prentice, H.G. (**2000**). A method for the preparation of highly purified adeno-associated virus using affinity column chromatography, protease digestion and solvent extraction. *J Virol Methods* 85(1-2), 23-34.

Atchison, R.W., Casto, B.C. and Hammon, W.M. (**1965**). Adeno-Associated Defective Virus Particles. *Science* 1, 754-756.

Aumailley, M., Gerl, M., Sonnenberg, A., Deutzmann, R. and Timpl, R. (**1990**). Identification of the Arg-Gly-Asp sequence in laminin A chain as a latent cell-binding site being exposed in fragment P1. *FEBS Lett* 262(1), 82-6.

Auricchio, A., Hildinger, M., O'Connor, E., Gao, G.P. and Wilson, J.M. (**2001**). Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. *Hum Gene Ther* 12(1), 71-6.

Avanzi, G.C., Lista, P., Giovinazzo, B., Miniero, R., Saglio, G., Benetton, G., Coda, R., Cattoretti, G. and Pegoraro, L. (**1988**). Selective growth response to IL-3 of a human leukaemic cell line with megakaryoblastic features. *Br J Haematol* 69(3), 359-66.

Balague, C., Kalla, M. and Zhang, W.W. (**1997**). Adeno-associated virus Rep78 protein and terminal repeats enhance integration of DNA sequences into the cellular genome. *J Virol* 71(4), 3299-306.

Bantel-Schaal, U., Delius, H., Schmidt, R. and zur Hausen, H. (**1999**). Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. *J Virol* 73(2), 939-47.

Baranowski, E., Ruiz-Jarabo, C.M. and Domingo, E. (**2001**). Evolution of cell recognition by viruses. *Science* 292(5519), 1102-5.

Bartlett, J.S., Kleinschmidt, J., Boucher, R.C. and Samulski, R.J. (**1999**). Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)2 antibody. *Nat Biotechnol* 17(2), 181-6.

Bartlett, J.S., Wilcher, R. and Samulski, R.J. (**2000a**). Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* 74(6), 2777-85.

Bartlett, J.S., Wilcher, R. and Samulski, R.J. (**2000b**). Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* 74(6), 2777-85.

Becerra, S.P., Koczot, F., Fabisch, P. and Rose, J.A. (**1988**). Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. *J Virol* 62(8), 2745-54.

Berns, K.I. (**1990**). Parvovirus replication. *Microbiol Rev* 54(3), 316-29.

Berns, K.I. and Linden, R.M. (**1995**). The cryptic life style of adeno-associated virus. *Bioessays* 17(3), 237-45.

Berns, K.I., Pinkerton, T.C., Thomas, G.F. and Hoggan, M.D. (**1975**). Detection of adeno-associated virus (AAV)-specific nucleotide sequences in DNA isolated from latently infected Detroit 6 cells. *Virology* 68(2), 556-60.

Blacklow, N.R. (1988). Adeno-associated virus of humans. *In* "Parvoviruses and human disease" (J. Pattison, Ed.), pp. 165-174. CRC Press, Boca Raton, FL.

Blacklow, N.R., Hoggan, M.D., Kapikian, A.Z., Austin, J.B. and Rowe, W.P. (**1968a**). Epidemiology of adenovirus-associated virus infection in a nursery population. *Am J Epidemiol* 88(3), 368-78.

Blacklow, N.R., Hoggan, M.D. and Rowe, W.P. (**1968b**). Serologic evidence for human infection with adenovirus-associated viruses. *J Natl Cancer Inst* 40(2), 319-27.

Blacklow, N.R., Hoggan, M.D., Sereno, M.S., Brandt, C.D., Kim, H.W., Parrott, R.H. and Chanock, R.M. (**1971**). A seroepidemiologic study of adenovirus-associated virus infection in infants and children. *Am J Epidemiol* 94(4), 359-66.

Burguete, T., Rabreau, M., Fontanges-Darriet, M., Roset, E., Hager, H.D., Koppel, A., Bischof, P. and Schlehofer, J.R. (**1999**). Evidence for infection of the human embryo with adeno-associated virus in pregnancy. *Hum Reprod* 14(9), 2396-401.

Cantwell, M., Hua, T., Pappas, J. and Kipps, T.J. (**1997**). Acquired CD40-ligand deficiency in chronic lymphocytic leukemia. *Nat Med* 3(9), 984-9.

Cantwell, M.J., Sharma, S., Friedmann, T. and Kipps, T.J. (**1996**). Adenovirus vector infection of chronic lymphocytic leukemia B cells. *Blood* 88(12), 4676-83.

Carrel, S., Sordat, B. and Merenda, C. (**1976**). Establishment of a cell line (Co-115) from a human colon carcinoma transplanted into nude mice. *Cancer Res* 36(11 Pt 1), 3978-84.

Carter, B.J. and Rose, J.A. (**1974**). Transcription in vivo of a defective parvovirus: sedimentation and electrophoretic analysis of RNA synthesized by adenovirus-associated virus and its helper adenovirus. *Virology* 61(1), 182-99.

Carter, P.J. and Samulski, R.J. (**2000**). Adeno-associated viral vectors as gene delivery vehicles. *Int J Mol Med* 6(1), 17-27.

Chang, S.F., Sgro, J.Y. and Parrish, C.R. (**1992**). Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. *J Virol* 66(12), 6858-67.

Chao, H., Sun, L., Bruce, A., Xiao, X. and Walsh, C.E. (**2002**). Expression of human factor VIII by splicing between dimerized AAV vectors. *Mol Ther* 5(6), 716-22.

Chapman, M.S. and Rossmann, M.G. (**1993**). Structure, sequence, and function correlations among parvoviruses. *Virology* 194(2), 491-508.

Cheung, A.K., Hoggan, M.D., Hauswirth, W.W. and Berns, K.I. (**1980**). Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. *J Virol* 33(2), 739-48.

Chiorini, J.A., Kim, F., Yang, L. and Kotin, R.M. (**1999**). Cloning and characterization of adeno-associated virus type 5. *J Virol* 73(2), 1309-19.

Chiorini, J.A., Wendtner, C.M., Urcelay, E., Safer, B., Hallek, M. and Kotin, R.M. (**1995**). High-efficiency transfer of the T cell co-stimulatory molecule B7-2 to lymphoid cells using high-titer recombinant adeno-associated virus vectors. *Hum Gene Ther* 6(12), 1531-41.

Chiorini, J.A., Yang, L., Liu, Y., Safer, B. and Kotin, R.M. (**1997**). Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. *J Virol* 71(9), 6823-33.

Chipman, P.R., Agbandje-McKenna, M., Kajigaya, S., Brown, K.E., Young, N.S., Baker, T.S. and Rossmann, M.G. (**1996**). Cryo-electron microscopy studies of empty

capsids of human parvovirus B19 complexed with its cellular receptor. *Proc Natl Acad Sci U S A* 93(15), 7502-6.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R. and Wilson, J. (**1999**). Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther* 6(9), 1574-83.

Collaco, R.F., Cao, X. and Trempe, J.P. (**1999**). A helper virus-free packaging system for recombinant adeno-associated virus vectors. *Gene* 238(2), 397-405.

Cosset, F.L. and Russell, S.J. (**1996**). Targeting retrovirus entry. *Gene Ther* 3(11), 946-56.

Curiel, D.T. (**1999**). Strategies to adapt adenoviral vectors for targeted delivery. *Ann N Y Acad Sci* 886, 158-71.

Davidson, B.L., Stein, C.S., Heth, J.A., Martins, I., Kotin, R.M., Derksen, T.A., Zabner, J., Ghodsi, A. and Chiorini, J.A. (**2000**). Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc Natl Acad Sci U S A* 97(7), 3428-32.

de la Maza, L.M. and Carter, B.J. (**1980**). Molecular structure of adeno-associated virus variant DNA. *J Biol Chem* 255(7), 3194-203.

Douar, A.M., Poulard, K., Stockholm, D. and Danos, O. (**2001**). Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol* 75(4), 1824-33.

Duan, D., Li, Q., Kao, A.W., Yue, Y., Pessin, J.E. and Engelhardt, J.F. (**1999**). Dynamin is required for recombinant adeno-associated virus type 2 infection. *J Virol* 73(12), 10371-6.

Duan, D., Yue, Y. and Engelhardt, J.F. (**2001**). Expanding AAV packaging capacity with trans-splicing or overlapping vectors: a quantitative comparison. *Mol Ther* 4(4), 383-91.

Duan, D., Yue, Y. and Engelhardt, J.F. (**2003**). Dual vector expansion of the recombinant AAV packaging capacity. *Methods Mol Biol* 219, 29-51.

Dubielzig, R., King, J.A., Weger, S., Kern, A. and Kleinschmidt, J.A. (**1999**). Adeno-associated virus type 2 protein interactions: formation of pre-encapsidation complexes. *J Virol* 73(11), 8989-98.

Engelstadter, M., Bobkova, M., Baier, M., Stitz, J., Holtkamp, N., Chu, T.H., Kurth, R., Dornburg, R., Buchholz, C.J. and Cichutek, K. (**2000**). Targeting human T cells by retroviral vectors displaying antibody domains selected from a phage display library. *Hum Gene Ther* 11(2), 293-303.

Erles, K., Sebokova, P. and Schlehofer, J.R. (**1999a**). Update on the prevalence of serum antibodies (IgG and IgM) to adeno- associated virus (AAV). *J Med Virol* 59(3), 406-11.

Erles, K., Sebokova, P. and Schlehofer, J.R. (**1999b**). Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J Med Virol* 59(3), 406-11.

Ferrari, F.K., Xiao, X., McCarty, D. and Samulski, R.J. (**1997**). New developments in the generation of Ad-free, high-titer rAAV gene therapy vectors. *Nat Med* 3(11), 1295-7.

Fisher, K.J., Jooss, K., Alston, J., Yang, Y., Haecker, S.E., High, K., Pathak, R., Raper, S.E. and Wilson, J.M. (**1997**). Recombinant adeno-associated virus for muscle directed gene therapy. *Nat Med* 3(3), 306-12.

Fisher-Adams, G., Wong, K.K., Jr., Podsakoff, G., Forman, S.J. and Chatterjee, S. (**1996**). Integration of adeno-associated virus vectors in CD34+ human hematopoietic progenitor cells after transduction. *Blood* 88(2), 492-504.

Flotte, T.R., Afione, S.A., Conrad, C., McGrath, S.A., Solow, R., Oka, H., Zeitlin, P.L., Guggino, W.B. and Carter, B.J. (**1993**). Stable in vivo expression of the cystic

fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc Natl Acad Sci U S A* 90(22), 10613-7.

Fry, E.E., Lea, S.M., Jackson, T., Newman, J.W., Ellard, F.M., Blakemore, W.E., Abu-Ghazaleh, R., Samuel, A., King, A.M. and Stuart, D.I. (**1999**). The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. *Embo J* 18(3), 543-54.

Gao, G., Qu, G., Burnham, M.S., Huang, J., Chirmule, N., Joshi, B., Yu, Q.C., Marsh, J.A., Conceicao, C.M. and Wilson, J.M. (**2000**). Purification of recombinant adeno-associated virus vectors by column chromatography and its performance in vivo. *Hum Gene Ther* 11(15), 2079-91.

Gao, G.P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J. and Wilson, J.M. (**2002**). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci U S A* 99(18), 11854-9.

Gao, G.P., Qu, G., Faust, L.Z., Engdahl, R.K., Xiao, W., Hughes, J.V., Zoltick, P.W. and Wilson, J.M. (**1998**). High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. *Hum Gene Ther* 9(16), 2353-62.

Girod, A., Ried, M., Wobus, C., Lahm, H., Leike, K., Kleinschmidt, J., Deleage, G. and Hallek, M. (**1999a**). Genetic capsid modifications allow efficient re-targeting of adeno- associated virus type 2. *Nat Med* 5(9), 1052-6.

Girod, A., Ried, M., Wobus, C., Lahm, H., Leike, K., Kleinschmidt, J., Deleage, G. and Hallek, M. (**1999b**). Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med* 5(12), 1438.

Girod, A., Wobus, C.E., Zadori, Z., Ried, M., Leike, K., Tijssen, P., Kleinschmidt, J.A. and Hallek, M. (**2002**). The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *J Gen Virol* 83(Pt 5), 973-8.

Grifman, M., Trepel, M., Speece, P., Gilbert, L.B., Arap, W., Pasqualini, R. and Weitzman, M.D. (**2001**). Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. *Mol Ther* 3(6), 964-75.

Grimm, D., Kern, A., Pawlita, M., Ferrari, F., Samulski, R. and Kleinschmidt, J. (**1999**). Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. *Gene Ther* 6(7), 1322-30.

Grimm, D., Kern, A., Rittner, K. and Kleinschmidt, J.A. (**1998**). Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum Gene Ther* 9(18), 2745-60.

Grimm, D. and Kleinschmidt, J.A. (**1999**). Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical use. *Hum Gene Ther* 10(15), 2445-50.

Halbert, C.L., Allen, J.M. and Miller, A.D. (**2001**). Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *J Virol* 75(14), 6615-24.

Halbert, C.L., Allen, J.M. and Miller, A.D. (**2002**). Efficient mouse airway transduction following recombination between AAV vectors carrying parts of a larger gene. *Nat Biotechnol* 20(7), 697-701.

Halbert, C.L., Rutledge, E.A., Allen, J.M., Russell, D.W. and Miller, A.D. (**2000**). Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. *J Virol* 74(3), 1524-32.

Hallek, M. and Wendtner, C.M. (**1996**). Recombinant adeno-associated virus (rAAV) vectors for somatic gene therapy: recent advances and potential clinical applications. *Cytokines Mol Ther* 2(2), 69-79.

Handa, A., Muramatsu, S., Qiu, J., Mizukami, H. and Brown, K.E. (**2000**). Adeno-associated virus (AAV)-3-based vectors transduce haematopoietic cells not susceptible to transduction with AAV-2-based vectors. *J Gen Virol* 81(Pt 8), 2077-84.

Hansen, J., Qing, K. and Srivastava, A. (**2001**). Infection of purified nuclei by adeno-associated virus 2. *Mol Ther* 4(4), 289-96.

Hermens, W.T., ter Brake, O., Dijkhuizen, P.A., Sonnemans, M.A., Grimm, D., Kleinschmidt, J.A. and Verhaagen, J. (**1999**). Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum Gene Ther* 10(11), 1885-91.

Hermonat, P.L. (**1989**). The adeno-associated virus Rep78 gene inhibits cellular transformation induced by bovine papillomavirus. *Virology* 172(1), 253-61.

Hermonat, P.L., Labow, M.A., Wright, R., Berns, K.I. and Muzyczka, N. (**1984**). Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. *J Virol* 51(2), 329-39.

Hernandez, Y.J., Wang, J., Kearns, W.G., Loiler, S., Poirier, A. and Flotte, T.R. (**1999**). Latent adeno-associated virus infection elicits humoral but not cell-mediated immune responses in a nonhuman primate model. *J Virol* 73(10), 8549-58.

Hildinger, M., Auricchio, A., Gao, G., Wang, L., Chirmule, N. and Wilson, J.M. (**2001**). Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. *J Virol* 75(13), 6199-203.

Hinshaw, J.E. (**2000**). Dynamin and its role in membrane fission. *Annu Rev Cell Dev Biol* 16, 483-519.

Hinshaw, J.E. and Schmid, S.L. (**1995**). Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature* 374(6518), 190-2.

Hoque, M., Ishizu, K., Matsumoto, A., Han, S.I., Arisaka, F., Takayama, M., Suzuki, K., Kato, K., Kanda, T., Watanabe, H. and Handa, H. (**1999**). Nuclear transport of the major capsid protein is essential for adeno- associated virus capsid formation. *J Virol* 73(9), 7912-5.

Huttner, N.A., Girod, A., Perabo, L., Edbauer, D., Kleinschmidt, J.A., Büning, H. and Hallek, M. Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies (submitted for publication).

Huttner, N.A., Girod, A., Schnittger, S., Schoch, C., Hallek, M. and Buning, H. (**2003**). Analysis of site-specific transgene integration following cotransduction with recombinant adeno-associated virus and a rep encodingplasmid. *J Gene Med* 5(2), 120-9.

Inoue, N. and Russell, D.W. (**1998**). Packaging cells based on inducible gene amplification for the production of adeno-associated virus vectors. *J Virol* 72(9), 7024-31.

Jooss, K., Yang, Y., Fisher, K.J. and Wilson, J.M. (**1998**). Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol* 72(5), 4212-23.

Kaludov, N., Brown, K.E., Walters, R.W., Zabner, J. and Chiorini, J.A. (**2001**). Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J Virol* 75(15), 6884-93.

Kaplitt, M.G., Leone, P., Samulski, R.J., Xiao, X., Pfaff, D.W., O'Malley, K.L. and During, M.J. (**1994**). Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat Genet* 8(2), 148-54.

Kapturczak, M.H., Flotte, T. and Atkinson, M.A. (**2001**). Adeno-associated virus (AAV) as a vehicle for therapeutic gene delivery: improvements in vector design and viral

production enhance potential to prolong graft survival in pancreatic islet cell transplantation for the reversal of type 1 diabetes. *Curr Mol Med* 1(2), 245-58.

Kasamatsu, H. and Nakanishi, A. (1998). How do animal DNA viruses get to the nucleus? *Annu Rev Microbiol* 52, 627-86.

Kay, M.A., Glorioso, J.C. and Naldini, L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* 7(1), 33-40.

Khleif, S.N., Myers, T., Carter, B.J. and Trempe, J.P. (1991). Inhibition of cellular transformation by the adeno-associated virus rep gene. *Virology* 181(2), 738-41.

Kotin, R.M. (1994). Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum Gene Ther* 5(7), 793-801.

Kotin, R.M., Menninger, J.C., Ward, D.C. and Berns, K.I. (1991). Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. *Genomics* 10(3), 831-4.

Kotin, R.M., Siniscalco, M., Samulski, R.J., Zhu, X.D., Hunter, L., Laughlin, C.A., McLaughlin, S., Muzyczka, N., Rocchi, M. and Berns, K.I. (1990). Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A* 87(6), 2211-5.

Kronenberg, S., Kleinschmidt, J.A. and Bottcher, B. (2001). Electron cryo-microscopy and image reconstruction of adeno-associated virus type 2 empty capsids. *EMBO Rep* 2(11), 997-1002.

Labow, M.A. and Berns, K.I. (1988). The adeno-associated virus rep gene inhibits replication of an adeno-associated virus/simian virus 40 hybrid genome in cos-7 cells. *J Virol* 62(5), 1705-12.

Laughlin, C.A., Myers, M.W., Risin, D.L. and Carter, B.J. (1979). Defective-interfering particles of the human parvovirus adeno-associated virus. *Virology* 94(1), 162-74.

Laughlin, C.A., Tratschin, J.D., Coon, H. and Carter, B.J. (1983). Cloning of infectious adeno-associated virus genomes in bacterial plasmids. *Gene* 23(1), 65-73.

Lavillette, D., Russell, S.J. and Cosset, F.L. (2001). Retargeting gene delivery using surface-engineered retroviral vector particles. *Curr Opin Biotechnol* 12(5), 461-6.

Linden, R.M., Ward, P., Giraud, C., Winocour, E. and Berns, K.I. (1996a). Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A* 93(21), 11288-94.

Linden, R.M., Winocour, E. and Berns, K.I. (1996b). The recombination signals for adeno-associated virus site-specific integration. *Proc Natl Acad Sci U S A* 93(15), 7966-72.

Liu, X.L., Clark, K.R. and Johnson, P.R. (1999). Production of recombinant adeno-associated virus vectors using a packaging cell line and a hybrid recombinant adenovirus. *Gene Ther* 6(2), 293-9.

Lukashov, V.V. and Goudsmit, J. (2001). Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. *J Virol* 75(6), 2729-40.

Lusby, E.W. and Berns, K.I. (1982). Mapping of the 5' termini of two adeno-associated virus 2 RNAs in the left half of the genome. *J Virol* 41(2), 518-26.

Mandel, R.J., Spratt, S.K., Snyder, R.O. and Leff, S.E. (1997). Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc Natl Acad Sci U S A* 94(25), 14083-8.

Marcus, C.J., Laughlin, C.A. and Carter, B.J. (1981). Adeno-associated virus RNA transcription in vivo. *Eur J Biochem* 121(1), 147-54.

Margalit, H., Fischer, N. and Ben-Sasson, S.A. (1993). Comparative analysis of structurally defined heparin binding sequences reveals a distinct spatial distribution of basic residues. *J Biol Chem* 268(26), 19228-31.

Marks, B., Stowell, M.H., Vallis, Y., Mills, I.G., Gibson, A., Hopkins, C.R. and McMahon, H.T. (**2001**). GTPase activity of dynamin and resulting conformation change are essential for endocytosis. *Nature* 410(6825), 231-5.

Marsh, M. and Helenius, A. (**1989**). Virus entry into animal cells. *Adv Virus Res* 36, 107-51.

Mayor, H.D., Houlditch, G.S. and Mumford, D.M. (**1973**). Influence of adeno-associated satellite virus on adenovirus-induced tumours in hamsters. *Nat New Biol* 241(106), 44-6.

McKenna, R., Olson, N.H., Chipman, P.R., Baker, T.S., Booth, T.F., Christensen, J., Aasted, B., Fox, J.M., Bloom, M.E., Wolfinbarger, J.B. and Agbandje-McKenna, M. (**1999**). Three-dimensional structure of Aleutian mink disease parvovirus: implications for disease pathogenicity. *J Virol* 73(8), 6882-91.

McLaughlin, S.K., Collis, P., Hermonat, P.L. and Muzyczka, N. (**1988**). Adeno-associated virus general transduction vectors: analysis of proviral structures. *J Virol* 62(6), 1963-73.

McPherson, R.A., Rosenthal, L.J. and Rose, J.A. (**1985**). Human cytomegalovirus completely helps adeno-associated virus replication. *Virology* 147(1), 217-22.

Mendelson, E., Trempe, J.P. and Carter, B.J. (**1986**). Identification of the trans-acting Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. *J Virol* 60(3), 823-32.

Miller, A.D. (**1996**). Cell-surface receptors for retroviruses and implications for gene transfer. *Proc Natl Acad Sci U S A* 93(21), 11407-13.

Mizukami, H., Young, N.S. and Brown, K.E. (**1996**). Adeno-associated virus type 2 binds to a 150-kilodalton cell membrane glycoprotein. *Virology* 217(1), 124-30.

Monahan, P.E. and Samulski, R.J. (**2000a**). AAV vectors: is clinical success on the horizon? *Gene Ther* 7(1), 24-30.

Monahan, P.E. and Samulski, R.J. (**2000b**). Adeno-associated virus vectors for gene therapy: more pros than cons? *Mol Med Today* 6(11), 433-40.

Moskalenko, M., Chen, L., van Roey, M., Donahue, B.A., Snyder, R.O., McArthur, J.G. and Patel, S.D. (**2000a**). Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. *J Virol* 74(4), 1761-6.

Moskalenko, M., Chen, L., van Roey, M., Donahue, B.A., Snyder, R.O., McArthur, J.G. and Patel, S.D. (**2000b**). Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. *J Virol* 74(4), 1761-6.

Muramatsu, S., Mizukami, H., Young, N.S. and Brown, K.E. (**1996**). Nucleotide sequencing and generation of an infectious clone of adeno- associated virus 3. *Virology* 221(1), 208-17.

Myers, M.W., Laughlin, C.A., Jay, F.T. and Carter, B.J. (**1980**). Adenovirus helper function for growth of adeno-associated virus: effect of temperature-sensitive mutations in adenovirus early gene region 2. *J Virol* 35(1), 65-75.

Nicklin, S.A., Buening, H., Dishart, K.L., de Alwis, M., Girod, A., Hacker, U., Thrasher, A.J., Ali, R.R., Hallek, M. and Baker, A.H. (**2001**). Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells. *Mol Ther* 4(3), 174-81.

Ohno, K., Sawai, K., Iijima, Y., Levin, B. and Meruelo, D. (**1997**). Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. *Nat Biotechnol* 15(8), 763-7.

Owens, R.A. (**2002**). Second generation adeno-associated virus type 2-based gene therapy systems with the potential for preferential integration into AAVS1. *Curr Gene Ther* 2(2), 145-59.

Perabo, L., Büning, H., Kofler, D., Ried, M.U., Girod, A., Enssle, J. and Hallek, M. (**2003**). In vitro Evolution of Receptor Specific Gene Vectors: The Adeno-Associated Virus Display. *Mol Ther*.

Pfeifer, A. and Verma, I.M. (**2001**). Gene therapy: promises and problems. *Annu Rev Genomics Hum Genet* 2, 177-211.

Philpott, N.J., Giraud-Wali, C., Dupuis, C., Gomos, J., Hamilton, H., Berns, K.I. and Falck-Pedersen, E. (**2002**). Efficient integration of recombinant adeno-associated virus DNA vectors requires a p5-rep sequence in cis. *J Virol* 76(11), 5411-21.

Ponnazhagan, S., Erikson, D., Kearns, W.G., Zhou, S.Z., Nahreini, P., Wang, X.S. and Srivastava, A. (**1997a**). Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. *Hum Gene Ther* 8(3), 275-84.

Ponnazhagan, S., Mukherjee, P., Wang, X.S., Qing, K., Kube, D.M., Mah, C., Kurpad, C., Yoder, M.C., Srour, E.F. and Srivastava, A. (**1997b**). Adeno-associated virus type 2-mediated transduction in primary human bone marrow-derived CD34+ hematopoietic progenitor cells: donor variation and correlation of transgene expression with cellular differentiation. *J Virol* 71(11), 8262-7.

Qing, K., Mah, C., Hansen, J., Zhou, S., Dwarki, V. and Srivastava, A. (**1999**). Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat Med* 5(1), 71-7.

Qiu, J. and Brown, K.E. (**1999a**). A 110-kDa nuclear shuttle protein, nucleolin, specifically binds to adeno-associated virus type 2 (AAV-2) capsid. *Virology* 257(2), 373-82.

Qiu, J. and Brown, K.E. (**1999b**). Integrin alphaVbeta5 is not involved in adeno-associated virus type 2 (AAV2) infection. *Virology* 264(2), 436-40.

Qiu, J., Mizukami, H. and Brown, K.E. (**1999**). Adeno-associated virus 2 co-receptors? *Nat Med* 5(5), 467-8.

Rabinowitz, J.E., Rolling, F., Li, C., Conrath, H., Xiao, W., Xiao, X. and Samulski, R.J. (**2002**). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* 76(2), 791-801.

Rabinowitz, J.E. and Samulski, R.J. (**2000**). Building a better vector: the manipulation of AAV virions. *Virology* 278(2), 301-8.

Rabinowitz, J.E., Xiao, W. and Samulski, R.J. (**1999**). Insertional mutagenesis of AAV2 capsid and the production of recombinant virus. *Virology* 265(2), 274-85.

Ried, M.U., Girod, A., Leike, K., Buning, H. and Hallek, M. (**2002**). Adeno-associated virus capsids displaying immunoglobulin-binding domains permit antibody-mediated vector retargeting to specific cell surface receptors. *J Virol* 76(9), 4559-66.

Rivadeneira, E.D., Popescu, N.C., Zimonjic, D.B., Cheng, G.S., Nelson, P.J., Ross, M.D., DiPaolo, J.A. and Klotman, M.E. (**1998**). Sites of recombinant adeno-associated virus integration. *Int J Oncol* 12(4), 805-10.

Rohde, V., Erles, K., Sattler, H.P., Derouet, H., Wullich, B. and Schlehofer, J.R. (**1999**). Detection of adeno-associated virus in human semen: does viral infection play a role in the pathogenesis of male infertility? *Fertil Steril* 72(5), 814-6.

Rolling, F. and Samulski, R.J. (**1995**). AAV as a viral vector for human gene therapy. Generation of recombinant virus. *Mol Biotechnol* 3(1), 9-15.

Romanczuk, H., Galer, C.E., Zabner, J., Barsomian, G., Wadsworth, S.C. and O'Riordan, C.R. (**1999**). Modification of an adenoviral vector with biologically selected peptides: a novel strategy for gene delivery to cells of choice. *Hum Gene Ther* 10(16), 2615-26.

Root, C.N., Wills, E.G., McNair, L.L. and Whittaker, G.R. (**2000**). Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor. *J Gen Virol* 81(Pt 11), 2697-705.

Ruffing, M., Zentgraf, H. and Kleinschmidt, J.A. (**1992**). Assembly of viruslike particles by recombinant structural proteins of adeno-associated virus type 2 in insect cells. *J Virol* 66(12), 6922-30.

Ruoslahti, E. (**1996**). RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* 12, 697-715.

Russell, D.W. and Kay, M.A. (**1999**). Adeno-associated virus vectors and hematology. *Blood* 94(3), 864-74.

Rutledge, E.A., Halbert, C.L. and Russell, D.W. (**1998**). Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J Virol* 72(1), 309-19.

Samulski, R.J., Berns, K.I., Tan, M. and Muzyczka, N. (**1982**). Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci U S A* 79(6), 2077-81.

Samulski, R.J., Zhu, X., Xiao, X., Brook, J.D., Housman, D.E., Epstein, N. and Hunter, L.A. (**1991**). Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *Embo J* 10(12), 3941-50.

Sanlioglu, S., Benson, P.K., Yang, J., Atkinson, E.M., Reynolds, T. and Engelhardt, J.F. (**2000**). Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. *J Virol* 74(19), 9184-96.

Sanlioglu, S., Duan, D. and Engelhardt, J.F. (**1999**). Two independent molecular pathways for recombinant adeno-associated virus genome conversion occur after UV-C and E4orf6 augmentation of transduction. *Hum Gene Ther* 10(4), 591-602.

Schlehofer, J.R., Ehrbar, M. and zur Hausen, H. (**1986**). Vaccinia virus, herpes simplex virus, and carcinogens induce DNA amplification in a human cell line and support replication of a helpervirus dependent parvovirus. *Virology* 152(1), 110-7.

Seiler, M., Halbert, C.L., Chiorini, J.A., Miller, A.D. and Zabner, J. (**2002**). AAV5 and AAV6 mediate gene transfer to human airway epithelia via different receptors. *Mol. Ther.* 5, abstract No. 117.

Seisenberger, G., Ried, M.U., Endress, T., Buning, H., Hallek, M. and Brauchle, C. (**2001**). Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science* 294(5548), 1929-32.

Sever, S., Damke, H. and Schmid, S.L. (**2000**). Dynamin:GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. *J Cell Biol* 150(5), 1137-48.

Shi, W., Arnold, G.S. and Bartlett, J.S. (**2001**). Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. *Hum Gene Ther* 12(14), 1697-711.

Shi, W. and Bartlett, J.S. (**2003**). RGD inclusion in VP3 provides adeno-associated virus type 2 (AAV2)-based vectors with a heparan sulfate-independent cell entry mechanism. *Mol Ther* 7(4), 515-25.

Siegl, G., Bates, R.C., Berns, K.I., Carter, B.J., Kelly, D.C., Kurstak, E. and Tattersall, P. (**1985**). Characteristics and taxonomy of Parvoviridae. *Intervirology* 23(2), 61-73.

Sinha, P., Sengupta, J. and Ray, P.K. (**1999**). Functional mimicry of protein A of Staphylococcus aureus by a proteolytically cleaved fragment. *Biochem Biophys Res Commun* 260(1), 111-6.

Smith, T.J. (**2001**). Antibody interactions with rhinovirus: lessons for mechanisms of neutralization and the role of immunity in viral evolution. *Curr Top Microbiol Immunol* 260, 1-28.

Smuda, J.W. and Carter, B.J. (**1991**). Adeno-associated viruses having nonsense mutations in the capsid genes: growth in mammalian cells containing an inducible amber suppressor. *Virology* 184(1), 310-8.

Somia, N. and Verma, I.M. (**2000**). Gene therapy: trials and tribulations. *Nat Rev Genet* 1(2), 91-9.

Stacchini, A., Aragno, M., Vallario, A., Alfarano, A., Circosta, P., Gottardi, D., Faldella, A., Rege-Cambrin, G., Thunberg, U., Nilsson, K. and Caligaris-Cappio, F. (**1999**). MEC1 and MEC2: two new cell lines derived from B-chronic lymphocytic leukaemia in prolymphocytoid transformation. *Leuk Res* 23(2), 127-36.

Strassheim, M.L., Gruenberg, A., Veijalainen, P., Sgro, J.Y. and Parrish, C.R. (**1994**). Two dominant neutralizing antigenic determinants of canine parvovirus are found on the threefold spike of the virus capsid. *Virology* 198(1), 175-84.

Summerford, C., Bartlett, J.S. and Samulski, R.J. (**1999**). AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med* 5(1), 78-82.

Summerford, C. and Samulski, R.J. (**1998**). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 72(2), 1438-45.

Summerford, C. and Samulski, R.J. (**1999**). Viral receptors and vector purification: new approaches for generating clinical-grade reagents. *Nat Med* 5(5), 587-8.

Tal, J. (**2000**). Adeno-associated virus-based vectors in gene therapy. *J Biomed Sci* 7(4), 279-91.

Tamayose, K., Hirai, Y. and Shimada, T. (**1996**). A new strategy for large-scale preparation of high-titer recombinant adeno-associated virus vectors by using packaging cell lines and sulfonated cellulose column chromatography. *Hum Gene Ther* 7(4), 507-13.

Thomson, B.J., Weindler, F.W., Gray, D., Schwaab, V. and Heilbronn, R. (**1994**). Human herpesvirus 6 (HHV-6) is a helper virus for adeno-associated virus type 2 (AAV-2) and the AAV-2 rep gene homologue in HHV-6 can mediate AAV-2 DNA replication and regulate gene expression. *Virology* 204(1), 304-11.

Tratschin, J.D., Miller, I.L. and Carter, B.J. (**1984**). Genetic analysis of adeno-associated virus: properties of deletion mutants constructed in vitro and evidence for an adeno-associated virus replication function. *J Virol* 51(3), 611-9.

Tsao, J., Chapman, M.S., Agbandje, M., Keller, W., Smith, K., Wu, H., Luo, M., Smith, T.J., Rossmann, M.G., Compans, R.W. and et al. (**1991**). The three-dimensional structure of canine parvovirus and its functional implications. *Science* 251(5000), 1456-64.

Vincent, K.A., Piraino, S.T. and Wadsworth, S.C. (**1997**). Analysis of recombinant adeno-associated virus packaging and requirements for rep and cap gene products. *J Virol* 71(3), 1897-905.

Walter, W. and Stein, U. (**1996**). Cell type specific and inducible promotors for vectors in gene therapy as an approach for cell targeting. *J Mol Med* 74, 379-392.

Walz, C., Deprez, A., Dupressoir, T., Durst, M., Rabreau, M. and Schlehofer, J.R. (**1997**). Interaction of human papillomavirus type 16 and adeno-associated virus type 2 co-infecting human cervical epithelium. *J Gen Virol* 78 (Pt 6), 1441-52.

Wang, K., Huang, S., Kapoor-Munshi, A. and Nemerow, G. (**1998**). Adenovirus internalization and infection require dynamin. *J Virol* 72(4), 3455-8.

Weichert, W.S., Parker, J.S., Wahid, A.T., Chang, S.F., Meier, E. and Parrish, C.R. (**1998**). Assaying for structural variation in the parvovirus capsid and its role in infection. *Virology* 250(1), 106-17.

Weitzman, M.D., Kyostio, S.R., Kotin, R.M. and Owens, R.A. (**1994**). Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc Natl Acad Sci U S A* 91(13), 5808-12.

Wendtner, C.M., Kofler, D.M., Theiss, H.D., Kurzeder, C., Buhmann, R., Schweighofer, C., Perabo, L., Danhauser-Riedl, S., Baumert, J., Hiddemann, W., Hallek, M. and Buning, H. (**2002**). Efficient gene transfer of CD40 ligand into primary B-CLL cells using recombinant adeno-associated virus (rAAV) vectors. *Blood* 100(5), 1655-61.

White, J.M. (**1993**). Integrins as virus receptors. *Curr Biol* 3, 596-599.

Wierda, W.G., Cantwell, M.J., Woods, S.J., Rassenti, L.Z., Prussak, C.E. and Kipps, T.J. (**2000**). CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. *Blood* 96(9), 2917-24.

Wistuba, A., Kern, A., Weger, S., Grimm, D. and Kleinschmidt, J.A. (**1997a**). Subcellular compartmentalization of adeno-associated virus type 2 assembly. *J Virol* 71(2), 1341-52.

Wistuba, A., Kern, A., Weger, S., Grimm, D. and Kleinschmidt, J.A. (**1997b**). Subcellular compartmentalization of adeno-associated virus type 2 assembly. *J Virol* 71(2), 1341-52.

Wistuba, A., Weger, S., Kern, A. and Kleinschmidt, J.A. (**1995**). Intermediates of adeno-associated virus type 2 assembly: identification of soluble complexes containing Rep and Cap proteins. *J Virol* 69(9), 5311-9.

Wobus, C.E., Hugle-Dorr, B., Girod, A., Petersen, G., Hallek, M. and Kleinschmidt, J.A. (**2000a**). Monoclonal antibodies against the adeno-associated virus type 2 (AAV-2) capsid: epitope mapping and identification of capsid domains involved in AAV-2-cell interaction and neutralization of AAV-2 infection. *J Virol* 74(19), 9281-93.

Wobus, C.E., Hugle-Dorr, B., Girod, A., Petersen, G., Hallek, M. and Kleinschmidt, J.A. (**2000b**). Monoclonal antibodies against the adeno-associated virus type 2 (AAV-2) capsid: epitope mapping and identification of capsid domains involved in AAV-2-cell interaction and neutralization of AAV-2 infection. *J Virol* 74(19), 9281-93.

Wu, P., Xiao, W., Conlon, T., Hughes, J., Agbandje-McKenna, M., Ferkol, T., Flotte, T. and Muzyczka, N. (**2000**). Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol* 74(18), 8635-47.

Xiao, W., Chirmule, N., Berta, S.C., McCullough, B., Gao, G. and Wilson, J.M. (**1999**). Gene therapy vectors based on adeno-associated virus type 1. *J Virol* 73(5), 3994-4003.

Xiao, W., Chirmule, N., Schnell, M.A., Tazelaar, J., Hughes, J.V. and Wilson, J.M. (**2000**). Route of administration determines induction of T-cell-independent humoral responses to adeno-associated virus vectors. *Mol Ther* 1(4), 323-9.

Xiao, W., Warrington, K.H., Jr., Hearing, P., Hughes, J. and Muzyczka, N. (**2002**). Adenovirus-facilitated nuclear translocation of adeno-associated virus type 2. *J Virol* 76(22), 11505-17.

Xiao, X., Li, J. and Samulski, R.J. (**1996**). Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 70(11), 8098-108.

Xiao, X., Li, J. and Samulski, R.J. (**1998**). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 72(3), 2224-32.

Xie, Q., Bu, W., Bhatia, S., Hare, J., Somasundaram, T., Azzi, A. and Chapman, M.S. (**2002**). The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc Natl Acad Sci U S A* 99(16), 10405-10.

Yakinoglu, A.O., Heilbronn, R., Burkle, A., Schlehofer, J.R. and zur Hausen, H. (**1988**). DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. *Cancer Res* 48(11), 3123-9.

Yakobson, B., Koch, T. and Winocour, E. (**1987**). Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. *J Virol* 61(4), 972-81.

Yalkinoglu, A.O., Zentgraf, H. and Hubscher, U. (**1991**). Origin of adeno-associated virus DNA replication is a target of carcinogen-inducible DNA amplification. *J Virol* 65(6), 3175-84.

Yang, Q., Mamounas, M., Yu, G., Kennedy, S., Leaker, B., Merson, J., Wong-Staal, F., Yu, M. and Barber, J.R. (**1998**). Development of novel cell surface CD34-targeted recombinant adenoassociated virus vectors for gene therapy. *Hum Gene Ther* 9(13), 1929-37.

Young, S.M., Jr., McCarty, D.M., Degtyareva, N. and Samulski, R.J. (**2000a**). Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. *J Virol* 74(9), 3953-66.

Young, S.M., Jr. and Samulski, R.J. (**2001**). Adeno-associated virus (AAV) site-specific recombination does not require a Rep-dependent origin of replication within the AAV terminal repeat. *Proc Natl Acad Sci U S A* 98(24), 13525-30.

Young, S.M., Jr., Xiao, W. and Samulski, R.J. (**2000b**). Site-specific targeting of DNA plasmids to chromosome 19 using AAV cis and trans sequences. *Methods Mol Biol* 133, 111-26.

Zabner, J., Seiler, M., Walters, R., Kotin, R.M., Fulgeras, W., Davidson, B.L. and Chiorini, J.A. (**2000**). Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *J Virol* 74(8), 3852-8.

Zadori, Z., Szelei, J., Lacoste, M.C., Li, Y., Gariepy, S., Raymond, P., Allaire, M., Nabi, I.R. and Tijssen, P. (**2001**). A viral phospholipase A2 is required for parvovirus infectivity. *Dev Cell* 1(2), 291-302.

Ziady, A.G., Perales, J.C., Ferkol, T., Gerken, T., Beegen, H., Perlmutter, D.H. and Davis, P.B. (**1997**). Gene transfer into hepatoma cell lines via the serpin enzyme complex receptor. *Am J Physiol* 273(2 Pt 1), G545-52.

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J. and Muzyczka, N. (**1999**). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* 6(6), 973-85.

ABBREVIATIONS

aa	amino acid
AAV	adeno-associated virus, specifically adeno-associated virus type 2
AAVS1	AAV integration site 1 (located in human chromosome 19)
Ab	antibody
Ad	adenovirus
AFM	atomic force microscopy

Amino acids:

A (Ala)	alanine
C (Cys)	cysteine
D (Asp)	aspartate
E (Glu)	glutamate
F (Phe)	phenylalanine
G (Gly)	glycine
H (His)	histidine
I (Ile)	isoleucine
K (Lys)	lysine
L (Leu)	leucine
M (Met)	methionine
N (Asn)	asparagine
P (Pro)	proline
Q (Gln)	glutamine
R (Arg)	arginine
S (Ser)	serine
T (Thr)	threonine
V (Val)	valine
W (Trp)	tryptophan
Y (Tyr)	tyrosine

Bases:

A	adenin
C	cytosin
G	guanin
T	thymin

B19	B19 human parvovirus
bp	base pair
BSA	bovine serum albumin
Cap	capsid protein
cDNA	complementary DNA
ch	chromosome
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
CPV	canine parvovirus
CTL	cytotoxic T-lymphocyte
Cy3, Cy5	indocarbocyanine dyes
Da	Dalton
DMEM	Dulbecco's Modified Eagle Medium
e.g.	for example (Lat.: exempli gratia)
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor 1
Fig.	figure
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FPV	feline panleukopenia virus
GFP	green fluorescence protein
Gy	Gray
h	hour
HA	hemagglutinin
HSPG	heparan sulfate proteoglycan
i.e.	that is (Lat.: id est)
i.m.	intra muscular
ITR	inverted terminal repeat
kb	kilobases
K_d	dissociation constant
mAb	monoclonal antibody
MHC	major histocompatibility complex
min	minute
MOI	multiplicity of infection
NPC	nuclear pore complex

nt	nucleotide
ori	origin of replication
ORF	open reading frame
PLL	poly-L-lysine
rAAV	recombinant AAV
RBS	Rep binding site
Rep	viral regulatory protein
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
Stav	streptavidin
Stpl	Streptavidin linked to polylysine
T	triangulation number
TRS	terminal resolution site
VP	viral protein (AAV capsid protein)
wtAAV	wild-type AAV

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Fluent in **English** and **German.** Mother language: **Italian.**

Publications

Perabò L., Buning H., Kofler DM., Ried M., Girod A., Wendtner CM., Enssle J. and Hallek M. (2003) *In vitro selection of viral vectors with modified tropism: the adeno-associated virus display*. ***Molecular Therapy*** (2003) 8(1),151-157.

Wendtner CM, Kofler DM, Theiss HD, Kurzeder C, Buhmann R, Schweighofer C, Perabo L, Danhauser-Riedl S, Baumert J, Hiddemann W, Hallek M, Buning H. (2002) *Efficient gene transfer of CD40 ligand into primary B-CLL cells using recombinant adeno-associated virus (r-AAV) vectors*. ***Blood*** 100(5),1655-61.

Buning H., Ried M.U., Perabo L., Gerner F.M., Huttner N.A., Enssle J. and Hallek M. (2002) *Receptor targeting of adeno associated virus vectors*. ***Gene Therapy*** 10(14),1142.

Huttner N., Girod A., Perabo L.,and Hallek M. (2003) *Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies*. Gene Therapy, submitted.

Patents

Patent pending: TCT/EP02/14750 World Intellectual Property Organization *In vitro Evolution of Adeno Associated Virus: a Combinatorial Library Approach for the Selection of Retargeted Vectors*

Awards

- Excellence in Research Award of the American Society of Gene Therapy (2002)
- 2nd price in the Young Masters Tournament at the German, Swiss and Austrian Society of Hematology and Oncology Meeting (2002)

Oral Presentations at International Meetings

- V Annual Meeting of the American Society of Gene Therapy, Boston, USA (2002)
- IX International Parvovirus Workshop, Bologna, Italy (2002)
- German, Swiss and Austrian Society of Hematology and Oncology, Munich, Germany (2002)
- Vector Targeting Strategies for Gene Therapy, Cold Spring Harbor, USA (2003)

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