Selection and characterization of targeted vector capsids from random adeno-associated virus type 2 (AAV-2) display peptide libraries

Dissertation

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Stefan Michelfelder
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Referenten: Prof. Dr. Marcel Leist
Prof. Dr. Dr. Thomas Hartung
Prof. Dr. Martin Trepel

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1 Summary

Selection of viral vectors by screening viral display peptide libraries is an auspicious approach to improve safety and efficiency of gene vectors. The screening of random AAV peptide libraries occurs *via* the amplification of viruses from a multitude of potential targeting peptides each presented within an AAV capsid that are internalized into target cells, mediated by the peptide displayed on their surface.

The aim of this thesis was the selection of cell type- or tissue-directed gene vectors from random peptide libraries displayed on adeno-associated virus (AAV) and their characterization.

Immature malignant blood progenitor cells causing acute myeloid leukemia (AML) are generally considered to be transduction-resistant to most conventional gene vectors. We screened random AAV serotype 2 peptide libraries on AML cells to select vector capsids with optimized leukemia transduction capacity. The screening revealed a distinct peptide sequence motif displayed on the selected viral capsids. The capsid mutant displaying the peptide NQVGSWS transduced the leukemia cell line Kasumi-1 with up to 90% efficiency, in contrast to vectors displaying a random unselected peptide (0.2% efficiency). Transduction assays on a panel of cell lines showed that the NQVGSWS capsid was able to overcome resistance to AAV-transduction especially in hematopoietic cancer cells. We further showed that NQVGSWS transduction of leukemia cells is independent of the primary attachment receptor heparin sulfate proteoglycan that is used for infection by wild-type AAV-2. Finally, leukemia targeted NQVGSWS-AAV vectors harboring a suicide gene conferred selective killing to Kasumi-1 AML cells. Therefore, we concluded that the selected vector capsids are a suitable and valuable tool to target therapeutic genes to AML cells.

Screening AAV peptide libraries *in vivo* provides much more appropriate conditions to select for tissue-targeted gene vectors than mere cell-based *in vitro* approaches. In the second part of this thesis we developed a PCR-based amplification method allowing for adenovirus independent screening of AAV libraries. We performed *in vivo* selections applying several kinetic approaches in animals over multiple rounds after intravenous administration. The polyoma middle T-transgenic murine breast cancer and murine lung tissue were used as prototype targets. The peptide sequences of AAV clones yielded distinct sequence motifs unique for the target

tissue. Selected capsid mutants conferred gene expression in the target tissue which was not detectable in animals injected with control vectors. However, most of the clones also transduced heart tissue in addition to the target tissue. We therefore conclude that this approach may be particularly useful if the tropism of the intended gene transfer *in vivo* has to be extended to rather than confined to the tissue of interest, indicating that targeting AAV to certain tissues *in vivo* seem to require more than one capsid modification. This impact the further development and improvement of AAV peptide libraries.

Taken together, the work presented here demonstrates that random AAV displayed peptide libraries can be used to select for improved gene delivery vectors *in vitro* and, which is entirely novel, *in vivo*. Our results broaden the knowledge of transduction behavior of vectors isolated from AAV-2 libraries on different targets *in vitro* and *in vivo* and showed that such vectors have the potential to be used for therapeutic gene transfer.

2 Zusammenfassung

Die Selektion viraler Vektoren aus randomisierten, auf Viruskapsiden exprimierten Peptidbanken, ist ein vielversprechender Ansatz zur Steigerung der Sicherheit und Effizienz von Genvektoren. Bei AAV Peptidbanken handelt es sich um eine virale Peptidbank mit einer Diversität von $2x10^8$, bei der jeweils ein randomisiertes Peptid in der Rezeptorbindung vermittelten Region des AAV Kapsids präsentiert wird. Ein Screening dieser Peptidbanken auf Zielzellen ermöglicht eine Anreicherung zielzelltransduzierenden Kapsidvarianten. Ziel dieser Arbeit war die Isolierung und Charakterisierung von zelltyp- bzw. gewebespezifisch transduzierender adeno-assoziiert-viraler (AAV) Vektoren mittels Screening randomisierter AAV Peptidbanken.

Die zur Entstehung der akuten myeloischen Leukämie (AML) führenden malignen Vorläuferzellen der Hämatopoese, sind durch herkömmliche Vektorsysteme nur in geringem Maße transduzierbar. In der vorliegenden Arbeit wurden mittels Screening randomisierter AAV Peptidbanken auf AML-Zellen virale Kapside mit einem eindeutigen Peptidmotif auf der Kapsidoberfläche angereichert. Rekombinante AAV Vektoren der Kapsidvariante NQVGSWS transduzierten die AML-Zelllinien auf denen sie selektiert worden waren mit einer Effizienz von bis zu 90% (Kontrolle mit unselektiertem Peptid ca. 0,2%). Bei Transduktionsexperimenten auf einer Vielzahl verschiedener Zelllinien zeigte sich, dass die Kasidvariante NQVGSWS die Transduktionsresistenz vor allem in hämatopoetischen Tumorzellen überwindet. In weiteren Versuchen wurde gezeigt, dass der Transduktionsmechanismus von NQVGSWS in Leukämiezellen unabhängig vom natürlichen zellulären Rezeptor Heparansulfat-Proteoglykan ist, welche für die Infektion von Wildtyp-AAV des verwendeten Serotyps 2 benötigt wird. Mittels der Kapsidvariante NQVGSWS wurde ein zielgerichteter zytotoxischer Gentherapieansatz auf Leukämiezellen etabliert und durchgeführt. Hieraus folgerten wir, dass die selektierte NQVGSWS-Mutante ein vielversprechender Vektor zum zielgerichteten Einbringen von therapeutischen Genen in Zellen der akuten myeloischen Leukämie darstellt.

Die Selektion von AAV-Peptidbanken *in vivo* ist im Vergleich zu einem rein zell-basierten *in vitro*-Ansatz weitaus geeigneterer zur Selektion Gewebe-gerichteter Vektoren. Im zweiten Teil dieser Arbeit wurde daher ein Verfahren entwickelt welches auf PCR-Amplifikation der Peptidinsertinformation basiert. Dieser Ansatz

ermöglichte eine *in vivo* Selektion nach systemischer Gabe der AAV-Banken über mehrere Runden auf Polyoma Mittel-T-induziertem Brustkrebsgewebe und Lungengewebe als Zielorgane im Mausmodell. Abhängig vom jeweiligen Zielorgan wurden verschiedene Vektorkapside angereichert, welche das entsprechende Zielgewebe nach systemischer Gabe transduzierten. Bei Tieren, die mit Kontrollvektoren injiziert wurden, konnte keine Genexpression in den jeweiligen Zielgeweben nachgewiesen werden. Doch war bei den selektierten Vektoren auch neben dem Zielgewebe eine zusätzliche Transduktion des Herzgewebes detektierbar. Diese Befunde werden einen großen Einfluss auf die Weiterentwicklung von AAV-Peptidbanken haben, da die Notwenigkeit deutlich wird, das AAV-Kapsid zusätzlich zu modifizieren, um eine gewebsspezifische Transduktion *in vivo* zu erreichen.

Zusammenfassend zeigen die in dieser Arbeit geschilderten Ergebnisse, dass AAV-Peptidbanken sowohl *in vitro* als auch *in vivo* dazu geeignet sind, Genvektoren mit verbesserten Transduktionseigenschaften zu selektieren. Diese hier gewonnenen Erkenntnisse erweitern das grundlegende Verständnis für aus AAV-Peptidbanken isolierte Vektoren und zeigen zugleich, dass diese auch potenziell zum therapeutischen Einsatz geeignet sind.

3 Introduction

3.1 Promises and problems in human gene therapy

Gene therapy holds great promise for the treatment of a broad spectrum of inherited and acquired human diseases. The basic concept of gene therapy is the insertion and expression of a functional gene into cells or tissues with the aim either to cure a disease or to stop its progression. Over the last years, about 1350 phase 1-3 clinical trials based on gene delivery have been conducted. Promising results in the treatment of severe combined immune deficiency (SCID) [48], cardiovascular diseases [108, 110] and cancer [84, 127] have generated great hopes in the emerging field of gene therapy, but the agents evaluated in these studies are not yet eligible for broad clinical application.

While the spectrum of potential therapeutic genes rapidly expands with our understanding of molecular mechanisms in cell biology and in the development of diseases, the generation of safe and efficient gene delivery systems remains the biggest challenge in gene therapy [196, 262].

The majority of gene-based clinical trials are cancer related, reflecting the urgent need for novel therapeutic approaches for this disease. Particularly in the treatment of disseminated cancer, targeted gene delivery approaches are mandatory because of their potential to reach malignant cells after systemic application by targeting specific biological features of cancer cells not amenable to conventional therapies. For this purpose, several viral and non-viral gene delivery systems are the subject of intensive basic and translational research [172].

3.1.1 Vectors for gene delivery

The most simple gene delivery systems are based on local administration of either naked DNA or DNA mixed with poly-lysine or cationic lipids. Such non-viral gene delivery approaches offer several advantageous safety aspects including their non-inflammatory, non-toxic and non-infectious properties. Furthermore, they have the capacity to transfer large genes without the risk for unwanted integration into the host genome and are easily amenable to large-scale production. Efforts have been made in the development of ligand-modified carrier systems that allow for tissue-directed gene transfer after intravenous injections in animal models [103, 120, 149]. However, the

use of non-viral vectors for clinical applications is limited by their low transduction efficiency and the tendency to mediate only short term gene expression [263].

Viral gene vectors appear to be more promising for therapeutic gene transfer. Viruses can deliver genes with high efficiency and have the potential to mediate long term gene expression since they have evolved over millions of years to optimally enter a broad range of cells and to transfer their genetic material into their nucleus. The most commonly applied gene vectors are based on retroviruses, adenoviruses, herpes simplex virus and on adeno-associated viruses (AAV) [263], either one of which being used in 70% of all gene-based clinical trials so far [295].

Since each viral vector system offers a specific set of properties, the choice of the vector system depends largely on the therapeutic requirements. Retroviral vectors that stably integrate into the host genome and therefore mediate sustained expression of the affected protein are advantageous for the treatment of genetic disorders (*e.g.* SCID). Adenoviral vectors that confer high but transient expression would be preferred in cancer related therapies [122]. Recently, gendicine, the first commercially available gene therapy drug based on the human p53 as a transgene delivered by an adenoviral vector, has been licensed for the local treatment of several cancers in China [193]. However, the potential risks of unwanted insertional mutagenesis [48] or strong immune reactions against the viral vector [159] reported from clinical trials raised several safety concerns precluding the the broad application of viral gene therapy vectors.

In view of these concerns, vectors derived from adeno-associated virus (AAV) have emerged as a promising tool for a wide field of clinical applications due to their desirable safety and efficiency profile. AAV as a virus is non-pathogenic, only mildly immunogenic, and has the potential to integrate site-specifically into the host genome, while its broad host tropism allows for efficient transduction and long term gene expression in various target tissues [47, 275]. Its properties are introduced in more detail below.

3.2 Adeno-associated virus (AAV)

Adeno-associated virus (AAV) has first been described in 1965 [12] as a contaminant of adenoviral stocks. AAV is a small non-enveloped DNA virus of the genus *dependovirus* that belongs to the *parvoviridae* family. To date, 14 different serotypes

(AAV-1 to AAV-14) isolated from human or primate tissues have been distinguished by their phenotypes and tissue tropisms [40, 79, 80, 169, 224, 287]. From all serotypes described so far, AAV-2 is the best characterized one. A seroprevalence of up to 80 percent [65] reflects a widespread distribution among the human population where AAV infections seem to occur mainly *via* the respiratory or gastrointestinal tract [101]. So far, AAV infection has not been associated with any human disease [23, 298]. Based on this, AAV-2 has been the first serotype used as a vector for experimental and therapeutic gene delivery.

3.2.1 Genomic organization and capsid structure of AAV

The single stranded (ss) AAV-2 genome with a length of 4.7 kb comprises two open reading frames (*rep* and *cap*) flanked by inverted terminal repeats (ITRs) (Figure 1A). ITRs are the only required *cis*-acting regulatory elements for viral genome replication and packaging. Their palindromic GC-rich nucleotide sequence with a length of 145 bases forms a characteristic T-shaped hairpin structure comprising a Rep-binding element (RBE) and a terminal resolution site (trs) flanked by a single stranded segment termed as a D-sequence. In addition to their regulatory functions, ITRs serve as an origin of replication and are essential for genome packaging and site-specific integration [83, 217].

The *rep* gene products Rep78, Rep52 and their respective splice variants Rep68 and Rep40 are under transcriptional control of two promoters (p5 and p19). Rep78 and Rep68 are site-specific DNA binding proteins that exhibit site- and strand- specific endonuclease activity. Rep52 and Rep40 exhibit helicase and ATPase activities [22, 107]. The Rep proteins encode for the regulatory proteins involved in DNA replication, regulation of gene expression, packaging and site-specific integration processes [83] .

The *cap* gene encodes for three structural capsid proteins VP1, VP2 and VP3 (90, 72, 62 kDa) that share the same C-terminal amino acid (aa) sequences, while VP1 und VP2 contain additional N-terminal sequences of 65 and 202 amino acids. Structural proteins are transcribed under the control of the p40 promotor. Alternative splicing at two acceptor site originates two transcripts. The larger transcript encodes for the biggest capsid subunit VP1, the shorter mRNA possesses two initiation start codons (ACG, AUG) that are utilized to translate the capsid subunits VP2 and VP3 (Figure 1A) [83]. Under permissive conditions allowing viral replication, VP1, VP2 and VP3 are expressed at a molar ratio of approximately 1:1:20 [204]. This ratio is also

maintained within the assembled capsid were 60 copies of VP proteins form a T=1 icosahedral capsid structure with 18-30 nm in diameter. VP1 contains a phospholipase 2 (PLA2) domain at its N-terminus necessary for endosomal escape and nuclear entry [32, 82, 237]. The N-terminus of VP1-VP3 contains 4 basic regions (BR) that constitute putative nuclear localization sequences (NLS) involved in the nuclear transfer of the virus (Figure 1B) [86, 87, 265].

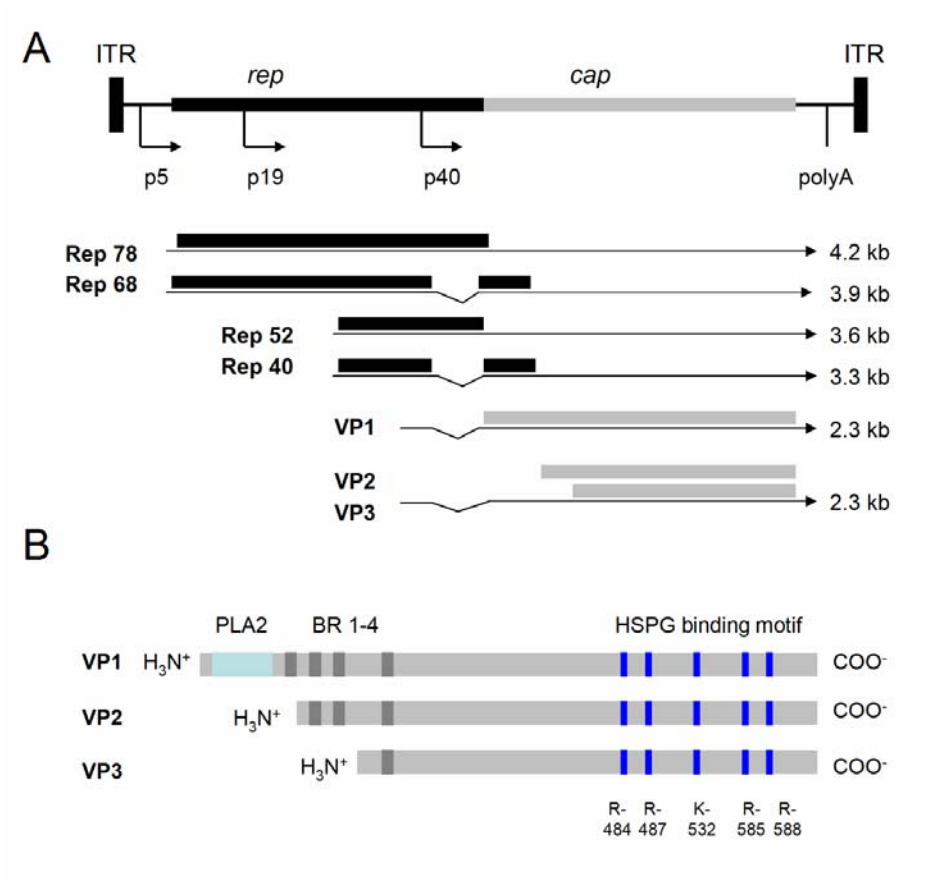

Figure 1: Organization of the genome and the resulting structural proteins VP1-VP3 of AAV-2.
A: The 4.7 kb AAV genome encodes for the 2 open reading frames *rep* and *cap*, flanked by inverted terminal repeats (ITR`s) under control of the three promoters p5, p19, and p40. The generated RNA transcripts are shown as arrows and the ORFs as boxes. The presence of an intron is indicated by the open triangle (modified from Büning *et al.* 2004 [39]). **B:** Schematic depiction of the functional domains of the three structural proteins VP1-VP3. VP1 contains a phospholipase A2-domain (PLA2), the four basic regions (BR1-4) are located at the N–terminus of VP1-VP3. The heparin sulfate proteoglycan binding domain is generated by the basic residues at positions R484, R487, K532, R585, and R588 located at the C-terminus of the VP proteins (modified from Grieger *et al.* 2006 [87]).

The atomic structure of AAV-2 has recently been resolved by X-ray crystallography [292]. The core structure of each VP protein comprises a conserved eight-stranded antiparallel β-barrel motif. Large loop insertions between the β-strands of adjacent VP subunits contribute to the formation of the surface structure. This leads to the formation of characteristic protrusions arranged in groups of three (“threefold spikes”,

formed by GH loops contributed from three capsid subunits) clustering around the threefold axis of symmetry and a cylindrical pore structure clustered around the fivefold axis of symmetry. They are surrounded by characteristic depressions termed as canyon, plateau, and dimple (Figure 2A, B) [124, 154, 186]. Mutagenesis-based approaches and structural data recently led to the identification of several functional sites of the capsid that determine the tropism and antigenicity of the virus. The single or at least one of the primary cellular attachment receptors for AAV-2 is heparin sulfate proteoglycan (HSPG) [245]. The HSPG binding domain on the AAV capsid surface is generated by the basic residues at positions R484, R487, K532, R585, and R588 (VP numbering) presented within two adjacent VP protein subunits forming protrusions on the threefold spike region (Figure 1B, 2C, D) [124, 186, 292]. Binding of negatively charged sulfate and carboxyl groups of HSPG and positively charged amino acid residues occurs mainly *via* electrostatic interactions.

The epitopes of two AAV-2 neutralizing antibodies C37-B and A20 are both mapped to regions adjacent to the threefold proximal-peak. While the monoclonal antibody C37-B inhibits binding of AAV to the host cell, A20 does not block receptor binding but neutralizes AAV infection at a post-binding step, possibly by interfering with internalization, endosomal release or viral uncoating [283]. These findings suggest that the capsid region adjacent to threefold axis of symmetry act as receptor binding site and additionally has other important viral functions.

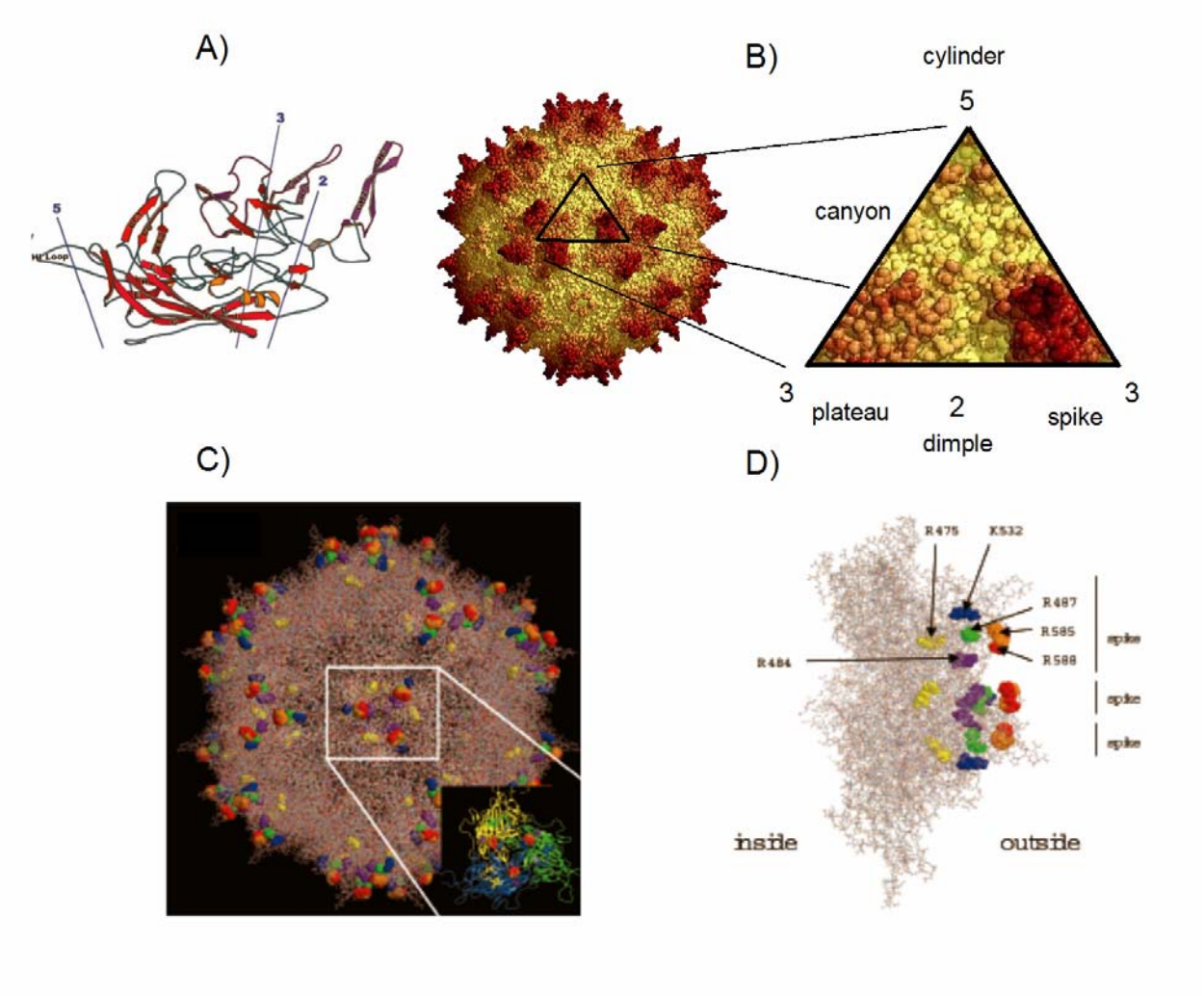

Figure 2: Structure of the AAV-2 capsid.
A) Ribbon drawing of the VP3 protein of AAV-2. The position of the 2-fold, 3-fold, and 5-fold axis of symmetry is indicated as 2, 3, or 5, respectively. Between the strands of the β-barrels core, there are large loop insertions which form the capsid surface (adopted from Xie *et al.*, 2002 [292]) **B)** Surface topology of the AAV capsid. One of the 60 triangular asymmetric subunits shows the surface structure of the capsid forming characteristic threefold spikes clustering around the 3-fold axis and a cylindrical pore structure clustered around the 5-fold axis of symmetry, surrounded by characteristic depressions termed as canyon, plateau, and dimple (image adopted from www.virology.wisc.edu and modified from Lochrie *et al.*, 2006 [154]) **C)** and **D)** Localization of amino acids involved in binding to HSPG on the AAV-2 capsid. The basic residues R484, R487, K532, R585, and R588 cluster at the 3-fold axis of symmetry and are presented at the surface of the capsid (C and D adopted from Kern *et al.*, 2003 [124]).

3.2.2 Replicative cycle

The infection cycle of AAV-2 is initiated by attachment to its primary receptor HSPG which is widely expressed on many cell types and tissues. This might explain the broad host tropism of AAV-2 although it has been shown that HSPG on the host cell membrane is not a prerequisite for AAV-2 infection [36]. For efficient cellular uptake and transduction, HSPG-bound AAV-2 requires further receptors assisting the binding and initiating the internalization process. Integrin αvβ5 [244], integrin α5β1 [11],

fibroplast-growth factor receptor-1 (FGFR1) [202], the hepatocyte growth factor receptor (HGFR1) [118], and the 37/67-kDa laminin receptor (LamR) [1] have been identified as co-receptors for AAV-2 infection. AAV-2 binding to Integrin αvβ5 triggers endocytosis of the virions *via* clathrin-coated pits in a dynamin-dependent process into the early endosome [18, 57]. Integrin binding by AAV activates Rac1, a GTP-binding protein which triggers the phosphatidylinositol-3-kinase pathway that initiates intracellular movement of the endosome to the nucleus along microtubules and microfilaments [219]. Due to a conformation change the VP1/VP2 N-termini of the AAV capsid get exposed at the fivefold cylinder region leading to the activation of the PLA2 domain that triggers endosomal escape of the AAV-2 virion into the cytoplasm. Since endosomal acidification is not sufficient to trigger the conformation change of VP1/VP2, further unknown mediators seem to be involved in this process [139, 237]. The rate of infection is influenced by proteosomal degradation of virions located in the cytoplasm. The ubiquitin-proteasome pathway plays an essential role in this process [57, 60]. Consequently, proteasome inhibitors have been demonstrated to increase the rate of AAV-2 transduction in some cell types and tissues [60]. Recently, several capsid residues have been identified that seem to mediate the proteasomal mediated degradation. Point mutations let to a protection from intracellular ubiquitinylation resulting in increased transduction rates of rAAV-2 vectors [302]. After entry into the cytoplasm, AAV accumulates perinuclearly and translocates into the nucleus *via* the nuclear pore complex (NPC) where finally viral uncoating occurs [237].

When latent, AAV-2 persists either by site-specific integration into the q-arm of chromosome 19 (AAVS1) in a Rep protein-dependent process [137, 218] or as circular extrachromosal episomes [225]. For productive replication, AAV requires helper viral proteins delivered by adenovirus (Ad) or herpes simplex virus (HSV) [176] that enable the rescue of the AAV genome, DNA replication and gene expression of the viral proteins. Capsid assembly takes place in the nucleoli of infected cells that are finally redistributed to the nucleoplasm [105, 282]. There, virions are co-localized with Rep 78/68-tagged viral ss DNA. Rep 52/40 proteins are involved in unwinding and transfer of the viral DNA into the empty capsid through pores located at the fivefold axes of symmetry [31, 128]. Finally, replicated viruses are released within the lysis of the host cell.

3.3 AAV as vector for gene therapy

3.3.1 AAV in clinical trials

AAV vectors have emerged as a safe and efficient and therapeutic gene delivery system for a variety of genetic and acquired diseases. A large number of preclinical studies in animal models revealed promising results ranging from substantial correction to complete cure in hemophilia, α1-anti-trypsin deficiency, cystic fibrosis, Duchenne muscular dystrophy, rheumatoid arthritis and others. Furthermore, AAV has been employed for a variety of anti-cancer gene therapy approaches. Common strategies are based on the delivery of cytotoxic genes, reconstitution of tumor suppressor genes, inhibition of drug resistance, immunotherapy and anti-angiogenesis [198]. So far, at least 40 clinical trails have been approved or completed with AAV-2 based vectors [2, 47, 173, 275].

3.3.2 Production of recombinant AAV vectors

Recombinant AAV (rAAV) vectors are constructed by replacement of the viral DNA containing the two open reading frames *rep* and *cap* flanked by an expression cassette encoding the gene of interest under transcriptional control of a suitable promoter. From the native wild-type virus, only the ITR sequences required for replication and packaging remain. For vector production, the structural and non-structural Rep and Cap proteines can be provided in *trans*. Vectors are usually obtained by transfection of a suitable cell line with three vector plasmids [307] (Figure 3). 1.) The expression cassette flanked by the ITRs 2.) the *rep cap* helper sequences and 3.) the adenoviral helper plasmid that encodes for the adenoviral E2a, E4, VA helper genes [89, 291]. This allows the production of replication deficient, wild-type-free and adenovirus-free rAAV vectors stocks at adequate titers. To enable easy scaling up of vector production and to generate Good Manufacturing Practice (GMP) compliant rAAV vector stocks for clinical or commercial use, novel techniques are under investigation [61, 306]. Such approaches are based on the generation of stably transfected producer cell lines [33, 52], suspension cell transfection and transduction techniques [61, 162, 190] and even cell-free production [304] of rAAV. Innovative purification protocols using iodixanol gradients and heparin affinity chromatography have contributed to making production and purity of stable rAAV vector stocks feasible even on a large scale.

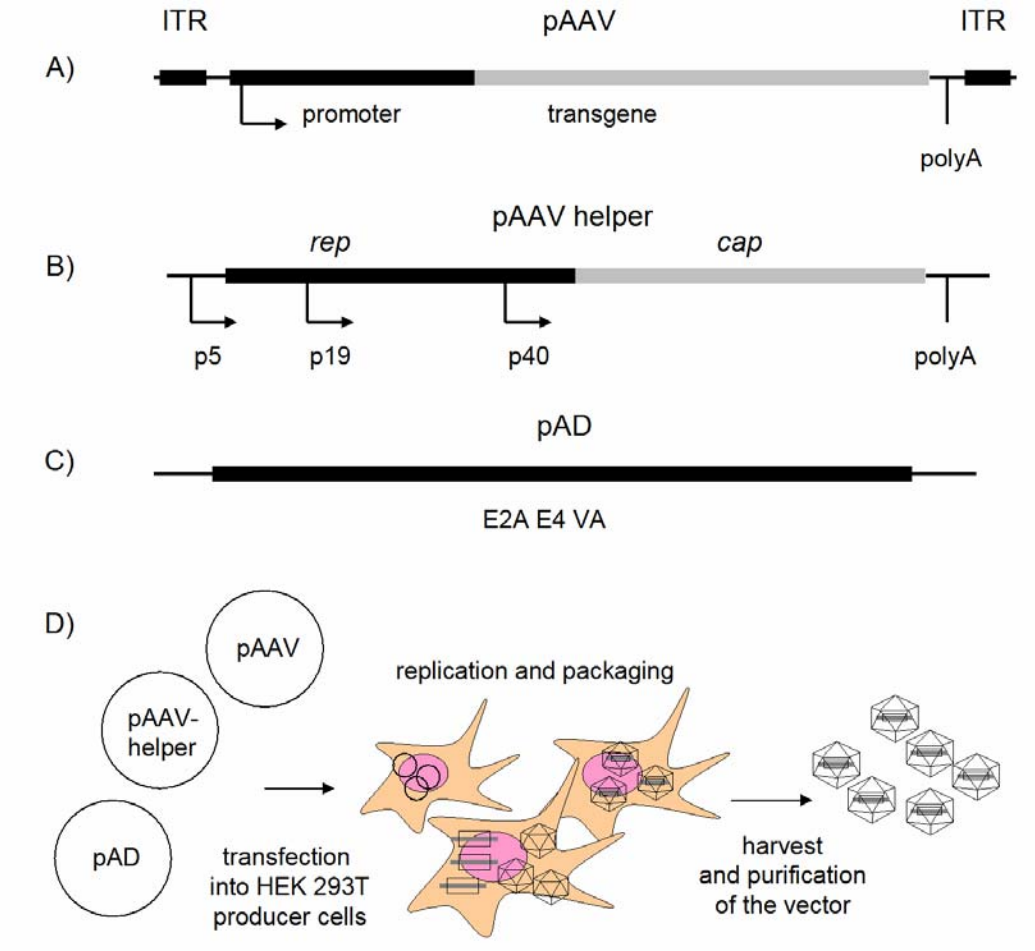

Figure 3: Genomic structure and production of rAAV vectors.
A) AAV vector expression cassette pAAV, containing the ITRs of the wild-type virus, the transgene of interest and its promoter. **B)** the pAAV helper cassette contains the viral *rep* genes required for virus replication and packaging and the *cap* gene encoding for the structural proteins of the virus capsid. **C)** the adenoviral helper plasmid pAD, contains adenoviral helper genes required for virus replication. **D)** For production of rAAV vectors HEK 293T cells are transfected with pAAV vector plasmids, AAV helper plasmids and adenoviral helper plasmids. Vectors are obtained after producer cell lysis and purification using density gradient ultracentrifugation and/or affinity chromatography (A, B, C and D modified from Merten *et al.*, 2005 [164]).

3.3.3 Site-specific integration

One challenge for human gene therapy is to generate vectors that integrate at a certain site of the genome because random integration can contribute to the development of secondary cancers by insertional mutagenesis. In this regard, it is of considerable interest that rAAV vectors have the potential for site-specific integration into the host genome [163, 296]. Since rAAV vectors lack its parental *AAV rep* gene they have lost their ability of targeted integration and vector genomes mainly persist as episomal entities within the transduced cells. However, since the ITRs are still present in rAAV vectors, integration yet occurs, but at a low rate and at apparently

random sites. Levels of random integration are comparable to spontaneous mutations in the human genome, thus the risk of unwanted insertional mutagenesis is rare [47]. One novel approach aims at restoring the site-specific integration capability of rAAV vectors by providing *rep* in *trans* [145]. A large study in adult mice revealed no evidence for tumorgenesis after vector administration [21]. This issue has been discussed controversially since AAV vectors led to an elevated frequency of hepatocellular carcinomas in neonatal mice due to random vector integration [59, 121].

3.3.4 AAV and immune responses

Humoral and cellular immune responses against a viral gene vector can limit sustained gene expression or vector re-administration. In particular, strong inflammatory responses can evoke life-threatening complications in patients.

AAV vectors do not contain viral genes that elicit substantial cellular immune responses and generally appear to induce only mild inflammatory processes in the host organism [298]. Inefficient transduction of professional antigen presenting cells (APC) *in vivo* seems to prevent the induction of cellular T- cell responses [99], although the generation of cytotoxic T-cells *via* MHC-I class molecules and cross presentation pathways has been observed [50, 270]. The bigger challenge remains to minimize humoral immunity that occurs against AAV capsids. About 18-67.5 % of human sera contain pre-existing neutralizing antibodies that could markedly reduce or impair AAV-2 mediated gene transduction [65, 220]. The use of vectors originating from different serotypes or with modified AAV capsid epitopes may have the potential to escape pre-existing humoral responses [81, 170, 247, 283, 298, 106].

3.3.5 Limited packing capacity and rate-limited transduction

For some genetic disorders where the delivery of large transgene expression cassettes is essential, the packing capacity of AAV vectors remains a major obstacle. The coding capacity for AAV vectors is restricted to approximately 4.5 kb pairs [58]. The size of the transgene has been increased by the development of so-called trans-splicing gene vectors. This approach takes advantage of the property of AAV genomes to form concatemers by head-to-tail recombination, thus allowing the reconstitution of a functional gene delivered by two vectors into the same target cell, as demonstrated by successful gene transfer to the retina [207] and into human cystic

fibrosis lung epithelia [153]. Another strategy to minimize the size of the transgene is the use of minigenes that code only for essential regions of a protein of interest. Gene delivery of a truncated version of the dystrophin gene by AAV vectors led to restoration of the muscle contractile property in mdx mice [294].

AAV vector transduction is characterized by a delayed onset of gene expression that limits its use for acute clinical applications. Addicted to the host cell DNA synthesis machinery, second strand synthesis in the host nucleus has been shown to be the major rate-limiting step in AAV vector transduction [71, 303]. Self-complementary AAV (scAAV) vectors have the potential to counteract this rate-limiting step. ScAAV are generated by deletion of the D-sequence or mutation of the trs sequence of one ITR leading to a high percentage of self-complementary vectors. Independent of the need for *de novo* DNA synthesis or annealing of sense and antisense strands, dimeric AAV transgenes allow for rapid and increased expression of the transgene in several tissues *in vivo*, although the packing capacity of scAAV vectors is reduced to half of the size of conventional vectors [161, 208, 272].

3.3.6 Host tropism

AAV vectors have the capacity to deliver genes to a broad spectrum of dividing and non- dividing cell types and tissues *in vitro* and *in vivo*. Efficient and long term gene transfer has been demonstrated in skeletal muscle fibers [10, 74, 100, 290], cardiac tissue [189, 261], airway epithelial cells [75], hepatocytes [236, 288] brain [17, 115, 289] and several cancer cell lines [92]. On the other hand, some preferable gene delivery target cells are only moderately or not permissive for AAV-2 transduction, including embryonic stem cells [234], hematopoietic cells [16, 194, 199, 200, 210], and endothelial cells [227]. However, to obtain adequate gene transduction in the tissue of interest, the broad host tropism of AAV would require high vector doses if used for systemic gene therapy. This would result in undesirable transduction of nontarget tissues increased toxicity and immune-mediated side effects.

If applied systemically, AAV-2 mainly transduce the liver, but also additional tissues [305]. Thus vector application is limited to administration of the vector to a defined cell type *ex vivo* or for local administration. Vector targeting can be performed at two levels. Transcriptional targeting has been attained by the use of tissue-specific promoters [175, 271]. However, specific promoters that generate adequate expression levels are not available for particular cell types and do not allow for gene transduction

in cells that are non-permissive for AAV infection. Alternative approaches aim to modify the vector's capsid structure to generate receptor targeted vectors.

3.4 Targeting AAV vectors to certain cell types

The receptor diversity among different cell types, tissues and malignant cancer cells offers the potential to specifically target a cell type or tissue of interest. Such targeted delivery could highly improve the clinical benefit of therapeutic compounds by preventing their action in non-target tissues, thereby increasing therapeutic efficiency while diminishing adverse effects. The principle of receptor-targeted therapy has been exploited in several clinical applications and some have recently begun to prove their value. Moreover, therapeutic antibodies or small molecule ligands have the potential to block carcinogenesis and cell proliferation or even to specifically kill target cells if conjugated to cytotoxic agents.

While viral vectors are most promising candidates for targeted gene therapy, targeting viral vectors *in vivo* faces several challenges that have not been overcome yet. These include the capacity of a vector to ensure sufficiently strong receptor-ligand interaction under circulation conditions *in vivo*, the ability to escape clearance by the host immune system and the reticuloendothelial system, and to overcome physical barriers as the endothelial cell layer and the extracellular matrix [279].

To generate targeted AAV vectors, several attempts aim to modify the capsid surface to improve interaction with cell type-specific surface molecules that would allow for efficient and specific gene delivery.

3.4.1 Exploration of AAV serotypes, pseudotyping and mosaic capsids

One opportunity to expand the tropism of AAV-2 is the exploitation of the variety of serotypes that differ in their transduction efficiencies for several tissues and cell types [40, 287, 305]. Several studies have verified that AAV-1 is the most appropriate serotype for muscle cell transduction, AAV-8 for liver and AAV-9 for cardiac transduction [40]. Furthermore, pseudotyping AAV vectors by cross-packaging of an AAV genome into the capsid of another serotype could improve the *in vivo* transduction of certain tissues while circumventing problems of pre-existing immunity [140]. Although the isolation of novel serotypes enables vector delivery to otherwise refractory cell types, the number of vector serotypes is far lower than their potential target tissues. An

alternative approach is the generation of mosaic vectors from a mixture of different capsid subunits. This yielded vectors that combined the beneficial features of the originating vector capsids. Such vectors allow easy vector purification and have been shown to efficiently transduce muscle and liver [96] or vascular tissue [238]. In addition, unexpected synergistic transduction effects on various cell lines were observed when AAV-1 subunits were mixed with AAV-2 or AAV-3, these transduction effects suggested a potential approach to generate vectors with novel tropisms [203]. However, major drawbacks are the pre-existing antibodies against one of the parental serotypes and the difficulty to reproduce the exact stoichiometry of the generated capsid proteins in large scale vector production [140]. In addition, like all serotype-based targeted vectors, mosaic vectors do not seem to be capable of cell-type specific transduction.

3.4.2 Ligand directed receptor targeting

Several approaches aim to generate AAV vectors that display selective binding domains that enable a stringent interaction with specific target cell receptors. For this purpose, vectors have been modified basically in two ways, *i.e.* for indirect and direct targeting.

Indirect targeting of AAV vectors is achieved by conjugating receptor-binding ligands to the capsids. Using bi-specific $F(ab´\gamma)_2$ antibodies that are subsequently linked to the capsid, AAV vectors have been successfully retargeted to $\alpha_{IIb}\beta3$-expressing megakaryocytic cell lines [16]. Another approach used avidin-linked epidermal growth factor (EGF) or fibroblast growth factor (FGF) fusion proteins conjugated to biotinylated AAV capsids to transduce human ovarian cancer and megakaryocytic cell lines [199]. Although the use of conjugated ligands offers a high degree of versatility, such conjugates may increase immunogenicity, reduce infectivity, and lack of stability *in vivo*.

In the direct targeting approach, cell-specific targeting of the vector is mediated by a ligand coding sequence that is inserted into the VP capsid gene and presented within the viral capsid surface. By insertion of a 14 amino acid peptide containing an RGD-containing, integrin-binding domain at position 587, the first successful transductional retargeting of AAV vectors to $\alpha_v\beta5$ integrin expressing cells was demonstrated [81]. Since then, several sites in the AAV capsid have been identified that can tolerate the incorporation of even large peptides that may be designed to expand the tropism of

AAV-2 vectors [155, 157, 210, 228, 230, 274, 286]. However, among the tolerated insertion sites for ligands that have been investigated, the most promising is the region at amino acid positions 587/588 for several reasons. First, structural modelling revealed that a sequence inserted at this position into the *cap* gene is presented 60 times on the viral surface on the side of the peak at the threefold axis of symmetry. Peptides inserted at this position seem to be accessible for efficient receptor ligand interaction. Second, to generate selective and efficient retargeting of a vector, the binding to its natural receptor has to be eliminated. It has been shown that inserting peptides at positions adjacent to 585/588 interferes with the heparin binding motif composed of the five basic residues (at position 484, 487, 532, 585, 588) and therefore potentially abrogates the natural HSPG binding of AAV-2 capsids. This leads to a detargeting from the liver if vectors are applied systemically *in vivo* [124]. Third, AAV vectors modified at position 587 have the potential to escape the neutralizing effects of human antibodies with regard to their transduction efficiency without losing their ability to infect cells *via* the targeted receptors [106].

The design of the targeting peptide to generate targeted vectors is not an easy task. The use of phage display libraries allowing for the identification of targeted peptide ligands even without prior knowledge of their receptors has been a significant step forward in this field. For tissue targeting in particular, major advances have been made by the exploration of organ-specific “address molecules” expressed on endothelial surfaces by *in vivo* phage display [5, 93, 216, 253, 254]. Several peptide ligands have been identified for a variety of tissues and have subsequently been used for delivery of cytotoxic drugs or other therapeutic agents in relevant preclinical models *in vivo* [4, 6, 7, 134]. By incorporation of peptide ligands selected by phage display into the AAV capsid, AAV has been successfully retargeted to various tumor cell lines [228], CD13 expressing cells [88], endothelial cells [182] *in vitro* and to the vasculature in general [278] as well as vascular beds of lung and brain [284] and atherosclerotic lesions [277] *in vivo*. However, despite some success in this regard, the targeting capacity of ligands isolated in the structural context of phage display may suffer from a reduction of receptor-ligand affinity when incorporated into the AAV capsid. Furthermore, peptides isolated by phage display screenings are commonly selected only for cell binding and not for cellular internalization and subsequent gene transfer.

3.4.3 Random AAV display peptide libraries

Taking these limitations of conventional vector targeting into account, a ligand screening system based on the gene vector itself has recently been developed and validated [42, 174, 194]. Such libraries designated as random AAV display peptide libraries allow the isolation of targeted AAV vector capsids from a multitude of potential targeting motifs each presented within an AAV capsid (Figure 4).

AAV libraries are produced by cloning of a random oligonucleotide sequence encoding for a few random (*e.g.* seven) amino acids into the AAV *cap* gene at position N587 [194] or R588 [174] to generate a plasmid library. The virus library is obtained either by direct transfection of AAV producer cells with the library plasmids [194], or by using a three step strategy. For the latter, the random plasmid library and an ITR-less plasmid encoding for the wild-type *cap* gene are co-transfected to produce AAV library transfer shuttles carrying chimeric capsids containing wild-type and library subunits. These transfer shuttles are subsequently used to infect wild-type permissive AAV producer cells at the lowest possible MOI to generate the final viral library with a diversity of up to 10^8 different viral library particles [174]. This intermediate methodological step enables the production of AAV libraries that ensures the encoding of displayed peptides by the packaged AAV genome. Since each producer cell infected by a library shuttle can generate thousands of library particles, this technique furthermore allows the production of viral libraries titers comparable to rAAV vector stocks that would be mandoratory for *in vivo* selections. Due to homologous recombination processes, the final virus display library also contains contaminations with wild-type AAV that may reduce the diversity of the library and might interfere with the selection process on cell types or tissues partially susceptible to wild-type AAV2 infection. Waterkamp *et al.* further improved the quality of AAV libraries by using a novel constructed synthetic helper *cap* gene that allows for the production of entirely wild-type-free AAV random peptide display libraries [276].

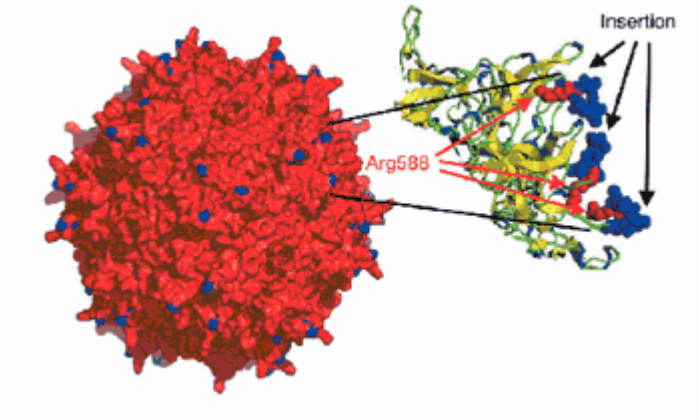

Figure 4: Principle of random AAV displayed peptide libraries.
An oligonucleotide encoding a peptide with random amino acids is cloned into the AAV *cap* gene and is presented 60 times within the surface-exposed GH-loop of VP proteins at position R588 (blue) at the top of each of the threefold spikes on the capsid surface. The natural tropism of the virus is abrogated and retargeted to alternative cellular receptors mediated by the peptide expressed on the capsid surface (adopted from Mueller *et al*. 2003 [174]).

The screening of AAV peptide libraries occurs *via* the amplification of viruses that are taken up by target cells, mediated by the peptide displayed on their surface. Amplification of library viruses in the target cells is initiated by adenoviral co-infection (Figure 5). So far, capsid mutants displaying striking peptide motifs have been isolated from human coronary artery endothelial cells [174], human megakaryocytic and chronic lymphocytic leukemia (B-CLL) cell lines [194], lung carcinoma, prostate cancer and rat cardiomyoblasts [276] by *in vitro* biopanning of AAV display libraries. Vectors displaying the selected peptide insert have been show to efficiently and selectively transduce the cell type they were selected on.

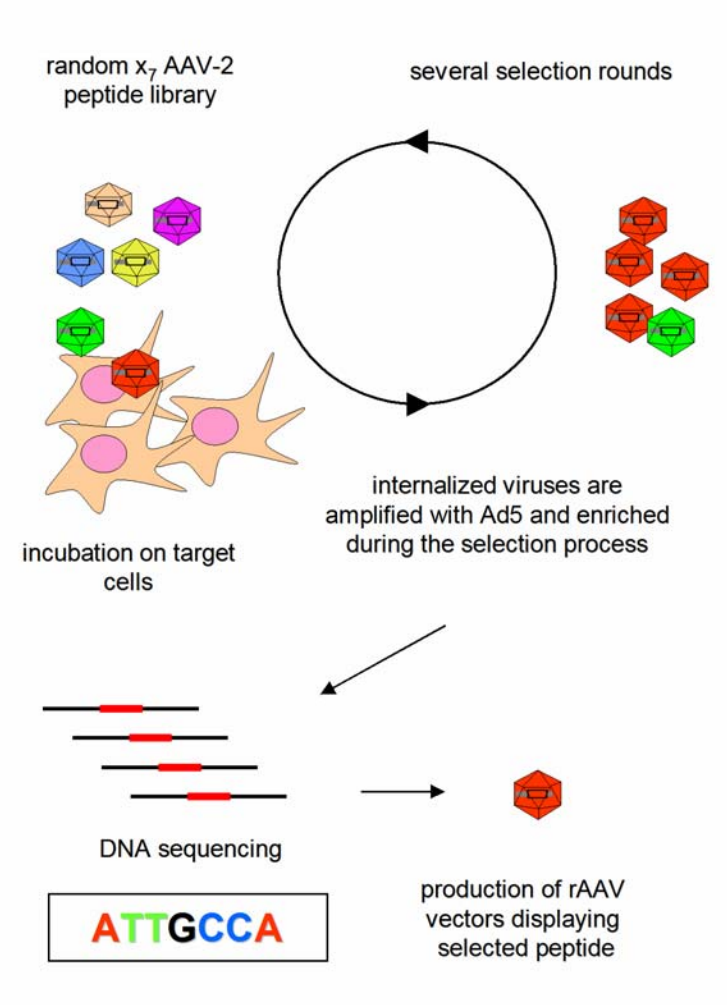

Figure 5: Principle of the adenovirus-type 5 (Ad5)-based selection using random AAV peptide libraries.
A random AAV peptide library is incubated on the target cells. Bound and internalized library viruses are subsequently amplified by superinfection with Ad5 and used for further rounds of selections to enrich cell type-directed virus capsids. Enriched peptide insertions are analyzed by DNA sequencing of recovered clones and rAAV vectors displaying selected peptide sequences can be produced.

3.5 Aim of the studies

Targeted gene therapy is of particular interest as a treatment for a variety of entities, especially for disseminated cancer or other diseases that are not accessible by surgery or refractory to conventional therapies. An unsolved and yet crucial issue in this field is the lack of safe and efficient gene delivery systems that specifically transduce their target cells after systemic application.

Vectors based on AAV-2 are a promising tool for therapeutic gene delivery since they meet several criteria in terms of safety and efficiency, but their tropism is unspecific. Screening AAV peptide libraries is a potential approach to select for targeted AAV vectors.

Acute myeloid leukemia (AML) cells are particularly resistant to wild-type AAV transduction. The aim of the first part of this thesis was the selection of AAV capsids from random AAV display peptide libraries that enable for efficient targeted transduction of AML cells. Further steps were to characterize the isolated clones with a focus on transduction efficiency, specificity, and binding properties. A targeted cytotoxic gene transfer on AML cells using a previously selected capsid mutant was to be established.

Screening AAV libraries *in vivo* may select for improved gene delivery vector capsids that target the tissue of interest under physiological conditions. The second part of this thesis was therefore to establish an adenovirus-free selection protocol and to perform *in vivo* selections of random AAV display peptide libraries to select for tissue-targeted vector capsids. As potential target tissues for *in vivo* screening, breast cancer tissue in a polyoma middle T (PymT) transgenic mouse model and lung tissue derived from wild-type mice were to be used. The final aim was the characterization of isolated AAV clones.

4 Vectors selected from adeno-associated viral display peptide libraries for leukemia cell-targeted cytotoxic gene therapy

Stefan Michelfelder[1]*, Mi-Kyung Lee[1]*, Elisete DeLima-Hahn[1], Thomas Wilmes[1], Felix Kaul[1], Oliver Müller[3], Jürgen A. Kleinschmidt[3], Martin Trepel[1, 2]

University of Freiburg Medical Center, [1] Dept. of Hematology and Oncology and [2] Institute for Molecular Medicine and Cell Research, Hugstetter Str. 55, D-79106 Freiburg, Germany

[3] Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

* these authors contributed equally to this work

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4.1 Abstract

For acute myeloid leukemia (AML), gene therapy may be used to treat patients refractory to conventional chemotherapy. However, availability of vectors sufficiently and specifically transducing this cell type is very limited. Here we report the selection of capsid modified adeno-associated viral (AAV) vectors targeting Kasumi-1 acute myeloid leukemia cells by screening random AAV displayed peptide libraries. The peptide inserts of the enriched capsid mutants share a common sequence motif. The same motif was selected in an independent library screening on HL 60 AML cells. Recombinant targeted vectors displaying the selected peptides transduced the target leukemia cells they have been selected on up to 500-fold more efficient compared to AAV vectors with control peptide inserts. One of the selected clones (NQVGSWS) also efficiently transduced all members of a panel of four other AML cell lines. Binding and blocking experiments showed that NQVGSWS binding to leukemia cells is independent of the wild-type AAV-2 receptor heparin sulfate proteoglycan. Transduction assays on a panel of hematopoietic and non-hematopoietic cell lines showed that the NQVGSWS capsid was able to overcome resistance to AAV-transduction especially in hematopoietic cancer cells, whereas normal peripheral blood mononuclear cells and CD34+ hematopoietic progenitor cells were not transduced. Consequently, recombinant targeted NQVGSWS AAV vectors harboring a suicide gene conferred selective killing to Kasumi cells but not to control cells. This suggests that the AAV mutant selected here may be used as a tool to target therapeutic genes to acute myeloid leukemia cells.

4.2 Introduction

The majority of patients with acute myeloid leukemia (AML) will die of their disease. Less than 40% of the patients diagnosed with AML younger than 60 years of age can be cured [242]. In the elderly, disease-free survival is rare and the available treatment options are limited [242]. The advances in our understanding of the pathophysiology of acute myeloid leukemia have not yet translated into substantial improvements of survival of patients with this dismal disease.

Gene therapy may be a valuable tool to treat AML refractory to chemotherapy as it has the potential to target specific biological features of cancer cells not being amenable to conventional chemotherapy. However, vectors that sufficiently and

specifically transduce this cell type are scarce. In fact, hematopoietic cells are considered particularly resistant to transduction compared to other cell types [16, 194, 199, 200, 210]. In turn, conventional viral and non-viral gene therapy vectors being able to transduce such cells usually do so unspecifically. Therefore, safety and efficacy of human gene therapy continue to be a subject of debate [14, 20, 41, 177, 183, 253]. Problems of current vectors include unintended transduction of certain tissues, adverse immune reactions, and lack of efficient transduction of the tissue of interest [41, 177, 256]. Ablating the endogenous unspecific tropism of the vector and retargeting it to a specific tissue may overcome many of these safety and efficacy concerns.

Recombinant adeno-associated virus vectors are promising because of their ability to mediate stable and efficient gene expression with a favourable biological safety profile [168, 249]. Targeting AAV-2 vectors to alternative receptors can be achieved by insertion of specific peptide ligands into certain sites of the AAV capsid [41, 81, 88, 155, 174, 177, 228, 229, 231, 253, 278, 285]. Several sites in the AAV capsid were identified to be amenable to manipulation and incorporation of peptides but only a few of them have been systematically evaluated for insertion of targeting ligands to alter the cellular tropsim of AAV-2 [155, 205, 228, 230, 286]. While the unique VP1 and VP2 regions of the AAV capsid proteins seem to be suitable to express and display even large incorporated targeting peptides on the vector surface [228, 274], the majority of publications address sites in the VP3 protein of AAV-2 for targeting purposes. Girod *et al.* described three sites in the AAV-2 capsid at which an inserted integrin targeting peptide was exposed on the capsid surface [81]. One of these, adjacent to an arginin at amino acid position 588 (R588), showed preferential transduction of integrin-expressing [81] or CD13-expressing cells [88]. This capsid site has since been the most often used site to insert targeting ligands, leading to targeted transduction of various cell types such as endothelial cells or certain other cell lines [88, 182, 228, 278, 284, 285], particularly since peptide insertions at this site diminish heparin binding of the mutant particles and therefore abrogate their natural tropism. In fact, R588 has recently been shown to be one of four arginines that mediate attachment of AAV-2 to its natural receptor [124, 186]. Inserting peptides adjacent to R588 most likely interferes with this heparin binding motif and therefore abrogates but not fully eliminates the natural tropism of AAV-2 capsids. This allows for detargeting of the AAV-2 from the liver and retargeting to alternative tissues *in vivo* [278, 284]

Based on this work, we [174] and others [194] have developed a novel vector capsid screening system based on random peptide libraries displayed on adeno-associated virus (AAV) type 2. Such libraries consist of viruses that display a potential targeting peptide with random sequence in a capsid region mediating virus binding to cellular receptors. Targeted AAV specifically transducing the cell type of interest can be selected from such libraries [174, 194, 195, 276].

Here we used an AAV library to select vectors targeted to AML cells. The library was screened on Kasumi-1 AML cells and enrichment of a distinct peptide motif was observed. Vectors displaying one of the enriched peptides transduced also AML cell lines other than the one used for selection. This leukemia targeting effect is independent of heparin-sulfate proteoglycan. Suicide gene therapy vectors encoding for the herpes simplex virus thymidine kinase gene packaged in the selected leukemia targeting capsids specifically kill the target leukemia cells upon treatment with the prodrug gancyclovir.

This is the first report of selection and validation of AML-targeted AAV from a random vector display system and the use of such targeted vectors for cell type-directed cytotoxic gene therapy. This may have broad implications for the development of targeted vectors as a novel treatment option in this devastating disease.

4.3 Materials and Methods

4.3.1 Cell culture, transfection, virus production and titering

Kasumi-1, U937, HL60, SKNO, K562, KG1a, NB4 myeloid leukemia cells were a gift from Michael Lübbert, University of Freiburg Medical Center. SiHa and U-2 OS cells were obtained from Jens Hasskarl, University of Freiburg Medical Center. L1236, LCL-GK, KMH-2, cells were a gift from Ursula Kapp, University of Freiburg Medical Center. LNCAP-C42 were obtained from Ursula Elsässer-Beile, University of Freiburg Medical Center. RPMI 8226 und Jurkat cells were obtained from ATCC (Manassas, IN). 293T cells were used with kind permission of Dr. David Baltimore, California Institute of Technology, Pasadena, California. Cells growing in suspension or semi-adherent cells (Kasumi-1, SKNO-1, U937, HL60, K562, L1236, RPMI 8226, LCL-GK, Jurkat, KMH-2, NB4, KG1a, LNCAP-C42) and adherent cells (293T, U-2 OS, SiHa) were maintained in the appropriate media (DMEM for adherent or RPMI for suspension cells) containing 1% penicillin/streptomycin and 10% fetal calf serum

(20% for Kasumi-1 cells). Primary human peripheral blood mononuclear cells (PBMC) were obtained from voluntary healthy donors and isolated by ficoll density gradient centrifugation (LSM1077 Lymphocyte separation medium, PAA, Pasching, Austria). Primary CD34+ hematopoietic cells were isolated from leukapheresis material of a patient with Ewing's sarcoma in remission by using the CD34+ Progenitor Cell Isolation Kit (Miltenyi Biotec, Germany) following to manufacturer's instructions (the patient gave informed consent to use this material). Primary cells were cultivated in RPMI containing 1% penicillin/streptomycin and 10% fetal calf serum. Transfections were performed by calcium phosphate precipitation [97] or PolyFect Transfection Reagent (Qiagen, Hilden, Germany). For production of AAV, 293T cells were transfected with the pSub201 plasmid [217], or its mutant derivatives, respectively, along with pXX6 [291] containing the adenovirus helper functions. After 72 h cells were harvested and viruses were purified by using iodixanol gradient ultracentrifugation [97]. Wild-type adenovirus type 5 (Ad5, generously supplied by the Laboratoire de Thérapie Génique, Nantes, France) was used for library particle amplification and inactivated at 55 °C for 30 min after harvest of the cell lysate. The AAV capsid, and replicative titers were determined as described [89]. The genomic titer was determined by quantitative PCR (SYBR Green, MyiQ apparatus, Biorad, Munich, Germany), using the primers 5'-GGCGGAGTTGTTACGACAT-3' and 5'-GGGACTTTCCTACTTGGCA-3' [212] using vector plasmid DNA as a standard.

4.3.2 Heparin binding

$5x10^9$ AAV capsid particles in PBS containing 1 mM $MgCl_2$ and 2.5 mM KCl (PBS-MK) were bound to 1 ml heparin agarose (Sigma; St. Louis, MO), washed twice with 5 ml PBS-MK, and eluted with 4 mL PBS containing 1 M NaCl. Fractions were collected and analyzed with the A20-enzyme-linked immuno assay (ELISA) [89].

4.3.3 AAV library production

The random X_7 AAV display peptide library was produced as described previously [174]. Briefly, the degenerate oligonucleotide encoding the random seven residue peptide insert at position 3967 in the AAV genome was synthesized as follows (University of Freiburg Oligonucleotide Synthesis Core Facility): 5'-CAGTCGGCCAGAGAGGC$(NNK)_7$GCCCAGGCGGCTGACGAG-3'. The second

strand was added by using sequenase (Amersham; Freiburg, Germany) and the second strand primer 5'-CTCGTCAGCCGCCTGG-3'. The double stranded insert was purified using the QIAquick Nucleotide Removal Kit (Qiagen; Hilden, Germany). The 15 bp stuffer within pMT187-0-3 was cleaved by *Sfi*I digestion and plasmid and insert were ligated at a 1:15 molar ratio. Ligated plasmids were transformed into electrocompetent *DH5α* bacteria using the Gene Pulser (Biorad; Munich, Germany). Plasmid library diversity was determined by the number of clones growing from a representative aliquot of the transformed bacteria on agar plates containing 150 µg/mL ampicillin ($1x10^8$ clones). Transformed bacteria were grown to saturation and the library plasmids were purified using Qiagen's Plasmid Preparation Kit. The AAV display peptide library was made from plasmids in a two-step system as described [174]. First, the AAV library genomes were packaged into chimeric wild-type and mutant AAV capsids ("AAV library transfer shuttles"). Therefore, $2.2x10^8$ 293T cells were transfected using a 1:1:2 ratio of the pXX2 plasmid (containing the wild-type *cap* gene without ITRs) [291] and the library plasmids along with the pXX6 helper plasmid [291]. The resulting AAV library transfer shuttles were harvested, purified and titered. The random AAV display peptide library was obtained by infection of 293T cells with the AAV library transfer shuttles at an MOI of 0.5 replicative units per cell and superinfection with Ad5 at an MOI of 7 plaque-forming units (pfu)/cell. The AAV library was harvested from the supernatant after 48 h, corresponding to approx. 50% cytopathic effect. The supernatant was concentrated using VivaSpin columns (Viva Science, Hannover, Germany) and the library viruses were purified using iodixanol gradient ultracentrifugation as described [174].

4.3.4 AAV peptide library biopanning

$1.5x10^7$ Kasumi-1 cells or $1.5x10^6$ HL60 cells, respectively, were infected with the AAV display peptide library at an MOI of 100 capsids/cell (Kasumi -1 cells) or 1000 capsids/cell (HL60 cells), respectively. After 5 h, cells were washed with PBS followed by incubation with Ad5 at an MOI of 100 pfu/cell (Kasumi-1 cells) or 500 pfu/cell (HL60 cells), respectively. Replicated AAV particles were harvested from supernatant and from cell lysates (obtained by 3 freeze-thaw cycles) after 48 h. For each subsequent selection round, 90% of the preselected AAV library particles recovered from the preceding selection round were reapplied to the target cells.

4.3.5 PCR and sequencing of AAV library clones

DNA extracted by the QIAamp Tissue Kit (Qiagen) from harvested cells containing internalized AAV served as template for a PCR using the primers 5´-GGTTCTCATCTTTGGGAAGCAAG-3´ and 5´-TGATGAGAATCTGTGGAGGAG-3´. PCR products were analyzed by gel electrophoresis, digested with *Bgl*I and cloned into the *Sfi*I-digested pMT187-0-3 plasmid [174]. Randomly assigned clones were sequenced using the reverse primer 5´-CAGATGGGCCCCTGAAGGTA-3´.

4.3.6 Production of capsid-modified rAAV

The pXX2-187 plasmid is an AAV *rep-cap* construct containing the library cloning site required for peptide insertions in the capsid amino acid position R588 and lacking the flanking inverted terminal repeats. The pXX2-187 construct was cloned as follows: pXX2 [148] and pMT187-0-3 [174] were each digested with *Xba*I, releasing the *rep-cap* cassette from both plasmids. The *rep-cap* cassette from pXX2 was discarded and replaced by the *rep-cap* cassette derived from pMT187-0-3 containing the *Sfi*I restriction site required for oligonucleotide cloning. To obtain rAAV-vectors carrying reporter genes, the cap gene region encoding the peptide insert was amplified by PCR as described for sequencing. The PCR product was digested with *Bgl*I and cloned into pXX2-187. 293T cells were co-transfected with the modified pXX2-187 or pXX2 for wild-type, respectively, pXX6 and the pUF2-GFP[307] derivative pTRUF-CMV-eGFP or pUF2-CMV-luc [276] or the HSV-tymidine kinase mutant SR39 [94], respectively, as described above.

4.3.7 Flow cytometric analysis of gene transduction

To analyze gene transduction by AAV vectors harboring the gene encoding enhanced green fluorescent protein (GFP), 10^4 cells per well of each indicated cell type were seeded in 24-well plates and incubated with AAV GFP vectors at an MOI of 7500 capsids/cell. After three days, cells were harvested and GFP reporter gene expression was determined by FACS analysis (FACS Calibur, BD Biosciences, Heidelberg, Germany). CD34+ hematopoietic progenitor cells were stained with CD34-PE (BD Biosciences) and CD4-APC (DacoCytomation, Denmark) labelled antibodies. In co-cultivation experiments, populations of Kasumi-1 cells and CD34+ hematopoietic progenitor cells were gated in the CD34 and CD4 plot and the amount

of GFP positive cells was analyzed. Isotype controls were IGg1κ (BD Biosciences, Germany).

4.3.8 Luciferase gene transduction

To analyze luciferase gene transduction, $5x10^3$ Kasumi-1 cells per well were seeded in 96-well plates and incubated with AAV-luciferase vectors at an MOI of 7500 capsids/cell. After three days, cells were harvested and reporter gene activity was determined using the firefly luciferase assay (Promega, Mannheim, Germany) according to the manufacturer's instructions.

4.3.9 Heparin competition assay

$7.5x10^7$ rAAV-vector capsids carrying the GFP reporter gene were incubated for 45 min with 0, 5, 50, 150, 500 µg/mL heparin sodium salt (Sigma) in medium containing supplements. 10^4 cells per well were incubated with the AAV-heparin mix. After 12 h, heparin and AAV vectors were removed, cells were washed with PBS and fresh medium was added. Transgene expression was determined after 72 h by FACS analysis (FACS Calibur, BD Biosciences, Heidelberg, Germany).

4.3.10 Suicide gene transfer and gancyclovir treatment

$5x10^3$ cells per well were seeded in 96-well plates and transduced with rAAV-SR39 vectors at an MOI of 1000 vector genomes per cell (vg/cell). After two cycles of 10 µM gancyclovir treatment (24 h and 72 h post transduction), the number of viable cells was assessed by MTT assay (Sigma, St. Louis, MO).

4.4 Results

4.4.1 Screening of a random AAV display peptide library yields enrichment of a peptide motif

To select for AAV capsids with high transduction efficiency in acute myeloid leukemia cells, Kasumi-1 acute myeloid leukemia cells were infected with the AAV library at an MOI (multiplicity of infection) of 100 capsids per cell. Cells were superinfected with wild-type adenovirus type 5 (Ad5), allowing for amplification of internalized AAV

library clones. Amplified AAV were recovered and subjected to two more rounds of selection to enrich for AAV particles that bind to, are internalized by, and replicate within leukemia cells. The DNA region containing the oligonucleotide insert of AAV particles recovered from leukemia cells after each round of selection was amplified by PCR, verifying that exclusively AAV with cap genes containing the random insert were amplified (Figure 6). DNA sequencing of the subcloned PCR products revealed enrichment of peptides sharing common patterns after selection (Table 1). The sequenced clones were almost invariably characterized by an N in position 1, T or V in position 3 and L in position 3 or 4. Interestingly, a very similar pattern was observed upon selection on another acute myeloid leukemia cell line. In independent experiments, an X_7 AAV display peptide library was selected on HL60 acute myeloid leukemia cells and the oligonucleotide inserts of enriched clones were amplified by PCR (Figure 6). In this selection, in addition to the expected 359 base pair band containing the oligonucleotide insert, we also observed a smaller band corresponding to the size of the wild-type PCR product. This band most likely derived from the wild-type AAV particles which are an unavoidable part of the unselected library due to homologous recombination events taking place during library shuttle production [174]. The additional wild-type band faded from selection round to selection round indicating enrichment of AAV clones containing peptide inserts. The enriched clones were sequenced after three rounds of selection. Only two different peptide clones were identified: NAVTATS and NRVTDFP, showing sequence similarity especially in position 1 and 3 with the clones selected on Kasumi-1 cells. All of the enriched clones from each round of selection on Kasumi-1 cells were used for further analysis.

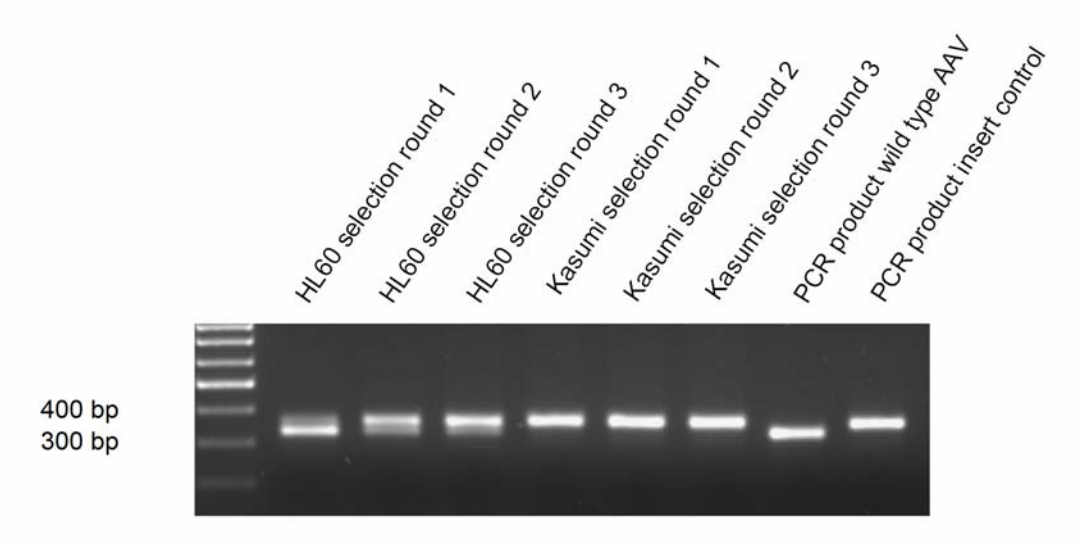

Figure 6: PCR amplification of the genomic AAV DNA fragment containing the modified *cap* gene region at different stages of selection.
DNA of AAV library pools obtained after 1, 2, or 3 rounds of selection on Kasumi-1 cells or HL60 cells served as templates; DNA of wild-type AAV as well as a clone randomly picked from the unselected library (*VRRPRFW*) were used as controls. The upper band corresponds to a fragment of the *cap* gene containing the library oligonucleotides. The lower band corresponds to the PCR product of the wild-type *cap* gene. The unselected library contains a certain amount of wild-type genomes (see text). No wild-type band was amplified after selection on Kasumi-1 cells, while a wild-type band fading from round to round was amplified in HL60 cells, indicating that the applied AAV particles were bound and propagated more efficiently than wild-type AAV.

Table 1: Amino acid sequences of enriched mutant AAV clones after selection of an X_7 random AAV display peptide library on Kasumi-1 acute myeloid leukemia cells.

clone (peptide sequence)*	round 1 †	round 2 †	round 3 †
NY**VL**GAD	78.4%	55.2%	51.5%
NDSR**L**SV	8.1%	-	9.1%
NSTLPL**S**	5.4%	13.8%	12.1%
V**NST**RQS	2.7%	-	3.0%
NQ**V**GSW**S**	2.7%	3.4%	12.1%
NVSF**L**RE	2.7%	-	-

* shared sequence patterns are highlighted in bold letters

† the observed frequency of each sequence is given in relation to the overall number of readable sequences in this round of selection (37 in round 1, 29 in round 2, 33 in round 3).

4.4.2 Targeted AAV efficiently transduce acute myeloid leukemia cells

To produce capsid modified rAAV, oligonucleotides encoding peptide inserts of the isolated clones from each round of selection were inserted into a helper plasmid without ITRs (pXX2-187) for vector production. Helper plasmids with the wild-type *cap* gene or a control insert randomly picked from the unselected library (VRRPRFW) were used as controls. Vector productions were performed as described in material and methods. The vector titers obtained ranged between $5x10^{10}$ and $1.0x10^{12}$ capsids/ml and $1x10^{10}$ and $1.0x10^{12}$ vector genomes (vg)/ml, respectively, after iodixanol density gradient purification (Table 2).

Table 2 : Titers of recombinant AAV-2 vectors.

clone (peptide sequence)	capsid titer* (capsids/ml)	genomic titer** (vector genomes (vg)/ml)		
	rAAV-eGFP	rAAV-eGFP	rAAV-luc	rAAV-SR39
wild-type	$5.56x\ 10^{11}$	5.79×10^{10}	1.2×10^{12}	1.1×10^{12}
random insert	5.37×10^{10}	2.13×10^{10}	8.5×10^{10}	1.1×10^{10}
N Y V L G A D	1.32×10^{11}	2.42×10^{10}	-	-
N D S R L S V	1.31×10^{11}	1.7×10^{10}	-	-
N S T L P L S	2.29×10^{11}	n.d. **	-	-
V N S T R Q S	3.37×10^{11}	2.2×10^{10}	-	-
N Q V G S W S	1.02×10^{12}	2.3×10^{11}	4.7×10^{11}	$8.3 x10^{10}$
N V S F L R E	2.29×10^{11}	8.74×10^{10}	-	-

* capsid titers of viral stocks were determined by AAV-2 ELISA.

** vector genomes of viral stocks were determined by quantitative real time PCR as described in material and methods.

To determine the transduction efficiency of the selected library clones, rAAV vectors displaying the selected peptide inserts or a control insert, Kasumi-1 cells were

transduced with GFP-vectors. FACS analysis revealed that the vectors displaying the selected peptide variants had a 5-fold to 60-fold better transduction efficiency compared to vectors carrying a wild-type AAV2 capsid, and up to 500-fold better transduction efficiency compared to vectors carrying a capsid with a random control insert (Figure 7A). The NQVGSWS clone invariably achieved the highest transduction efficiency in all experiments. In dose escalation experiments, the transduction efficiency of this clone could be increased to 90%, whereas control vectors displaying a random peptide did not show significant transduction in Kasumi-1 cells, even at high MOI (Figure 7B). Targeted transduction of Kasumi-1 cells by the NQVGSWS clone was also evaluated in an independent reporter gene assay using vectors harbouring a luciferase reporter gene. While no or weak luciferase activity was detectable in cells transduced with vectors carrying a random capsid insert or wild-type AAV capsid, respectively, cells transduced with AAV-NQVGSWS showed strong luciferase activity (Figure 7C).

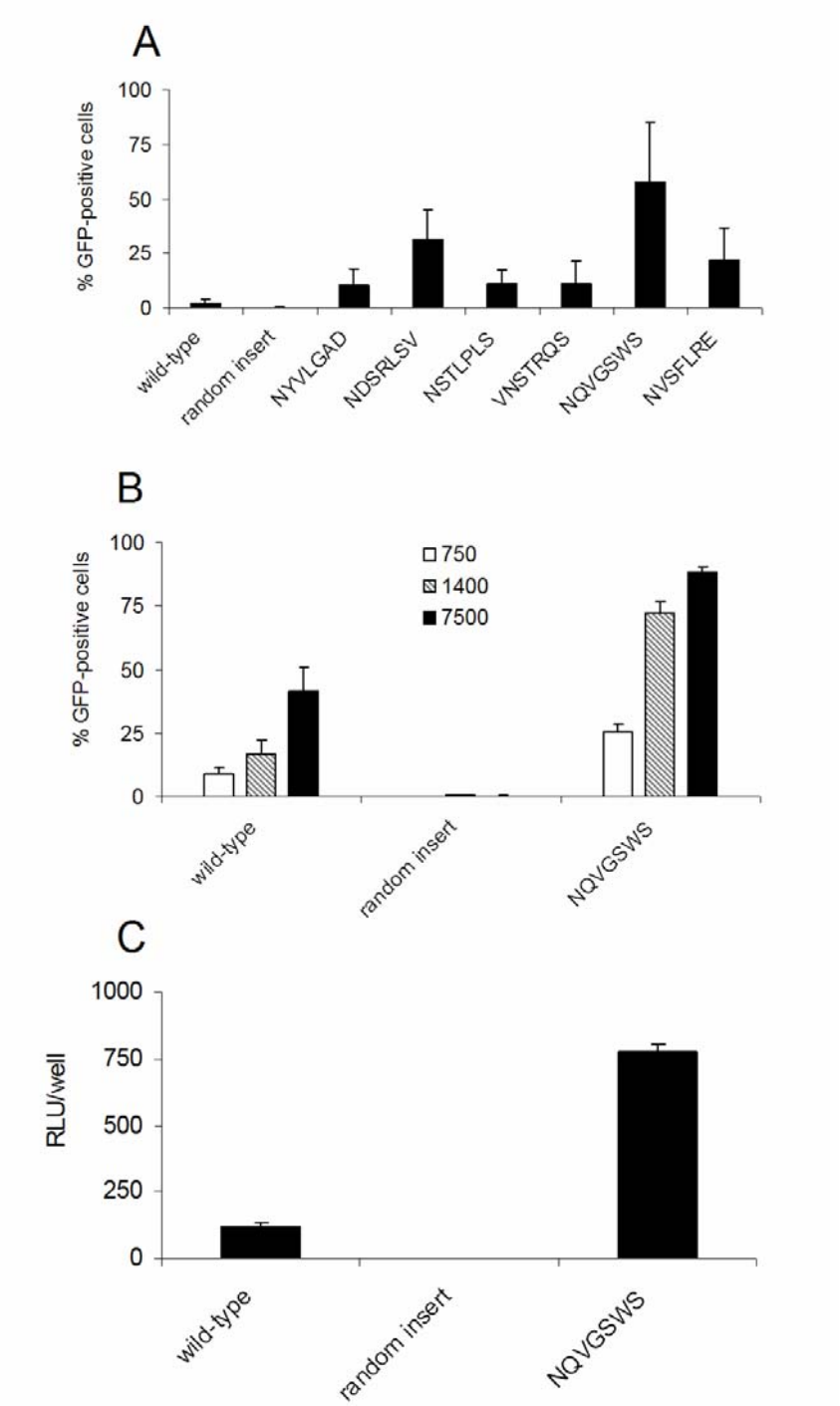

Figure 7: Transduction of Kasumi-1 cells with targeted AAV vectors.
A: The selected AAV clones harboring GFP transduce Kasumi-1 cells. Kasumi-1 cells were transduced at an MOI of $7.5x10^3$ capsids per cell with recombinant AAV vectors harboring a green fluorescent protein (GFP) reporter gene packaged into modified or wild-type AAV-2 capsids. The peptide insertion VRRPRFW served as a random control. Transduction efficiencies were evaluated 72 h later by FACS analysis. Values are shown in % fluorescent cells. Data represent mean values plus standard deviation from 3 independent experiments.
B: NQVGSWS-AAV transduce Kasumi-1 cells at very high efficiency. Transduction efficiency was evaluated as in A, using the NQSGSWS clone and controls in various MOIs (vg/cell). Data represent mean values plus standard deviation from triplicates.
C: Kasumi-1 cell transduction by selected AAV using an independent reporter gene system. Kasumi-1 cells were transduced using AAV- vectors carrying a luciferase reporter gene. Transduction efficiencies were evaluated 72 h later by measuring relative light units (RLU) after substrate edition to the cell lysate. Data represent mean values plus standard deviation from 12 wells in two independent experiments.

Next, we asked whether the targeted AAV clone NQVGSWS also transduces acute myeloid leukemia cells other than the cell line it was selected on. Four AML cell lines were transduced with AAV GFP-vectors at an MOI of $7.5x10^3$ capsids per cell which is the MOI needed to achieve submaximum transduction efficiency in Kasumi-1 cells by the NQVGSWS clone (Figure 8). In these cell lines, the transduction efficiency of wild-type rAAV vectors was only slightly above background level. Vectors displaying the NQVGSWS targeting peptide, however, transduced all of the AML cell lines at a level of approx. 3-fold to 40-fold more efficient than wild-type vectors. These data suggest that the NQVGSWS clone is suitable for targeting acute myeloid leukemia cells.

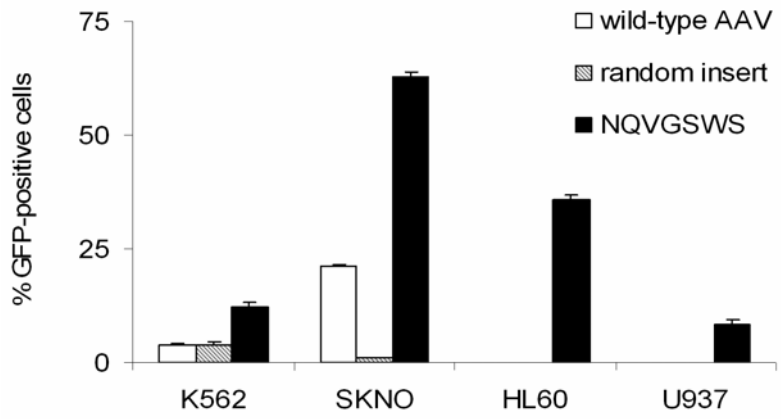

Figure 8: Transduction of AML-cell lines other than Kasumi-1 by leukemia-targeted AAV vectors.
Acute myeloid leukemia cells were transduced at an MOI of $7.5x10^3$ capsids per cell with recombinant AAV vectors harboring a green fluorescent protein (GFP) packaged into modified or unmodified AAV-2 capsids (NQVGSWS selected peptide insert, VRRPRFW random control insert, or wild-type AAV). Transduction efficiencies were evaluated 72 h later by FACS analysis. Values are shown in % fluorescent cells. Data represent mean values plus standard deviation from triplicates.

4.4.3 Transduction of target cells by the selected capsid mutants is independent of the natural AAV-2 receptor heparan sulfate proteoglycan

In the capsid of wild-type AAV-2, the region surrounding amino acid position 588 is involved in the binding to the primary AAV-2 receptor heparan sulfate proteoglycan (HSPG) [182, 245, 286]. Introduction of peptide ligands into this region abrogates binding of AAV-2 to its natural attachment receptor to a variable degree, largely depending on the peptide ligand used for insertion [81, 88, 174, 194, 228, 230]. Therefore, the role of heparan sulfate proteoglycan in the transduction of targeted AAV must be determined for each individual targeting peptide cloned into the 588 site of the AAV-2

capsid. Several experimental approaches were used to investigate this issue. First, we determined binding of wild-type and targeted AAV particles to heparin agarose. All capsid mutants selected on Kasumi-1 cells as well as a random control from the unselected library showed significantly reduced heparin binding compared to wild-type capsids (Table 3). Next, we evaluated transduction efficiency of the selected clones in pgsD677 cells, a heparan sulfate proteoglycan-deficient CHO cell mutant [151, 245]. As expected, neither wild-type nor rAAV-GFP vectors displaying a random control peptide transduced this cell line at a significant level (Figure 9A). In contrast, the NQVGSWS clone selected from Kasumi-1 cells, transduced pgsD677 cells at a level of up to 34% (Figure 9A). Finally, Kasumi-1 cells were transduced by the NQVGSWS clone in presence and absence of soluble heparin at various concentrations [245]. No inhibition but rather a slight enhancement of transduction was observed (Figure 9B), while a concentration-dependent inhibition of wild-type AAV transduction was observed in 293T cells, proving that the soluble heparin is functional. Taken together, these data clearly demonstrate that the AAV NQVGSWS clone selected on Kasumi-1 cells transduces its target cells by means of an attachment receptor other than heparan sulfate proteoglycan.

Table 3: Binding of leukemia-targeted AAV to immobilized heparin compared to binding of wild-type AAV-2 *.

Clone (peptide sequence)	% of wild-type AAV-2 binding
wild-type	100
random insert	13
NYVLDAG	13
NDSRLSV	18
NSTLPLS	21
VNSTRQS	5
NQVGSWS	23
NVSFLRE	36

* $5x10^9$ AAV-2 capsids with or without enriched peptide insertion (random control insert = VRRPRFW) were applied to immobilized heparin as described. Eluted AAV were quantified by capsid ELISA. Binding of wild-type AAV-2 without peptide insertion was set to 100%.

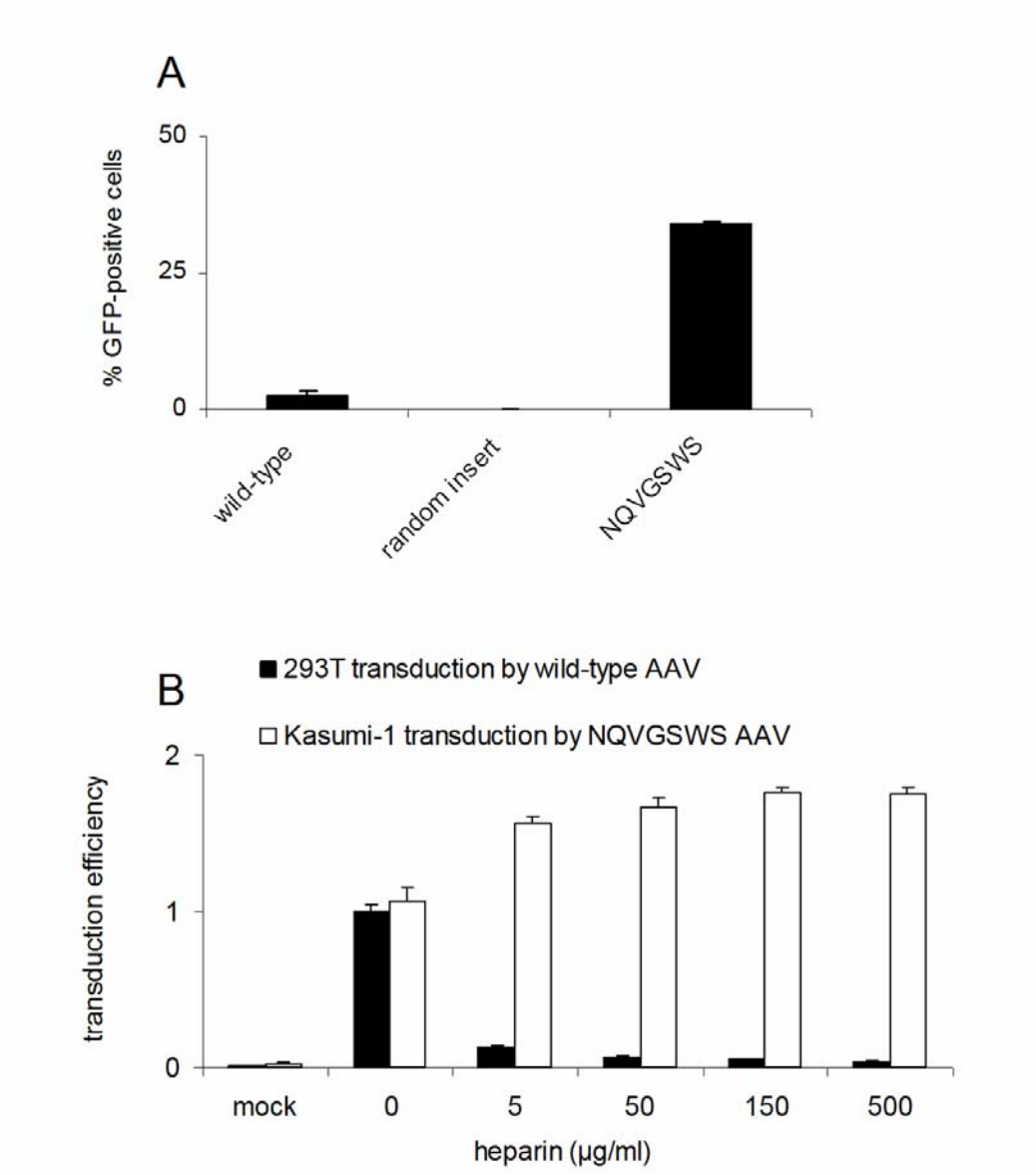

Figure 9: NQVGSWS-mediated targeting of AAV is independent of the natural AAV-2 attachment receptor heparan sulfate proteoglycan (HSPG).
A: Transduction of HSPG-negative pgsD677 cells by targeted and untargeted AAV. Cells were transduced at an MOI of $7.5x10^3$ capsids per cell with recombinant AAV vectors harboring a green fluorescent protein (GFP) packaged into modified or wild-type AAV-2 capsids as indicated. The control insert was VRRPRFW. Transduction efficiencies were evaluated 72 h later by FACS analysis. Values are shown in % fluorescent cells. Data represent mean values plus standard deviation from triplicates.
B: Kasumi-1 cell transduction by targeted AAV is independent of soluble heparin. Kasumi-1 or 293T control cells were transduced at an MOI of $7.5x10^3$ capsids/cell using NQVGSWS-AAV or wild-type AAV harboring a green fluorescent protein gene. Vectors were incubated with indicated amounts of heparin for 45 min prior to transduction. Transduction efficiencies were evaluated 72 h later by FACS analysis. Values are shown relative to % fluorescent cells in untreated controls which are set to 1. Data represent mean values plus standard deviation from triplicates.

4.4.4 Transduction efficiency of the NQVGSWS clone is superior to wild-type AAV transduction efficiency preferentially in hematopoietic cancer cells

The NQVGSWS AAV-peptide mutant was the one with the best transduction efficiency in all AML cell lines tested. Therefore this clone was chosen for further characterization. Our previous work suggested that the screening of random AAV display peptide libraries on distinct cell types reveals selection of distinct peptide motifs [174, 194, 276]. This selection of cell type specific peptide motifs even without negative selection strategies prompted us to screen a panel of hematopoietic and non-hematopoietic cell types for their susceptibility to transduction by the NQVGSWS clone. The panel included lymphoma cells (L1236, LCL, Jurkat, KMH-2), myeloid leukemia cells (NB4, KG1a), multiple myeloma cells (RPMI 8226), osteosarcoma cells (U-2 OS), cervical cancer cells (SiHa) and prostate cancer cells (LNCAP-C42). AAV-GFP vectors displaying the NQVGSWS peptide transduced 7 out of 11 cell lines better than wild-type AAV vectors (Figure 10). All of these cell lines were hematopoietic while all of the cell lines in which the NQVGSWS clone showed no advantage over or was inferior to wild-type AAV vectors were non-hematopoietic.

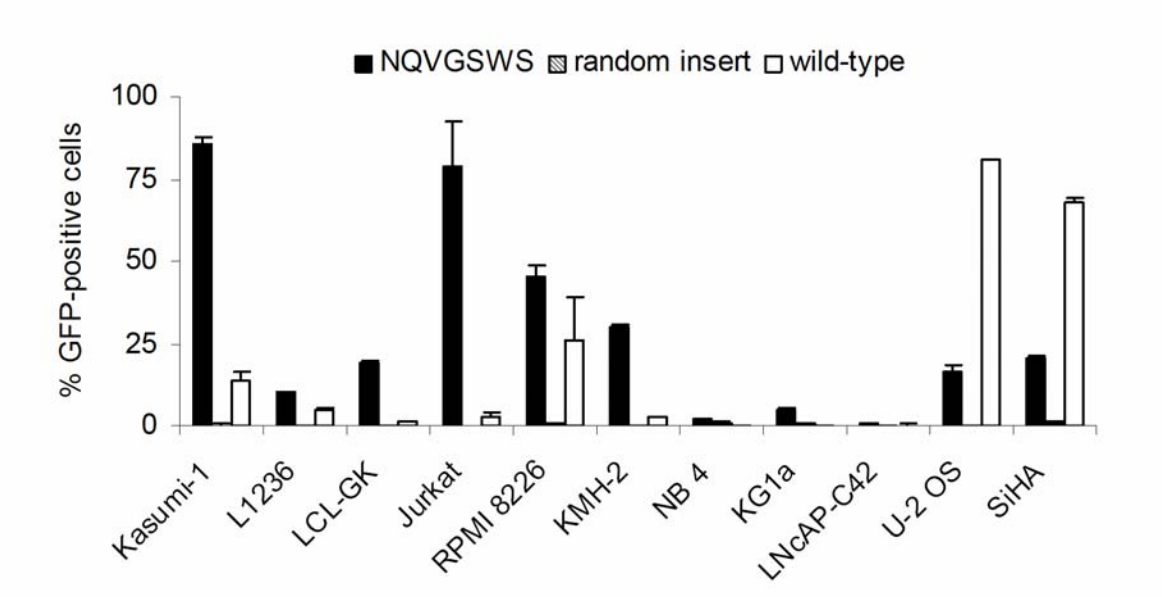

Figure 10: Transduction on different cell types by the AAV-NQVGSWS clone.
Various hematopoietic and non-hematopoietic cell lines were transduced at an MOI of $7.5x10^3$ capsids per cell with recombinant AAV-GFP vectors displaying the NQVGSWS insert, a random insert control (VRRPRFW) or wild-type capsid, respectively. Transduction efficiencies were evaluated 72 h later by FACS analysis. Values are shown in % fluorescent cells. Data represent mean values plus standard deviation from triplicates.

4.4.5 CD34+ primary hematopoietic progenitor cells and peripheral blood mononuclear cells are not susceptible to transduction with the NQVGSWS clone

To further test the specificity of the selected NQVGSWS clone, we determined its transduction efficiency on isolated primary human CD34+ progenitor cells (> 90%) and peripheral blood mononuclear cells (PBMCs). In contrast to Kasumi 1 cells, both of these non-neoplastic hematopoietic cell types were not transduced by the NQVGSWS clone (Figure 11A). To test the vector specificity in a coculture system, Kasumi-1 cells were co-cultivated with primary CD34+ hematopoietic progenitor cells and infected with AAV-GFP vectors displaying the NQVGSWS peptide. Here again, NQVGSWS vectors efficiently transduced Kasumi cells while normal CD34+ hematopoietic progenitor cells where not transduced (Figure 11B). In conjunction with the data presented above, these findings confirm that the NQVGSWS-mediated AAV-transduction is preferentially targeted to hematopoietic cancer cells and not to solid tumor cells or normal hematopoietic cell types.

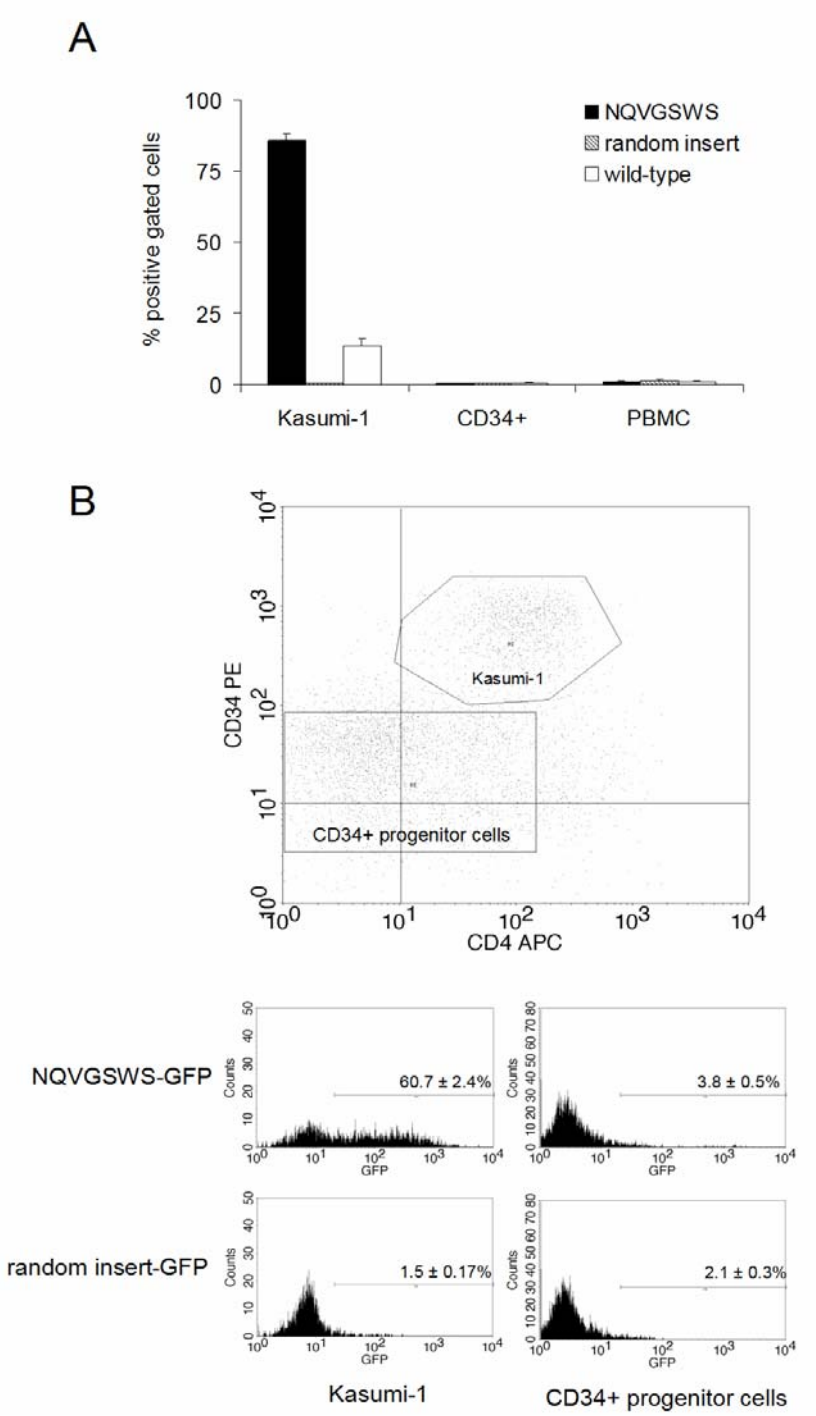

Figure 11: Lack of transduction of primary human CD34+ hematopoietic progenitor cells and peripheral blood mononuclear cells by the AAV-NQVGSWS clone.
A: AAV-NQVGSWS fails to transduce non-neoplastic hematopoietic cells. Kasumi-1, normal CD34+ hematopoietic progenitor cells and normal peripheral blood mononuclear cells from a healthy donor were transduced at an MOI of 1400 vg/cell with recombinant AAV-GFP vectors. Transduction efficiencies were evaluated after 72 h by FACS analysis. Values are shown in % fluorescent cells. Data represent mean values plus standard deviation from triplicates.
B: AAV-NQVGSWS specifically transduces Kasumi-1 cells in co-culture with non-neoplastic hematopoietic cells. Kasumi-1 cells and CD34+ hematopoietic progenitor cells were co-cultured at a 1:10 ratio in 24 well plates. The co-culture was infected at an MOI of 1400 vg/cell with capsid-modified AAV-GFP vectors. After 48h, cells were stained with CD34+ PE- and with CD4-APC conjugated antibodies (because approx. 20% of the normal progenitor cells turned CD34 negative after 48 h. The percentage of transduced Kasumi-1 or normal CD34+ cells was determined by analyzing the GFP positive cells gated for each corresponding cell type shown in C. Values are shown in % fluorescent cells. Data represent mean values plus standard deviation from triplicates.

4.4.6 NQVGSWS-mediated targeted expression of the HSV-TK suicide gene confers selective killing of Kasumi-1 cells after gancyclovir treatment

The ultimate goal of efficient and selective gene transfer into acute myeloid leukemia cells is selective killing of these cells by therapeutic gene delivery. Towards this end, we produced wild-type and tropism-modified AAV vectors harboring SR39 [29], a derivative of the HSV-TK suicide gene. We transduced Kasumi-1 cells with these AAV-SR39-vectors at an MOI of 1000 vector genomes (vg)/cell. Cells transduced by vectors carrying the wild-type capsid or the control capsid were almost resistant to gancyclovir prodrug treatment. In contrast, Kasumi-1 cells transduced by vectors with the NQVGSWS capsid insert showed strong cytotoxic effects upon gancyclovir treatment (Figure 12A). To show that AAV-NQVGSWS-mediated cell killing is specific for the cell type this clone was selected on, we compared AAV-NQVGSWS-mediated killing in Kasumi-1 cells with killing in SiHa cervical cancer control cells. Unlike in Kasumi-1 cells, cytotoxic effects in SiHa cells were only observed after wild-type AAV-mediated, but not after AAV-NQVGSWS-mediated SR39 gene transfer (Figure 12B). These findings confirm that the selected NQVGSWS AAV capsid allows for acute myeloid leukemia-directed cytotoxic gene transfer.

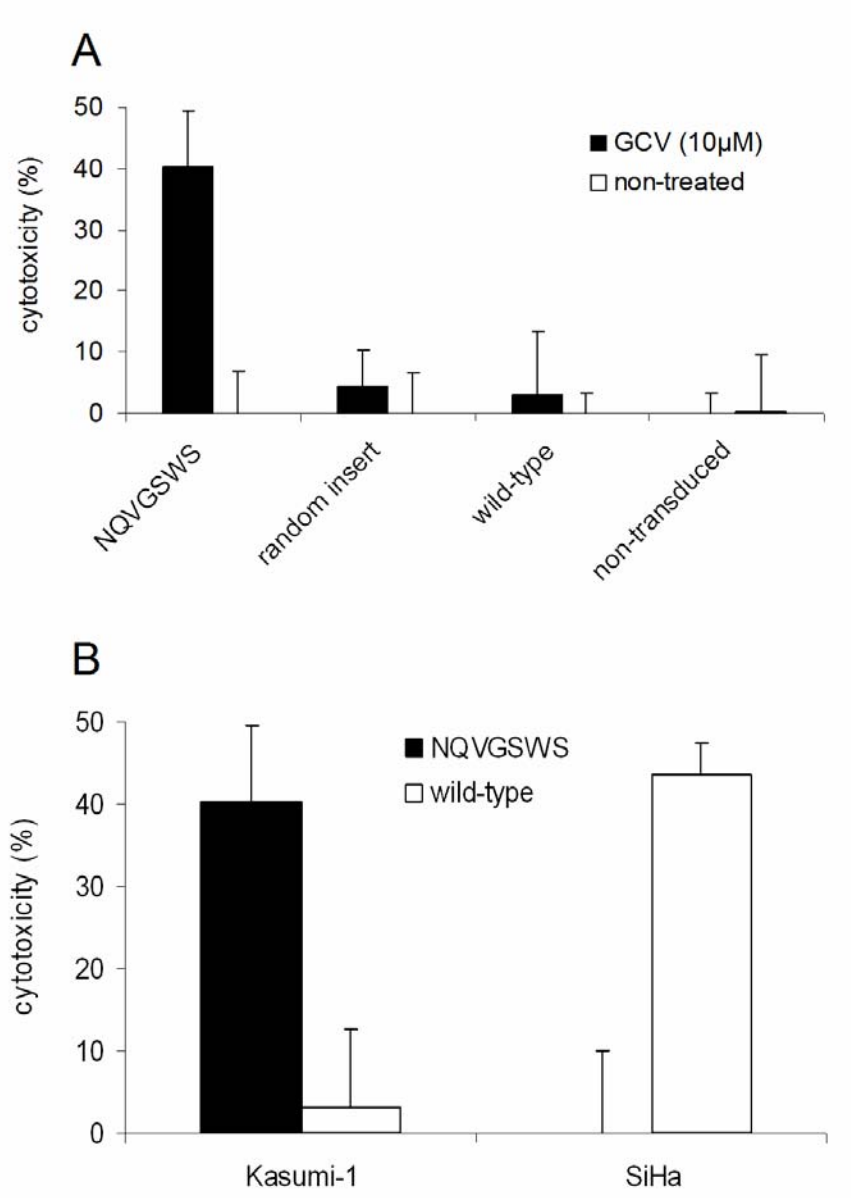

Figure 12: Leukemia-directed therapeutic suicide gene transfer using tropism-modified AAV capsid mutants.
A: Cells were transduced at an MOI of 10^3 vg/cell using recombinant AAV-SR39 vectors carrying wild-type capsids, or capsids with NQVGSWS insert or capsids with VRRPRFW control insert, respectively. Four days after initiation of gancyclovir (GCV) treatment, cytotoxic effects were evaluated by MTT assay. Values are shown in % cytotoxicity (determined as 100% minus % viable cells) compared to non-treated cells. Data represent mean values plus standard deviation from nine wells in three independent experiments.
B: Kasumi-1 and SiHa cells were transduced using wild-type AAV or NQVGSWS AAV vectors, respectively, and cytotoxicity was evaluated following GCV treatment as described in A. Data represent mean values plus standard deviation from nine wells in independent experiments.

4.5 Discussion

Targeting gene therapy vectors to specific cell types continues to be a major issue of interest [177, 183, 251]. Some of the hurdles due to the lack of tropism can be overcome by the use of vectors with specific viral serotypes like it has been shown for adeno-associated viral vector targeting to muscle tissue [30, 273] or airway epithelia [246]. Hematopoietic cells and leukemia cells in particular, are refractory to transduction using currently available gene transfer strategies. Therefore novel, more efficient vector systems are needed to transduce this cell type satisfactorily. A random screening system based on adeno-associated virus type 2 (AAV-2) allows for the selection of receptor-targeted AAV from a library of up to 10^8 different capsid mutants [174, 194]. Such combinatorial approaches have significant advantages over the introduction of known receptor binding peptides (*e.g.* selected from phage display libraries) into the viral capsids because targeting ligands are selected 1) that allow for sufficient assembly and production of the virus, 2) that take the unique protein context of the capsid surrounding the targeting ligand into account during selection, 3) that are selected by their ability to allow for virus internalization and expression of viral genes within the target cell. Comparable approaches have also been introduced for other vector systems [42]. We have used this system to select AAV capsid mutants that efficiently transduce Kasumi-1 acute myeloid leukemia cells, a cell line that is poorly susceptible to transduction by wild-type AAV-2 vectors. The selection revealed a peptide motif designated as $NS^{V}/_{T}LLXS$. One of the selected clones containing a variant of this motif (NQVGSWS) increased in frequency from one selection round to the next. Vectors displaying this NQVGSWS peptide also transduced acute myeloid leukemia (AML) cells other than Kasumi-1 at a degree considerably superior to wild-type AAV-based vectors, suggesting that the receptor targeted by NQVGSWS is expressed also in other AML cells and may characterize neoplastic hematopoietic cells in general. This assumption is supported by the fact that in a large screening experiment using a panel of hematopoietic and non-hematopoietic cancer cells the superiority of the NQVGSWS clone over wild-type AAV vectors was restricted to leukemia and lymphoma cells. Even though our selection method did not differentiate between malignant and non-malignant hematopoietic cells, the AAV-NQVGSWS specifically transduced AML cells but not normal hematopoietic cells. It remains to be determined in further studies whether the NQVGSWS clone also transduces leukemic stem cells. Further limitations of AAV as leukemia-targeted therapeutics

may be the need for repetitive application provoking immune responses. Here, however, targeted AAV may have considerable advantages over wild-type vectors, not only because the may evade the preexisting immunity against AAV [106] but also, because the targeted particles may be less exposed to the immune system as they specifically and efficiently transduce one cell type.

Our results indicate that the targeted transduction of leukemia cells mediated by the NQVGSWS clone is independent of the natural AAV-2 attachment receptor heparan sulfate-proteoglycan [245], as the clone showed abrogation of heparin binding in affinity chromatography experiments. Further, the clone was able to transduce the heparan sulfate deficient cell line pgsD677 and finally, the Kasumi-1-cell transduction could not be competed even with excessively high concentrations of heparin. It is generally assumed that AAV transduction involves binding of the capsid to an attachment receptor [245] followed by interaction with an independent cell entry receptor such as αVβ5 integrin [244], fibroblast growth factor receptor-1 [202], hepatocyte growth factor receptor (c-Met) [118], laminin receptor [1] or additional as yet unknown internalization-triggering receptors. It is unkown which and how many AAV capsid domains interact with such secondary receptors. Future research may reveal to which extend peptide insertions at the R588 site may influence AAV capsid interaction with the various secondary receptors described so far.

AAV has been targeted to a number of different cell types such as endothelial cells [174, 177, 182, 278] and also to hematopoietic cells in previous reports [16, 194, 210, 228]. Most of these studies used known ligands or antibodies binding to known receptors on leukemia cells. Earlier reports have used chemical conjugates to target AAV to leukemia cells [16, 199, 210]. These studies were a valuable proof of principle approach to show that AAV can be retargeted to alternative receptors [16, 199, 210]. Inserting the targeting ligand directly into the adeno-associated viral capsid was first reported by Girod et al. [81]. This strategy has significant advantages over using chemical conjugates in regard to handling, potential immunogenicity and particle size [174]. Most of the reports targeting hematopoietic cells used antibody ligands to known receptors on hematopoietic cells. Only one study used a combinatorial approach to select for viral capsids that are optimized for transduction while the potential targeting receptor is not predetermined [194]. In this study, two peptides (GENQARS und QNEGSRA) were selected for optimized transduction of human B-cell chronic lymphatic leukemia. One of these two peptides may show similarity to one of the clones presented here in

that it has an NQXXS pattern (X = any amino acid), even though the similarity is otherwise vague and it is speculative to assume that both peptides may bind a similar class of receptor. Our report complements these previous studies in that we selected capsid mutants for optimized transduction of a panel of acute myeloid leukemia cells. Of note, the multiplicity of infection (MOI) rates of targeted vectors needed in our study to achieve high level transduction efficiency in the targeted cell lines were considerably lower than in the above mentioned reports [16, 194, 199, 210] suggesting higher targeting efficiency of our vectors. The difference of transduction efficiency of the clones described here compared to the mutants described in previous reports [194] may be due to the different procedure in library making [174, 194] which may lead to different diversities of the libraries used for selection and therefore a different likelihood of selection of the optimal mutant for transduction. A direct side-by-side comparison of both libraries on the same cell type would be needed to confirm this assumption.

In our experiments, the observed frequency of clones after three rounds of selection does not fully correlate with transduction efficiency of vectors displaying the targeting peptides that were selected. Such mismatch of clonal frequency and targeting efficiency has been described before in a comparable setting using replication-based amplification of targeting capsid mutants [174]. Two reasons may contribute to this effect: 1) the process of replication is not equal to the process of transduction even though both processes may share common pathways. Therefore, a clone having an advantage in cell entry over another clone may not necessarily replicate faster if it, for instance, displays a targeting peptide that is disadvantagous for capsid assembly. 2) The superinfection with Ad5 allowing for replication of the clones internalized into the target cell is by far not 100% efficient, especially in leukemia cells. Thus, some clones may replicate more efficient in round 2 and 3 just because more of the leukemia cells infected by this clone were superinfected by Ad5 by chance.

This is the first report of selection and validation of transduction-optimized cytotoxic AAV vectors targeted to acute myeloid leukemia from a random vector display system. We conclude that the AAV mutant presented here may be used as a valuable tool to target therapeutic genes to acute myeloid leukemia cells as a novel treatment option in this and potentially other malignant hematopoietic diseases.

4.6 Acknowledgements

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5 Potential and limitations of random AAV display peptide libraries screenings *in vivo* for tissue targeted gene transfer

Stefan Michelfelder[1,2], Johannes Kohlschütter[3], Alexandra Skorupa[2], Sabrina Pfennings[2], Oliver Müller[4], Jürgen A. Kleinschmidt[4], Martin Trepel[1, 2, 3]

University of Freiburg Medical Center, [1] Dept. of Hematology and Oncology and [2] Institute for Molecular Medicine and Cell Research, Hugstetter Str. 55, D-79106 Freiburg, Germany

[3] University Medical Center Hamburg-Eppendorf, Department of Hematology and Oncology, Martinistr. 52, D-20246 Hamburg, Germany

[4] Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

Submitted to Molecular Therapy

5.1 Abstract

Targeting viral vectors to certain tissues *in vivo* has been a major challenge in gene therapy. Cell type-directed vector capsids can be selected from random peptide libraries displayed on viral capsids *in vitro* but so far this system could not easily be translated to *in vivo* applications. Using a novel amplification protocol for peptide libraries displayed on adeno-associated virus (AAV), we established proof of concept that vectors selected for optimized transduction of primary cells *in vitro* are not necessarily suitable for transduction of the same target cells under *in vivo* conditions. We therefore performed selections of AAV peptide libraries in living animals after intravenous administration using tumor and lung tissue as prototype targets. Analysis of peptide sequences of AAV clones after several rounds of selection yielded distinct sequence motifs for both tissues. The selected clones indeed conferred gene expression in the target tissue while gene expression was undetectable in animals injected with control vectors. However, all of the vectors selected for tumor transduction also transduced heart tissue and the vectors selected for lung transduction also transduced a number of other tissues, particularly and invariably the heart. This suggests that more than one capsid region of AAV must be modified to achieve tissue-specific transgene expression. While the approach presented here does not yield vectors whose expression is confined to one target tissue, it is a useful tool for *in vivo* tissue transduction when expression in tissues other than the primary target is uncritical.

5.2 Introduction

Efficient and specific delivery of therapeutic genes to the tissue of interest is a paramount and so far unsolved issue in gene therapy. Therefore, despite a wealth of potential transgenes evaluated *in vitro*, gene therapy has failed to evolve beyond the stage of an experimental therapeutic tool with considerable debate regarding its safety and efficacy.

Among the available viral vectors for gene delivery, adeno-associated virus (AAV) has gained particular attention recently. The low frequency of random integration into the genome [47] and the moderate immune response make AAV an attractive basis for gene therapy vector design [24, 54]. No substantial safety issues have been encountered in a number of clinical trials involving AAV vectors [47]. Like in almost all

other gene therapy vectors, the lack of target tissue specificity of AAV remains of concern. This may partly be circumvented by using AAV serotypes with an *in vivo* gene transduction pattern most closely fitting the needs of the application [305]. Also, the tropism of AAV capsids may be changed by combining parts of the natural serotype diversity (reviewed in [287]). Alternatively or in addition, peptides mediating binding to the cell type of interest can be identified by random phage display library screening and subsequently be introduced into an AAV capsid region critical for receptor binding [81, 88, 181, 228, 277, 278, 284]. Such peptide insertions into or other mutational manipulations of the heparin binding domain adjacent to VP capsid protein position R588 can abrogate the natural tropism of AAV-2 capsids and result in de-targeting from the liver *in vivo* [124, 174, 195]. The identification of numerous tissue-directed peptide ligands during the last decade [4-6, 43, 66, 114, 131, 133-135, 141, 156, 191, 201, 206, 255, 299] would seem to supply almost unlimited potential for the introduction of ligands into AAV capsids to establish targeted gene delivery *in vivo*. This approach has indeed been reported to be successful using certain peptides [277, 278, 284]. Yet, our own experience has been, that for many peptides cell tropism changes or gets lost after inserting them into the AAV capsid (author's unpublished observation). This may be due to a number of reasons. First and foremost, the peptide's conformation may change unpredictably when incorporated into the structural AAV capsid context, leading to a reduced receptor-ligand affinity and specificity. Further, peptides isolated by phage display screenings are commonly selected based on receptor binding but not on subsequent internalization, nuclear transfer, and transgene expression. To overcome these obstacles, we and others have developed a screening system based on random peptide libraries displayed directly on AAV capsids [174, 194]. In this system, the AAV library particles are amplified based on binding, uptake and viral gene expression in the target cell via adenoviral helper co-infection. Capsid mutants efficiently transducing various different cell types have been isolated from such libraries by *in vitro* biopanning on the cells of interest [165, 174, 194, 226, 276].

Despite the obvious importance of the question, it remains open for most of these vectors whether or not they are suited for targeted gene transfer after systemic administration *in vivo* as vector targeting *in vivo* faces several hurdles which are not present *in vitro.* These include ligand-receptor interactions under circulation conditions, host-anti-vector immune reactions and rapid vector clearance from the circulation by the reticuloendothelial system, and endothelial cell layers as well as the

extracellular matrix acting as physical barriers [279]. *In vivo* selection of random AAV peptide libraries for efficient and tissue-specific vector transduction after systemic administration *in vivo* has not been performed so far. In one recent report on screening of an AAV peptide library *in vivo* in mice, vectors were applied via the airways [90]. While the recovered vectors mediated lung gene transfer after topic application to the airways, they exhibited almost unchanged tropism compared to unselected vectors after systemic application via the blood stream. Among the limitations faced by *in vivo* AAV display library selection is the difficulty to rescue and amplify tissue-targeted library viruses for multiple selection rounds as the amplification systems used *in vitro* are based on adenoviral superinfection and can therefore not easily be applied in living animals.

Here we address the unsolved issues raised above. We set out to isolate tissue-directed AAV capsids from AAV display peptide libraries. Therefore, we established a novel adenovirus-free PCR based amplification protocol to select AAV libraries over multiple screening rounds after systemic application *in vivo*. While the selected vectors indeed transduced their target tissue orders of magnitude better than unselected vectors, we almost invariably observed unintended transduction of heart tissue, suggesting that more than one capsid region of AAV must be modified to achieve tissue-specific transgene expression mediated by vectors selected from AAV display peptide libraries.

5.3 Material and Methods

5.3.1 Cells and cell culture

HeLa, MCF-7, and 3T3 cells (all obtained from American Type Culture Collection ATCC, Manassas, VA) as well as 293T cells (kindly provided by David Baltimore, California Institute of Technology, Pasadena, CA), were maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 1% penicillin/streptomycin solution (Invitrogen) and 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). Primary mouse hepatocytes were prepared as previously described [129] and cultivated in Williams' Medium E (WME; Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS, 100 nM dexamethasone, 2 mM L-glutamine and 1% penicillin/streptomycin. Primary murine breast cancer cells were obtained from tumors growing in female transgenic FVB mice expressing the

polyoma middle T antigen under the control of the mouse mammary tumor virus promoter [91, 152] as previously described [260]. Briefly, tumors were cut into small pieces and digested for 1 h at 37 °C in collagenase 2 solution (Biochrom), dissolved in PBS, 10% 2 mM $MgCl_2/CaCl_2$ and 10% BSA. The cell suspension was passed through 100 µm and 40 µm cell strainers, washed twice with PBS, and cultured in Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) containing 10% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin, and 1.25 µg/ml amphotericin B (Invitrogen). All cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2.

5.3.2 Animals and tumor staging

The mouse strain FVB/N-TgN(MMTVPyVT)634-Mul (PymT) was purchased from Jackson Laboratory (Bar Harbor, ME). All procedures involving animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the German Animal Protection Code. Genotyping was performed by polymerase chain reaction (PCR) as described by Jackson Laboratory (www.jax.org). Starting at the age of 30 days, transgenic female mice were palpated weekly for early detection of mammary tumors. The animals were anesthetized by intraperitoneal injection of 100 mg/kg body weight 10% ketamine hydrochloride (115.34 mg/ml; Essex, Munich, Germany) and 5 mg/kg body weight 2% xylazine hydrochloride (23.32 mg/ml; Bayer, Leverkusen, Germany).

5.3.3 AAV library biopanning *in vitro* and *in vivo*

A random X_7 AAV display peptide library (random insert introduced at position R588 VP1 capsid protein numbering) with a diversity of $2x10^8$ random clones at the cloned plasmid level was produced using a three-step protocol as described previously [165, 174]. For *in vitro* biopanning (Figure 13, pathway A), $2x10^6$ primary PymT breast cancer cells were incubated with the AAV library at a multiplicity of infection (MOI) of 1,000 vector genomes (vg)/cell in selection round 1, 500 vg/cell in round 2, and 100 vg/cell in round 3. After 96 hours, unbound AAV library particles were removed by 3 washing steps in PBS. Surface-bound library viruses were detached by trypsin digestion for 20 minutes and subsequent washing. Extensive screening experiments on Kasumi-1 AML cells demonstrated that this additional trypsin digest is essential to

enrich clones for improved gene transduction (Ag Trepel, unpublished observations). Whole cellular DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany). The random oligonucleotides contained in AAV library particles internalized into tumor cells were amplified by PCR using the primers 5'-GGTTCTCATCTTTGGGAAGCAAG-3' and 5'-TGATGAGAATCTGTGGAGGAG-3'. For *in vivo*/*ex vivo* biopanning of AAV peptide libraries (Figure 13, pathway B), $1x10^{10}$ vg of an AAV library for selection round 1, or $2x10^{8}$ to $2x10^{9}$ vg per animal for round 2-4 were injected into the tail vein of female PymT transgenic mice bearing palpable breast tumors. After 24 hours, primary breast cancer cells were prepared as described above and grown *in vitro* for 96 hours. Oligonucleotide inserts of targeted AAV library particles were amplified by nested PCR using whole cellular DNA as template. Primers were 5'-ATGGCAAGCCACAAGGACGATG-3' and 5'-CGTGGAGTACGTTGTTAGGAAG-3' for the first PCR and 5'-GGTTCTCATCTTTGGGAAGCAAG-3' as well as 5'-TGATGAGAATCTGTGGAGGAG-3' for the second PCR. Pure *in vivo* library biopanning (Figure 13, pathway C) was performed along the same lines, except that the circulation time was 48 hours and that DNA extraction from the tumor tissue was done without prior *ex vivo* growth of the cells. To select for lung homing AAV, libraries were injected into the tail vein of 6-week-old female PycB/FVB wild-type mice (n=2 animals per selection round) as described for tumor selections (Figure 13, pathway C). DNA of whole lung tissue extracts from two animals was extracted, pooled and used as template to amplify the random oligonucleotide of lung-homing AAV. We varied the time of AAV blood circulation before lung harvest in 2 alternative selection approaches (5 minutes followed by a perfusion step, 48 hours in round 1, 48 hours or 6 days for round 2, and 6 days for round 3 to 4). For all selections, PCR products were analyzed by agarose gel electrophoresis to verify correct size, digested with *Bgl*I and cloned into the *Sfi*I-digested pMT-202-6 library backbone plasmid [165, 174]. Cloned AAV library plasmids were transformed into electrocompetent *E. coli* DH5-α (Invitrogen) using the Gene Pulser (Bio-Rad, Hercules, CA). Randomly assigned clones were sequenced using the reverse primer 5'-CAGATGGGCCCCTGAAGGTA-3'. For production of pre-selected AAV peptide libraries, $2x10^{8}$ 293T cells were transfected with the library plasmids at a ratio of 25 plasmids/cell using Qiagen's PolyFect reagent. PUC18 (Invitrogen) served as carrier DNA. Two hours after transfection, 293T cells were superinfected with wild-type

adenovirus type 5 (Ad5, supplied by the Laboratoire de Thérapie Génique, Nantes, France) at an MOI of 5 infectious particles/cell for library particle amplification. After 48 h, or when cell lysis became apparent, cells were detached from the culture dish in PBS-MK (140 mM NaCl, 5.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$) and pooled with supernatants. AAV library particles were harvested by cell lysis via three freeze-thaw cycles. Cellular DNA was removed by incubation with benzonase (Sigma) at 50 U/ml lysate at 37 °C for 30 min, followed by Ad5 inactivation at 55 °C for 30 min. Viral library preparations were purified using the iodixanol gradient centrifugation method as previously described [97, 98]. The 40% iodixanol fraction containing the purified AAV viruses was stored at -80 °C until further use.

5.3.4 Production of capsid modified recombinant AAV vectors

Recombinant AAV (rAAV) vectors displaying selected peptide sequences were generated by cloning the oligonucleotide inserts into the pXX2-187 plasmid (a derivative of the library backbone plasmid pMT-187-0-3 without ITRs) [165, 276]. 293T packaging cells were transfected with the modified pXX2-187 (or pXX2 for wild-type AAV-2 capsid controls), pXX6 [291], and a plasmid carrying a reporter gene or a toxic transgene of interest using PolyFect. Packaged reporter genes included the enhanced Green Fluorescent Protein (eGFP) gene contained in the plasmid pUF2-GFP, a derivative of pTRUF-CMV-eGFP [307], or the luciferase (luc) gene in the plasmid pUF2-CMV-luc [276]. The HSV thymidine kinase mutant SR39 [94] was used as a cytotoxic suicide gene. Cells were harvested 96 hours after transfection, and vectors were purified by iodixanol gradient centrifugation as described above.

5.3.5 AAV titration and evaluation of vector homing and serum distribution

The AAV capsid titers were determined as described [89] by ELISA (Progen, Heidelberg, Germany). The genomic titers of recombinant AAV vectors and AAV libraries were determined by quantitative PCR using the Absolute SYBR Green fluorescein master mix (Abgene, Epsom, UK) and the MyiQ cycler (Bio-Rad) as previously described [212, 213]. Vectors were quantified using the forward primer 5'-GGCGGAGTTGTTACGACAT-3' and the reverse primer 5'-GGGACTTTCCTACTTGGCA-3' specific for the CMV promoter sequence. The

genomic titer of AAV libraries was determined using the forward primer 5'-GCAGTATGGTGTATCTACCAA-3' and the reverse primer 5'-GCCTGGAAGAACGCCTTGTGT-3' specific for the AAV *cap* gene. Real-time PCR was done in 20 µl with 0.3 µM for each CMV primer, or 0.4 µM for each AAV primer, respectively, according to the manufacturer's protocol (Abgene). For CMV primers, annealing temperature was 64 °C for 15 seconds. For AAV primers, annealing temperature was 61 °C for 30 seconds. Fluorescence was measured at the end of each annealing phase. A standard curve for quantification was generated by serial dilutions of the respective vector plasmid DNA. Calculations were done using MyIQ analysis software (Bio-Rad). For quantification of vectors homing to lung tissue, $5x10^{10}$ capsid-modified rAAV-luciferase vectors were injected into the tail vein of female PycB/FVB wild-type mice (n=3 per group). After 8 days, lung tissue was removed. Whole DNA was extracted using the DNeasy tissue kit (Qiagen) and quantified using a 2100Pro spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). For real-time PCR, 500 ng of extracted genomic DNA were used as template to amplify vector specific DNA using CMV primers as described above. To determine the amount of circulating AAV library or wild-type viruses in the blood, $1x10^{10}$ vg were injected into the tail vein of PycB/FVB wild-type mice. Blood was obtained at indicated time points and centrifuged for 2 minutes at 10.000 rpm. Cell-free serum was diluted 1:100 in ddH_2O and used as template for real-time PCR using AAV specific primer pairs as described above.

5.3.6 Luciferase gene transduction

To analyze luciferase gene transduction *in vitro*, $2x10^4$ cells per well were seeded in 24-well plates or $5x10^3$ cells per well in 96-well plates and incubated with AAV-luciferase vectors at an MOI of 10^4 vg/cell for 72 h. For *in vivo* gene transfer, $5x10^{10}$ vg of rAAV-luciferase vectors were injected into the tail vein of anesthetized animals. After 8 or 28 days, respectively, the target tissue and representative control tissues were removed, snap frozen in liquid nitrogen, and stored at -80 °C. Frozen tissue samples and cell lysates were homogenized in reporter lysis buffer (RLB, Promega, Madison, WI) and luciferase reporter gene activity was determined in a luminometer (Centro LB 960, Berthold Technologies, Bad Wildbad, Germany) using Promega's luciferase assay according to the manufacturer's instructions. If required, values were normalized to protein levels in each probe determined by Bradford assay (Bio-Rad).

5.3.7 Flow cytometric analysis

To analyze transduction by AAV vectors harboring the gene coding for the enhanced green fluorescent protein (eGFP), $5x10^4$ PymT cancer cells per well were seeded in 24-well plates and incubated with AAV GFP vectors at an MOI of 10.000 vg/cell for 72 h. GFP reporter gene expression was determined using a flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, NJ) and CellQuest Pro analysis software. Cell viability was confirmed by counterstaining with propidium iodide (PI) solution (Sigma).

5.3.8 Immunostaining of primary PymT mouse tumor cells

Primary PymT cells were transduced with AAV GFP vectors at an MOI of 10.000 vg/cell. After three days, cells were fixed in 3.7 % paraformaldehyde for 10 minutes at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes and blocked with PBG (1x PBS, 0.5% BSA, and 0.2% fish gelatin) for 1 hour at room temperature. For cytokeratin staining, cells were incubated with a pan-cytokeratin antibody (Sigma) 1:50 in PBS/ 3% BSA for 1 hour at 37 °C, followed by incubation with a goat anti-mouse antibody conjugated to Texas Red (Sigma) for 1 hour at 37 °C. Cells were mounted in Vectashield™ mounting fluid containing DAPI. Images were acquired using an AxioVert200 microscope (Zeiss, Oberkochen, Germany) and AxioVision 4.5 analysis software.

5.3.9 Suicide gene transfer and toxicity assay

Cells were seeded at $5x10^3$ per well in 96-well plates and transduced with rAAV-SR39 vectors at an MOI of 10.000 vg/cell. After two cycles of 10 µM ganciclovir (GCV) treatment (24 h and 72 h after transduction), the number of viable cells was assessed as described [171, 233]. Cells were incubated with medium containing 500 µg/ml MTT (Invitrogen) for 4 h. Subsequently, absorbance of formazan crystals dissolved in SDS/HCl was measured at 570 nm in a SpectraMAX microplate reader (Molecular Devices, Sunnyvale, CA).

5.3.10 Statistics

Statistical analysis was performed using the GraphPad Prism program 3.0 (GraphPad Software, San Diego, CA). Parametric data were analyzed by one-way analysis of variance followed by a Bonferroni post test. Non-parametric data were analyzed by a Kruskal-Wallis test followed by a Dunns post test. p values < 0.05 were considered significant.

5.4 Results

5.4.1 PCR based screening of a random AAV display peptide library on primary PymT breast cancer cells yields enrichment of specific peptide motifs

To isolate AAV-2 capsids for targeted gene transfer in primary breast cancer cells of transgenic PymT mice, we prepared tumor cells and screened an X_7 random AAV display peptide library *in vitro* along the lines of pathway A in Figure 13. Because AAV-2 does not (or not to a detectable extent) replicate in these cells after superinfection with adenovirus type 5 (Ad5) (Figure 14), we developed a selection protocol by which internalized AAV library particles are amplified based on PCR amplification of their random oligonucleotide insert (Figure 13). The *cap* gene region containing the oligonucleotide insert of AAV recovered from breast cancer cells after each round of selection was amplified by nested PCR and correct size of the amplification product was verified by agarose gel electrophoresis (data not shown). The insert was cloned back into the library backbone plasmid pMT202-6 and the diversity of transformed library plasmids was at least $1x10^5$ clones for such secondary libraries in this and subsequent selections (Tables 4 and 5). New pre-selected AAV particle libraries were obtained by transfection of 293T cells with the generated secondary plasmid library in limiting dilution technique (25 library plasmid molecules per producer cell) to minimize the production of chimeric AAV library particles or mismatch of packaged DNA and displayed peptide due to uptake of multiple library genomes in one producer cell. The titers obtained with this approach were sufficient for further selection rounds (Tables 4 and 5). To increase the stringency of selection, MOIs of AAV libraries were decreased from 500 vg/cell to 100 vg/cell in rounds two and three, respectively. Sequence analysis showed enrichment of several clones after two rounds of selection compared to round 1,

functionally validating our novel selection protocol. Peptide sequences found after round 1 were RGDLGLS, RGDMSRE, DGLGRLV, and DRSPLSL. After three rounds of selection, RGDLGLS and RGDMSRE were the dominant clones (Table 6). Both peptides share the sequence motif RGDXXXX.

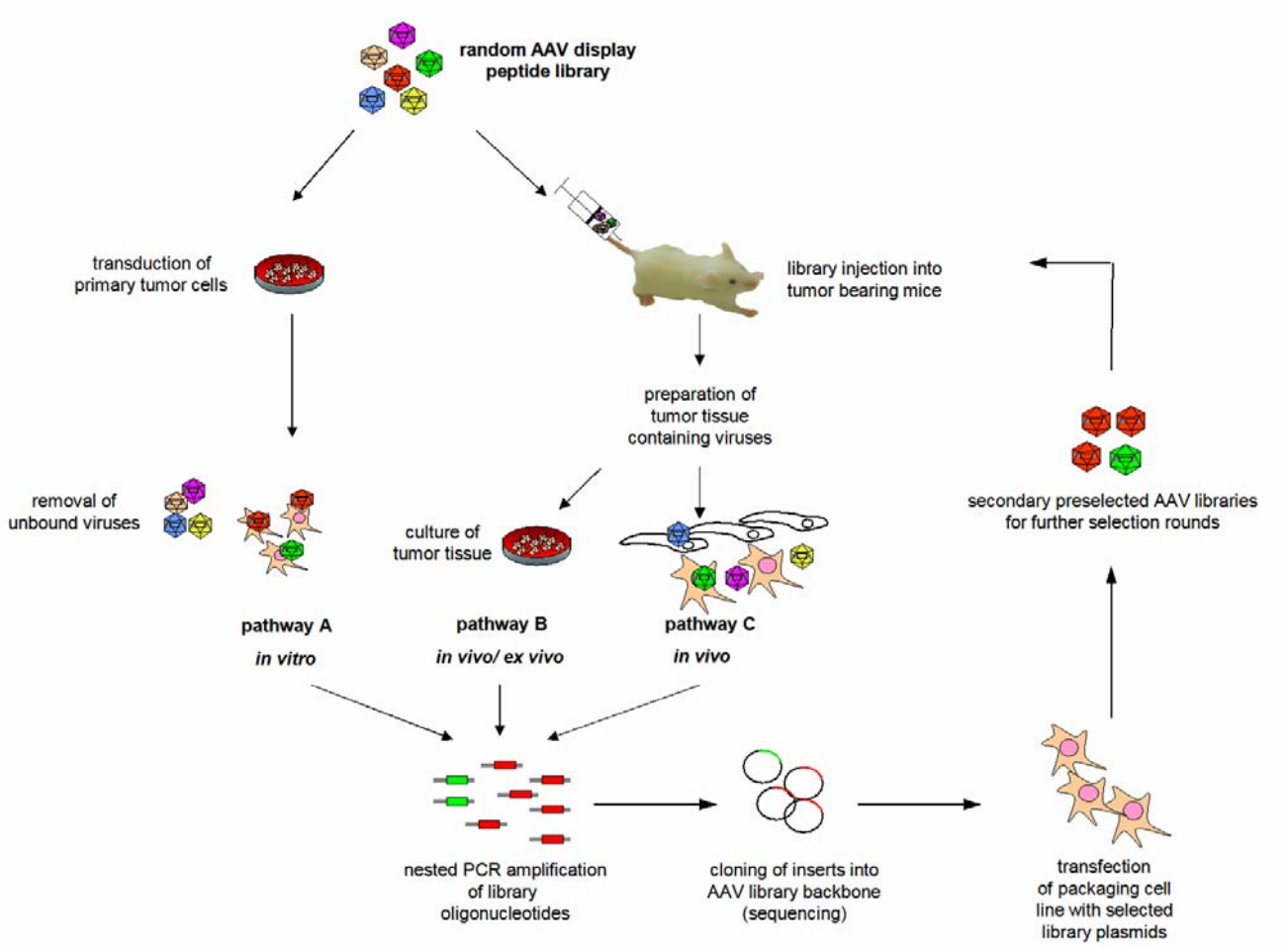

Figure 13: Pathways used for selection of targeted viral capsids by screening random AAV display peptide libraries.
For all selection pathways, genomic DNA containing *cap* gene fragments from internalized library viruses was extracted from the target cells or tissue. Library inserts were amplified by nested PCR and cloned back into the AAV library backbone plasmid pMT-202-6. The resulting pre-selected plasmid library was used to produce a secondary AAV library by transfection into 293T cells and subsequent superinfection with Ad5. Pre-selected AAV libraries were re-subjected to selection on the target cells *in vitro* or the target tissue *in vivo*. Preceding the amplification step, the library selection was done according to one of the following three pathways:
Pathway A, *in vitro* selection: A random AAV display peptide library was incubated on primary breast cancer dissociation cultures derived from female tumor-bearing PymT mice. Non-internalized but cell-bound AAV library particles were removed by extensive washing followed by trypsin digestion prior to DNA extraction and AAV insert amplification.
Pathway B, *in vivo*/*ex vivo* selection: A random AAV display peptide library was injected intravenously into female tumor-bearing PymT mice. After 24 hours, primary tumor cells of the injected mouse were prepared as in pathway A and grown *ex vivo* for 96 hours prior to DNA extraction and AAV insert amplification.
Pathway C, *in vivo* selection: A random AAV display peptide library was injected as in pathway B in tumor-bearing mice (for selection of tumor-homing AAV) or wild-type mice (for selection of lung homing AAV), respectively. After 48 hours, the target tissue (tumor or lung, respectively) was removed and lysed, and DNA was extracted for AAV insert amplification

Table 4: Characterization of AAV libraries used for selections on tumor tissue.

Selection pathway	Selection round			
	round 1	round 2	round 3	round 4
Pathway A (*in vitro*) on tumor cells				
Step 1: plasmid library				
Independent clones / library [a]	$1.1x10^6$	$5.5x10^6$	$5x10^7$	n.a.
Step 2: virus library [b]				
Genomic titer (vg/ml)	$1.4x10^9$	$2.7x10^9$	n.a.	n.a.
Capsid titer (capsids/ml)	n.d.	n.d.	n.a.	n.a.
Pathway B *(in vivo/ex vivo)* on tumor tissue				
Step 1: plasmid library				
Independent clones / library [a]	$2x10^6$	$4x10^6$	$5.5x10^6$	$3.5x10^6$
Step 2: virus library [b]				
Genomic titer (vg/ml)	$4.2x10^9$	$6.2x10^8$	$1.1x10^9$	n.a.
Capsid titer (capsids/ml)	n.d.	n.d.	n.d.	n.a.
Modified pathway B *(in vivo/ex vivo)* on tumor tissue (subsequent to 2 rounds of *in vitro* selection pathway A)				
Step 1: plasmid library				
Independent clones / library [a]	n.a.	n.a.	$1x10^6$	$1x10^5$
Step 2: virus library [b]				
Genomic titer (vg/ml)	n.a.	n.a.	$8.8x10^8$	n.a.
Capsid titer (capsids/ml)	n.a.	n.a.	n.d.	n.a.
Pathway C *(in vivo)* on tumor tissue				
Step 1: plasmid library				
Independent clones / library [a]	$1x10^6$	$4.1x10^6$	$1x10^5$	$2.5x10^5$
Step 2: virus library [b]				

Genomic titer (vg/ml)	$4.1x10^9$	$2.2x10^9$	$3.6x10^9$	n.a.
Capsid titer (capsids/ml)	n.d.	n.d.	n.d.	n.a.

[a] The diversity of plasmid libraries was determined by the amount of transformed bacterial clones.

[b] The genomic and capsid titer of the virus libraries was determined as described in material and methods.

n.d. not determined

n.a. not applicable because no virus solution was prepared

Table 5: Characterization of AAV libraries used for selections on lung tissue.

Selection approach	**Selection round**			
	round 1	round 2	round 3	round 4
5 min circulation in 1st round on lung tissue				
Step 1: plasmid library				
Independent clones / library [a]	$1x10^6$	$2.5x10^6$	$2.5x10^7$	$1x10^7$
Step 2: virus library [b]				
Genomic titer (vg/ml)	$9x10^8$	$6x10^8$	$1.2x10^9$	n.a.
Capsid titer (capsids/ml)	$3.3x10^9$	n.d.	n.d.	n.a.
2 days circulation in 1st round on lung tissue				
Step 1: plasmid library				
Independent clones / library [a]	$2.5x10^6$	$2.5x10^5$	$2.5x10^7$	$1.2x10^5$
Step 2: virus library [b]				
Genomic titer (vg/ml)	$2.4x10^9$	$5x10^8$	$2.5x10^8$	n.a.
Capsid titer (capsids/ml)	$9x10^9$	n.d.	n.d.	n.a.

[a] The diversity of plasmid libraries was determined by the amount of transformed bacterial clones.

[b] The genomic and capsid titer of the virus libraries was determined as described in material and methods.

n.d. not determined

n.a. not applicable because no virus solution was prepared

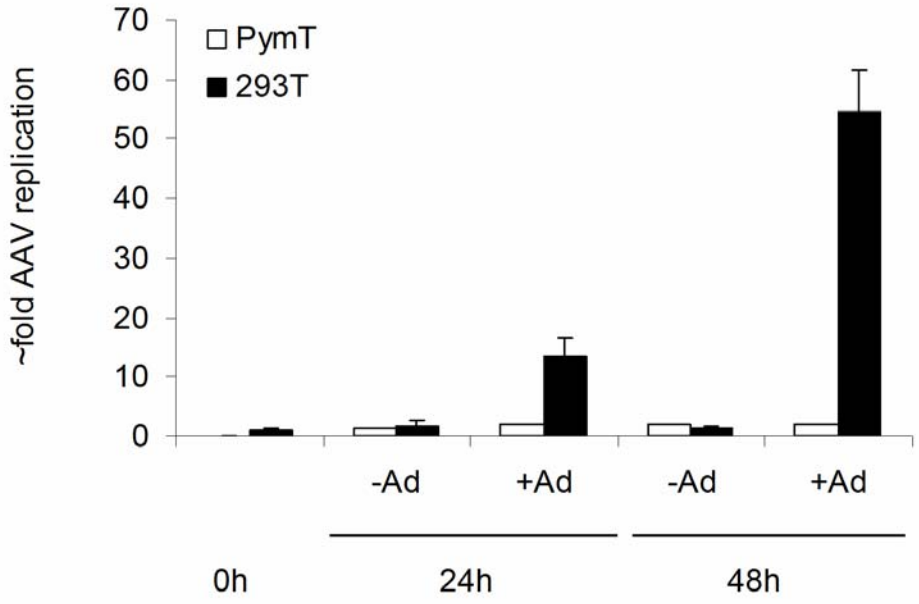

Figure 14: AAV-2 does not replicate in primary PymT cancer cells upon superinfection with Ad5.
Primary PymT breast cancer or 293T control cells were infected at an MOI of 1,000 vg/cell using wild-type AAV-2. Two hours after infection, cells were superinfected with Ad5 helper virus (100 infectious units/cell). Cells were harvested at 0, 24, and 48 hours after Ad5 infection. DNA was extracted and the amount of AAV genomes was determined by quantitative PCR. Data represent mean values plus ± SD from triplicates.

Table 6: Peptides enriched after PCR-based *in vitro* selection (pathway A) of AAV peptide libraries on primary breast cancer cells.

Peptide sequence [a]	**Frequency in selection round** [b]		
	round 1	round 2	round 3
RGDLGLS	-	3/10	6/9
RGDMSRE	-	1/10	3/9
DGLGRLV	-	3/10	-
DRSPLSL	1/6	2/10	-

[a] single letter code; shared amino acid patterns are highlighted in colored letters

[b] observed frequency relative to overall number of sequenced clones

5.4.2 Selected AAV capsids efficiently target primary breast cancer cells

To test whether the selected AAV capsid mutants allow for targeted gene delivery in primary PymT breast cancer cells, we produced rAAV luciferase vectors displaying the selected peptides RGDLGLS, RGDMSRE, and DGLGRLV for further analysis. These vectors transduced primary PymT breast cancer cells up to 17.8-fold better than wild-type AAV-2 vectors, and up to 3,500-fold better than vectors displaying an unselected random peptide (VRRPRFW) (Figure 15A).

Since primary tumor tissue consists of tumor parenchymal (epithelial), tumor stroma and vascular endothelial cells, we evaluated which cell type in the primary culture is transduced by the selected AAV. We produced RGDLGLS and DGLGRLV as well as control insert vectors harboring a GFP reporter gene and assessed transduction by FACS analysis, confirming gene expression data obtained from the luciferase transduction experiments. Fluorescence microscopy of transduced PymT cells with cytokeratin immunostaining revealed that the selected vectors only transduce cytokeratin-positive cells, suggesting that targeted cells are epithelium-derived, *i.e.* parenchymal (Figure 15B).

The targeted luciferase AAV clones RGDLGLS and DGLGRLV transduced MCF-7 human breast cancer cells at a rate comparable to primary PymT breast cancer cells, whereas HeLa cervical cancer cells, 3T3 mouse fibroblasts and primary mouse hepatocytes were not permissive for transduction with the selected capsid variants while they could be efficiently transduced with wild-type AAV (Figure 16), suggesting target specificity of the selected clones. These findings were further corroborated by experiments using modified vectors harboring SR39, a derivative of the HSV-tk suicide gene [29, 94, 132]. Primary PymT breast cancer cells transduced by vectors with the RGDLGLS capsid insert showed strong cytotoxic effects upon ganciclovir treatment, whereas cells transduced with control vectors were almost resistant to ganciclovir (Figure 15C). Taken together, these findings suggest RGDLGLS-AAV as a promising candidate for targeted gene transfer to breast cancer cells and demonstrate that our novel Ad5-free, PCR-based biopanning protocol allows for selection of targeted AAV vectors from random AAV display peptide libraries.

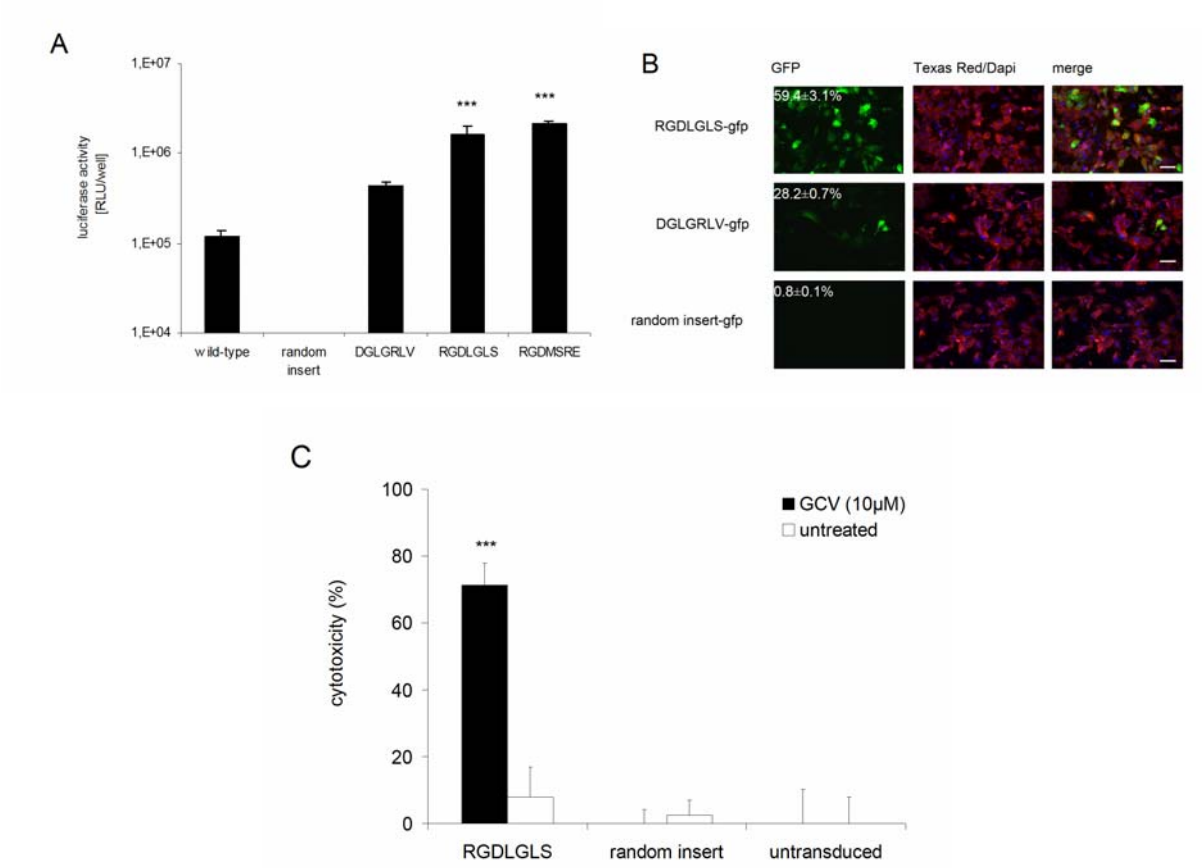

Figure 15: **Vectors selected from random AAV display peptide libraries for targeted gene transfer in primary breast cancer cells.**
A: Transduction of primary PymT breast cancer cells by selected AAV capsid variants. Primary PymT breast cancer cells were transduced by recombinant AAV-2 luciferase reporter gene vectors displaying the selected capsid peptide inserts RGDLGLS, RGDMSRE, or DGLGRLV, respectively. Capsids with no (wild-type) or random peptide insert (VRRPRFW) were used as controls. Transduction efficiency was determined after 72 hours by luciferase assay. Luciferase activities are shown in relative light units (RLU) per well. Data represent mean values ± standard deviation (SD) from one representative experiment (out of three) in triplicates (*** $p<0.001$ compared to wild-type and random insert controls).
B: Selected AAV-2 capsid mutants transduce epithelial cytokeratin-positive breast cancer cells. Primary breast cancer cells were transduced using selected AAV-2 vectors (RGDLGLS, DGLGRLV) or unselected controls (random insert displaying VRRPRFW) harboring a green fluorescent (eGFP) reporter gene. Transduction efficiencies in primary PymT cells were evaluated by FACS analysis 72 hours after transduction. The white numbers in the left panel show the percentage of GFP-positive-gated viable cells (quantitative data represent mean values ± SD from triplicates). Cells were stained with a pan-cytokeratin antibody and a secondary antibody conjugated to Texas Red. Nuclear counterstaining was done with DAPI. Scale bar: 100µm.
C: Breast cancer cell-targeted therapeutic suicide gene transfer using selected capsid mutants. Primary PymT cells were transduced using rAAV-SR39 vectors displaying RGDLGLS or a randomly selected control peptide (VRRPRFW). Four days after initiation of ganciclovir (GCV) treatment, cytotoxic effects were evaluated by MTT assay. Values are shown in % cytotoxicity (*i.e.* % killed cells). Untreated and untransduced cells served as controls. Data represent mean values ± standard error of the mean (SEM) from nine wells in three independent experiments (*** $p<0.001$ selected clone and treated cells vs. all controls).

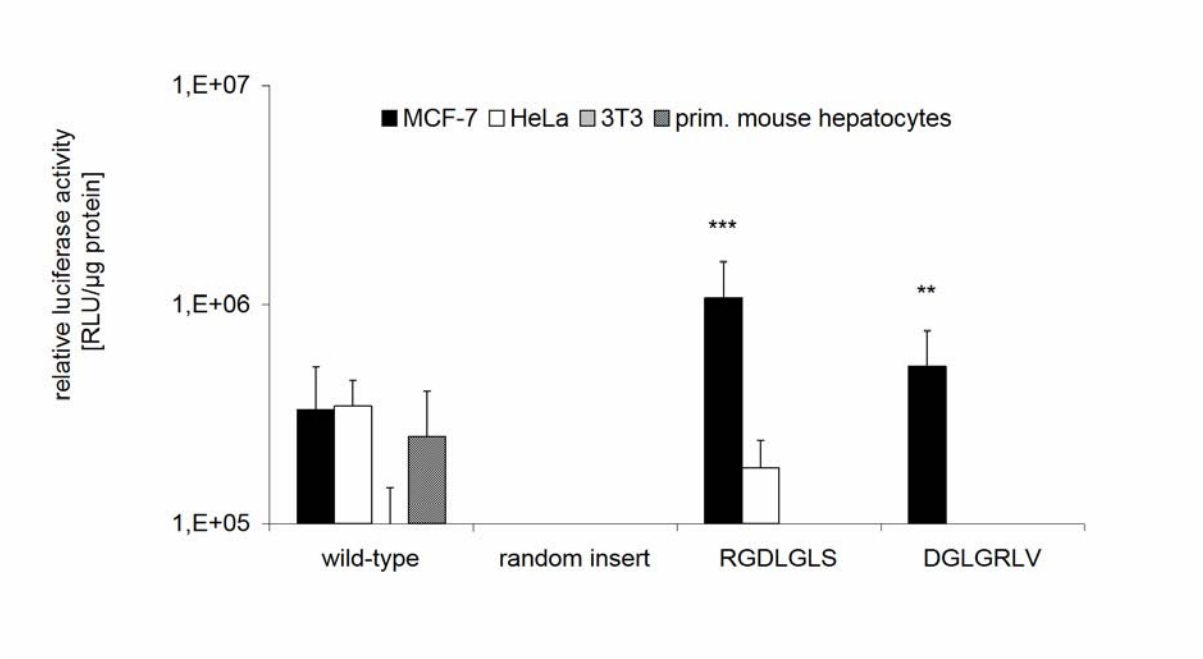

Figure 16: Selected AAV vectors efficiently transduce breast cancer derived cells.
MCF-7 breast cancer cells, HeLa cervical cancer cells, 3T3 mouse fibroblasts and primary mouse hepatocytes were transduced with rAAV vectors harboring a luciferase reporter gene packaged into selected AAV-2 capsids or controls (wild-type or VRRPRFW). Luciferase activities in cellular lysates were measured after 72 hours and were normalized to protein levels in each sample. Data represent mean values ± SD from at least 6 wells in two independent experiments. ** $p<0.01$; *** $p<0.001$ targeted vectors vs. random insert control.

5.4.3 Targeted AAV selected on PymT breast cancer cells *in vitro* fail to transduce PymT tumors *in vivo*

We investigated whether the capsid mutants selected *in vitro* can target PymT breast tumors *in vivo*. AAV luciferase vectors displaying the selected peptides RGDLGLS, RGDMSRE, DGLGRLV, an unselected control peptide, or no peptide (wild-type AAV), respectively, were injected intravenously into female PymT mice bearing breast cancers. After 8 days, tumor tissue and several control organs were harvested and reporter gene expression was analyzed. None of the applied vectors mediated transgene expression in the tumor tissue (Figure 17). Yet, interestingly, each clone showed a unique *in vivo* transgene expression pattern. While AAV-RGDLGLS mediated strong cardiac luciferase expression, no expression was observed in the liver tissue. In contrast, RGDMSRE mediated gene expression in heart and liver, showing that even variations in very few amino acids may have a considerable influence on the vector's *in vivo* transduction profile. A weak luciferase gene expression in mice injected with wild-type AAV vectors was exclusively found in liver tissue, while hardly any expression was observed after transduction with the random

insert control or the AAV mutant displaying the DGLGRLV peptide. These findings show that vectors selected for optimized transduction of a certain cell type *in vitro* do not necessarily transduce the same cells *in vivo* upon systemic administration. Thus we set out to take this into account during the selection process and established a protocol for screening AAV peptide libraries *in vivo* in living animals under physiological circulation conditions.

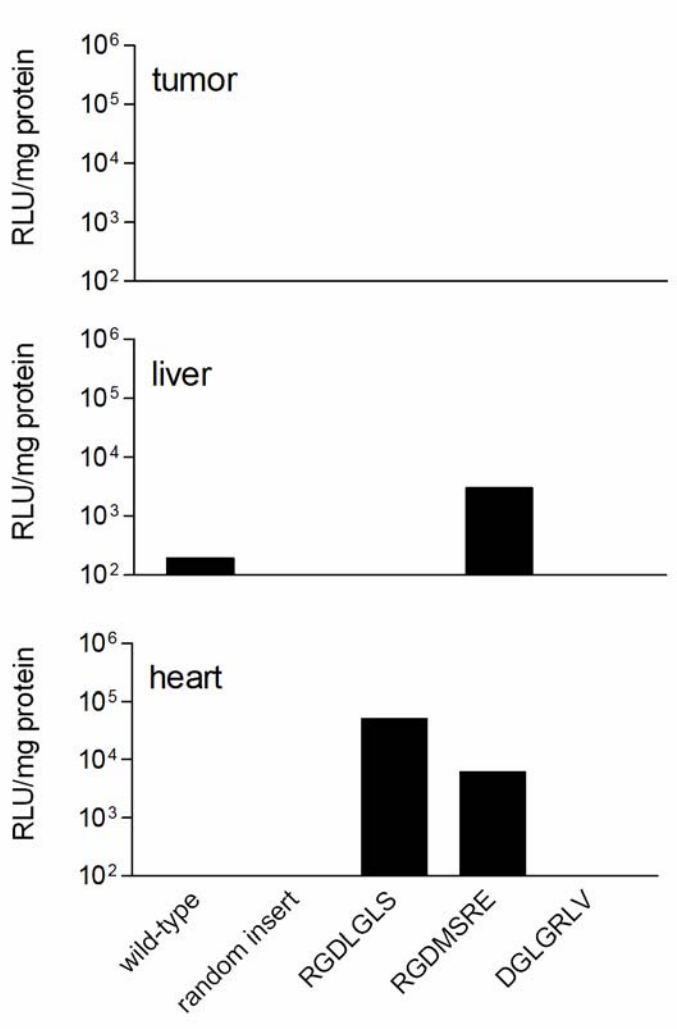

Figure 17: Targeted AAV selected *in vitro* on primary PymT tumor cells fail to transduce tumor cells *in vivo*.
AAV luciferase vectors displaying selected (RGDLGLS, RGDMSRE, DGLGRLV) or control capsids (wild-type or VRRPRFW) were injected intravenously into PymT transgenic female tumor bearing mice ($5x10^{10}$ vg per mouse). After 8 days, tumor and control tissues were harvested and luciferase activities were determined as relative light units (RLU) per mg protein. Data represent mean values from n=2 mice per clone.

5.4.4 Kinetics of circulating AAV display peptide library particles and wild-type AAV are similar

Based on the negative finding above, we hypothesized that selection under *in vivo* conditions is needed to enrich library clones that are able to bind cellular receptors in tumors, penetrate the tumor tissue and are internalized into tumor cells under physiological circulation conditions after intravenous administration. But we suspected that our novel PCR-based selection of AAV libraries may not be able to distinguish between library particles successfully internalized into target cells, and non-homing particles present in the circulation if the tissue is harvested too early after injection. To minimize the amount of circulating AAV library particles in our tissue samples at the time point of harvest, we analyzed the kinetics of circulating AAV library particles. AAV ($1x10^{10}$ vg per mouse) were injected intravenously, blood samples were collected at various time points, and the amount of circulating particles in the serum was quantified by real-time PCR. Clearance rates were comparable in AAV library particles and wild-type viruses (Figure 3). The amount of circulating genomes decreased in a straight proportional manner. We therefore decided to harvest tissues in AAV library selections 48 hours after virus administration.

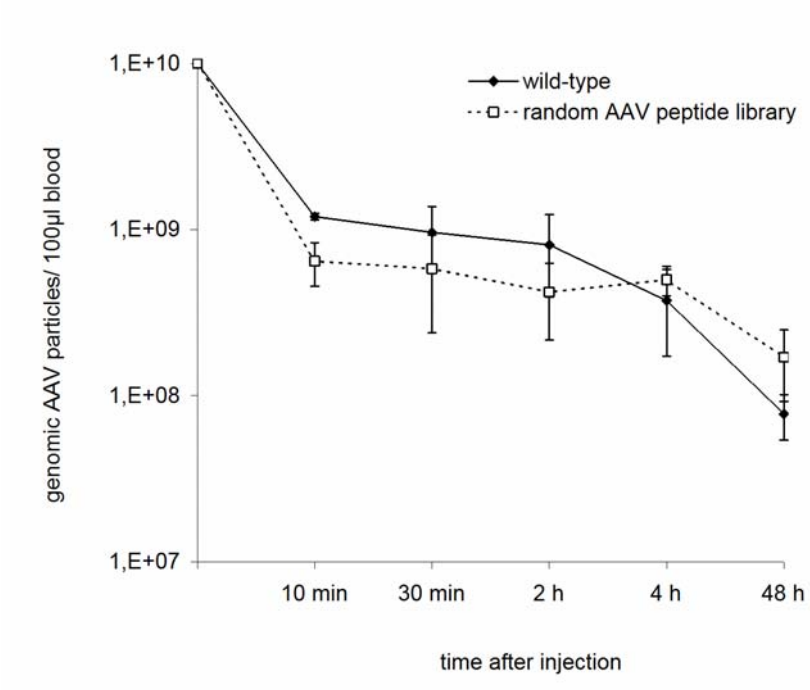

Figure 18: **Kinetics of circulating AAV peptide library particles is similar to wild-type AAV.**
A random X_7 peptide library or wild-type AAV-2 viruses were injected intravenously at $1x10^{10}$ vg per mouse. Blood samples were collected after indicated time points and the amount of circulating viral particles in the serum was determined by real-time PCR. Data represent mean values from n=3 mice per group, analyzed in triplicates ± SD.

5.4.5 *In vivo* selection of AAV display peptide libraries on tumor tissue results in enrichment of distinct peptide inserts

Two technical approaches were chosen (Figure 13, pathways B and C). Secondary libraries were produced and analyzed as for *in vitro* selections. Genomic titers of selected libraries allowed for injection of $2x10^8$ vg per mouse in selection rounds 2-4 (Table 4). After 4 rounds of selection, sequencing revealed the enrichment of serine and glycine-rich peptide motifs and repetition of several single clones. In particular, the motifs GGLSGXS and ESGXXXX, and the single clones EYRDSSG, QMSGGVA, EEPALRA, and APTLGLS were enriched during *"in vivo/ex vivo"* selections (Table 7). In a separate approach, we performed 2 further rounds of *ex vivo* selection with libraries pre-selected for 2 rounds on PymT cells *in vitro* (like in Figure 13, pathway A). Here, the only remaining clone following the *in vivo* part of this selection displayed the peptide DLGSARA (Table 7). During *in vivo* selections (Figure 13, pathway C), the peptide motifs enriched during four rounds of selection were XXSGVGS, GEARXXA, and SGNSGAA, as well as SSGSGGA and ESGIWVA (Table 7). The clones SGNSGAA and SSGSGGA shared the similar sequence pattern SSG or SGG, respectively, which also occurred in the EYRDSSG and QMSGGVA clones enriched during *ex vivo* selection. The motif ESGXXXX was highly enriched in both *in vivo/ex vivo* and pure *in vivo* selections. These data suggest that AAV library selection under circulation conditions is feasible and causes enrichment of a distinct pattern of displayed peptides after multiple rounds of biopanning. Therefore, we decided to evaluate *in vivo* gene transduction for all enriched clones.

Table 7: Peptides enriched in tumor tissue after selection for tumor-homing AAV.

Selection pathway	Peptide [a]	Frequency in selection round [b]			
		round 1	round 2	round 3	round 4
Pathway B *(in vivo/ex vivo)*	GGLSGVS	-/6	-/7	1/22	7/41
	GGLSGDS	-/6	-/7	-/22	1/41
	GSVSGSA	-/6	-/7	-/22	1/41
	EYRDSSG	-/6	-/7	-/22	7/41
	QMSGGVA	-/6	-/7	-/22	1/41
	ESGLSQS	-/6	1/7	1/22	2/41
	ESGIWVA	-/6	-/7	1/22	2/41
	EEPALRA	-/6	-/7	-/22	4/41
	APTLGSP	-/6	1/7	-/22	13/41
Pathway B, modified *(in vitro/in vivo/ex vivo)* [c]	RGDLGLS			5/16	-/10
	DLGSARA			2/16	10/10
	DGLGRLV			6/16	-/10
	DLRGLAS			1/16	-/10
	DRSPLSL			1/16	-/10
Pathway C *(in vivo)*	AISGVGS	-/6	1/15	2/24	2/32
	DRSGVGS	-/6	1/15	4/24	2/32
	SISGVGS	-/6	-/15	-/24	1/32
	SEGRSGV	-/6	-/15	-/24	1/32
	GEARSRA	-/6	-/15	-/24	1/32
	GEARISA	-/6	-/15	2/24	7/32
	SGNSGAA	-/6	1/15	4/24	8/32
	SSGSGGA	-/6	-/15	2/24	2/32
	ESGIWVA	-/6	-/15	-/24	2/32

[a]single letter code; only peptides occurring repetitively or sharing common sequence motifs are shown; shared amino acid patterns are highlighted in colored letters

[b]observed frequency relative to overall number of sequenced clones

[c]pathway B subsequent to 2 rounds of *in vitro* selection as in pathway A

5.4.6 Selected capsid-modified AAV clones transduce tumors *in vivo*

To assess whether the *in vivo*-selected AAV-2 vectors mediate gene expression in the tumor *in vivo*, we produced luciferase reporter vectors displaying the selected peptide sequences. All vectors could be produced to regular titers (Table 8). Luciferase gene expression in the breast tumor tissue was evaluated 8 days after intravenous injection in tumor-bearing PymT mice in a screening experiment to assess which clones should be investigated in detail. Five of the clones (GEARISA, SGNSGAA, ESGLSQS, EYRDSSG, and DLGSARA) showed an increased transduction of the breast tumor tissue compared to wild-type AAV vectors, whereas unselected control vectors did not mediate any gene expression (data not shown). We chose the most promising vectors for further experiments in a larger group of animals (n=5 mice per clone). Following intravenous injection, the selected clones transduced tumor tissue up to ~275-fold more efficient compared to wild-type AAV vectors (Figure 19A). To further investigate the specificity of selected AAV capsid mutants, luciferase expression in several control organs was evaluated (Figure 19B). Moderate de-targeting from the liver by clone ESGLSQS and the unselected control was observed, whereas clones GEARISA and EYRDSSG transduced the liver in a manner comparable to wild-type AAV. DLGSARA gene transduction in liver tissue was significantly increased compared to the unselected control vector. Further, we found a strongly enhanced cardiac luciferase expression for all clones, being significant for GEARISA, EYRDSSG and DLGSARA, and a weakly enhanced cardiac transduction of the unselected control vector compared to wild-type AAV. In regard to tissue specificity, the ESGLSQS clone had the most favorable profile as it transduced tumor tissue but not the liver. However, cardiac gene transduction was seen for this as for almost all the other clones as well. Reproduction of *in vivo* gene transduction with independent vector preparations for DLGSARA and ESGLSQS precisely confirmed our results (data not shown).

Table 8: Titers of recombinant AAV-2 vectors.

clone (peptide sequence)	genomic titer* (vector genomes (vg)/ml)		
	rAAV-luc	**rAAV-eGFP**	**rAAV-SR39**
wild-type	$6.8x10^{11}$	$2.1x10^{13}$	$1.2x10^{12}$
random insert (VRRPRFW)	$2.4x10^{11}$	$2.1x10^{11}$	$7.1x10^{10}$
RGDLGLS	$3.7x10^{11}$	$3.6x10^{13}$	$7.4x10^{10}$
RGDMSRE	$2.7x10^{11}$	n.a.	n.a.
DGLGRLV	$2.7x10^{11}$	$2.9x10^{13}$	n.a.
DRSGVGS	$1.7x10^{11}$	n.a.	n.a.
GEARISA	$7.0x10^{11}$	n.a.	n.a.
SSGSGGA	$7.8x10^{10}$	n.a.	n.a.
SGNSGAA	$3.3x10^{11}$	n.a.	n.a.
GGLSGVS	$1.9x10^{11}$	n.a.	n.a.
EYRDSSG	$1.8x10^{11}$	n.a.	n.a.
ESGLSQS	$1.4x10^{11}$	n.a.	n.a.
EEPALRA	$3.2x10^{11}$	n.a.	n.a.
APTLGSP	$4.3x10^{11}$	n.a.	n.a.
DLGSARA	$1.5x10^{11}$	n.a.	n.a.
PRSADLA	$3.0x10^{11}$	n.a.	n.a.
PRSTSDP	$2.8x10^{11}$	n.a.	n.a.

* genomic titers were quantified by real-time PCR as described in Material and Methods

n.d. not determined

n.a.not applicable because no virus solution was prepared

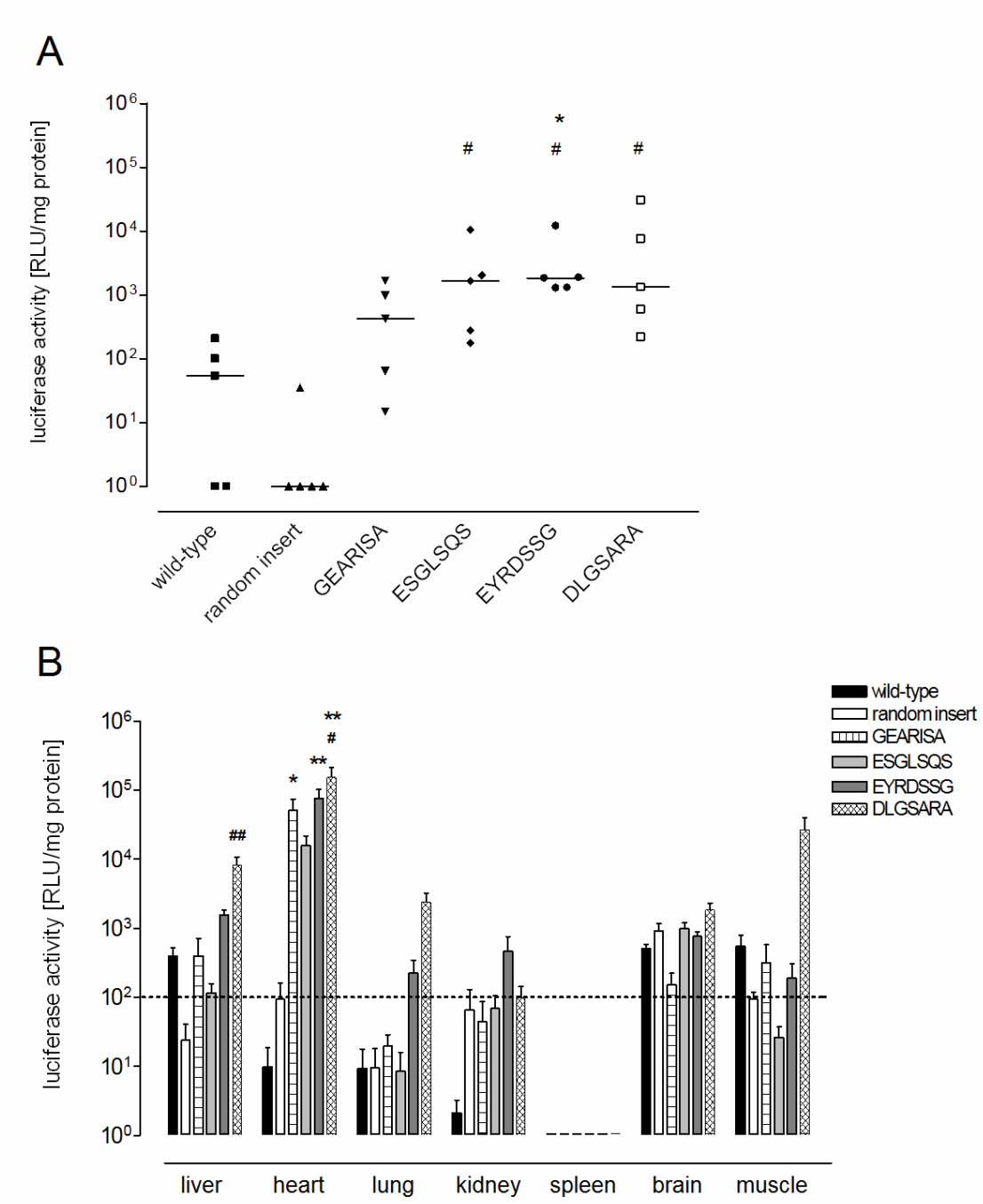

Figure 19: **Gene delivery by AAV mutants selected for breast cancer transduction *in vivo.***
AAV luciferase vectors displaying selected peptides or controls (wild-type or VRRPRFW) were injected intravenously into female PymT tumor-bearing mice. After 8 days, representative tissues were harvested and luciferase activities were determined in individual tissues as relative light units (RLU) per mg protein.
A: *In vivo* transduction of tumor tissue in PymT transgenic FVB mice by selected AAV mutants. Bars indicate the median, n=5 mice per group. * $p<0.05$ targeted vectors vs. wild-type. # $p<0.05$ targeted vectors vs. random insert control.
B: *In vivo* transduction of various tissues in PymT transgenic FVB mice by tumor-selected AAV mutants. The dotted line indicates the threshold beyond which luciferase expression data could be reliably delineated from background signal. Data represent mean values ± SEM, n=5 mice per group. * $p<0.05$; ** $p<0.01$ targeted vectors vs. wild-type AAV-2. # $p<0.05$; ## $p<0.01$ targeted vectors vs. random insert control.

5.4.7 *In vivo* selection of AAV capsids transducing lung tissue

To address the question whether the organ transduction pattern obtained by *in vivo* AAV library screenings depends on the target tissue the library was selected for, we also selected AAV libraries for preferential homing into lung tissue. The screening was done along the lines of the tumor targeting approach (Figure 13, pathway C). We varied the time of library circulation before tissue harvest in the first round (5 minutes and 2 days, respectively, in two independent approaches). For both selections, circulation time was increased to 6 days in selection rounds 2-4. After 4 rounds of *in vivo* selection for both approaches, sequencing of the peptide insert of the AAV clones recovered from the lung revealed a striking consensus sequence motif, PRSAD($^{D}/_{L}$)($^{A}/_{S}$), which was enriched independently in both selection procedures (Table 9). These data show that *in vivo* selection of AAV libraries *in vivo* in distinct tissues yields distinct peptide inserts, suggesting tissue specificity of the selection process.

Table 9: Peptides enriched in lung tissue during *in vivo* selection for lung-homing AAV after four rounds of selection

Selection approach	Peptide [a]
5 minutes circulation in 1st round	**PRSADLA**
	PRSADLA
	VRSAADI
	PRSTSDP
	PRSTSDP
	PRSVDLS
	RGDLGLS
2 days circulation in 1st round	**PRSADLA**
	PRSADLA
	PRSADLA
	VRSAADI
	PRSTSDP
	PRSVDLS
	PRSVDLS
	PASADLA
Consensus motif	**P R S A D ($^{D}/_{L}$) ($^{A}/_{S}$])**

[a]single letter code; shared amino acid patterns are highlighted in red letters

5.4.8 AAV clones displaying the PRSAD ($^{D}/_{L}$)($^{A}/_{S}$) motif transduce lung tissue *in vivo* after systemic administration

Reporter gene vectors were made carrying the PRSTSDP and PRSADLA peptides or controls and gene transduction *in vivo* was evaluated. In a first step, we investigated whether the selected AAV capsid variants home to lung tissue more efficiently than AAV control vectors (wild-type or random insert capsids). Vectors were administered intravenously, and DNA was recovered from lung tissue after 8 days. Quantitative PCR of the CMV promoter region of the vectors revealed an up to 63-fold higher yield for the selected capsid variants compared to AAV-2 wild-type vectors and up to 74-fold higher yield compared to random control insert vectors (Figure 20A). Evaluation of luciferase expression in the lung 28 days after intravenous administration revealed a 35-fold and 233-fold increased transduction efficiency of PRSADLA and PRSTSDP, respectively, compared to wild-type AAV (Figure 20B). To determine the specificity of lung-targeted capsids, luciferase expression in several control organs was evaluated. Both selected clones showed higher gene transduction in liver, heart, kidney, brain, and muscle, compared to unselected controls (Figure 20C), suggesting that the cellular target bound by the selected vectors *in vivo* is ubiquitously rather than lung-specifically expressed.

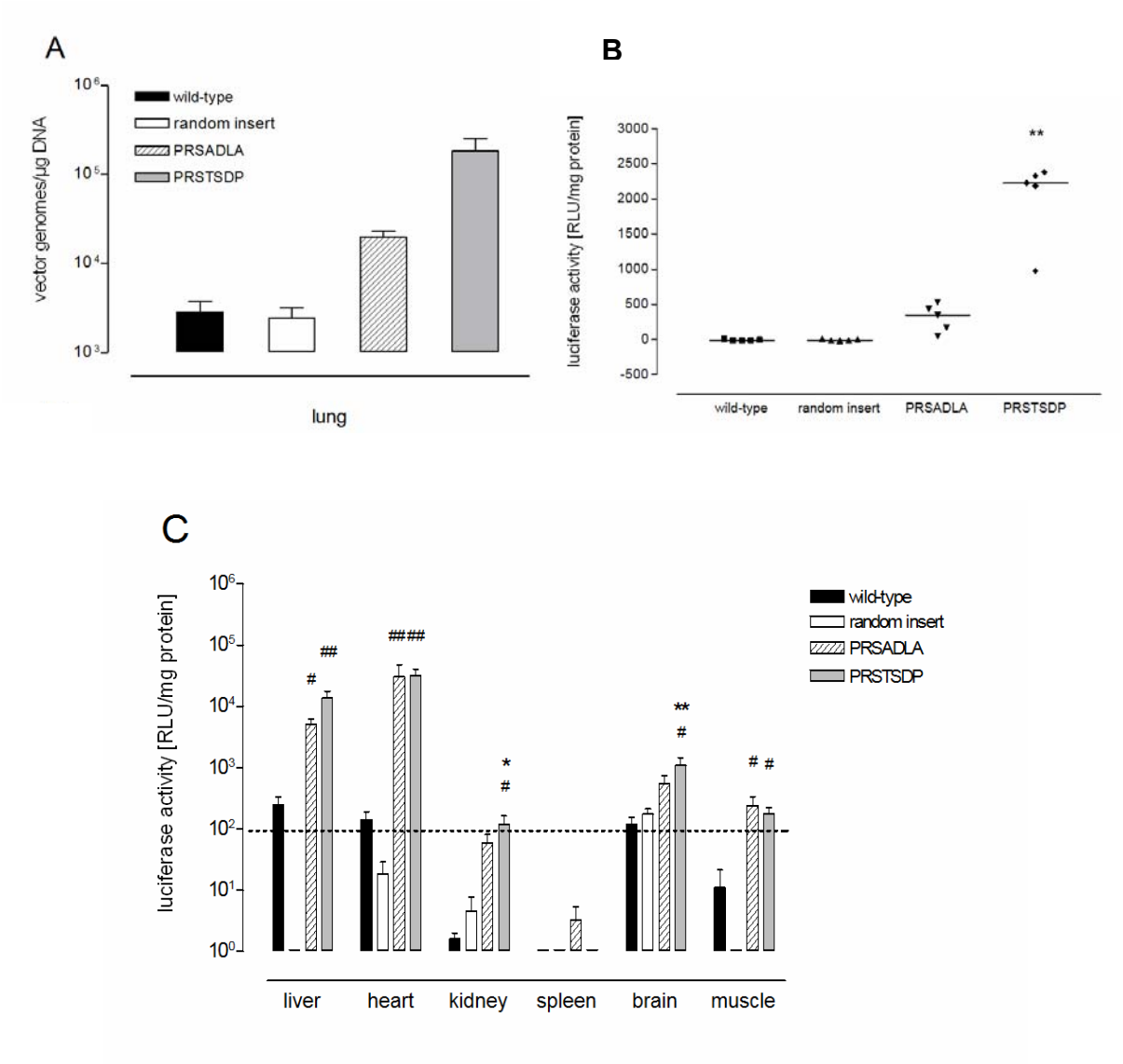

Figure 20: **Targeting of AAV capsid mutants selected on murine lung tissue *in vivo.***
AAV luciferase vectors displaying selected or control capsids (wild-type or random insert VRRPRFW) were injected intravenously into female FVB mice. Tissue was harvested after 8 or 28 days, respectively, and processed as indicated.
A: Evaluation of lung homing. Lung tissue was harvested 8 days after vector injection and the amount of AAV genomes was determined by quantitative PCR. Data represent mean values from n=3 mice per group, analyzed in triplicates ± SD.
B: *In vivo* lung gene transfer by selected AAV after intravenous injection. Lung tissue was harvested 28 days after vector injection, and luciferase activity was determined as relative light units (RLU) per mg protein. Bars indicate the median value, n=5 mice per group (** = p<0.001 targeted vectors vs. wild-type and random insert control).
C: *In vivo* transduction of various tissues in mice by AAV library mutants selected for lung transduction. Tissues were harvested and luciferase activity was determined as in 5B. The dotted line indicates the threshold beyond which luciferase expression data could be reliably delineated from background signal. Data represent mean values ± SEM, n=5 mice per group. * p<0.05; ** p<0.01 targeted vectors vs. wild-type AAV-2. # p<0.05; ## p<0.01 targeted vectors vs. random insert control.

5.5 Discussion

Vector targeting *in vivo* is of paramount importance in gene therapy. For adeno-associated virus (AAV), this issue has been addressed by the insertion of peptide ligands into the vector capsid [81, 88, 181, 228, 277, 278, 284] or by exploiting the diversity of the various capsid serotypes [287, 305]. Despite considerable progress in this field, the availability of tissue-directed vectors for *in vivo* use is very limited. The screening of random AAV display peptide libraries is an innovative tool to select for vectors efficiently transducing any cell type of interest. This approach has advantages over the introduction of known peptide ligands (*e.g.* selected from phage display) into the AAV capsid as it ensures selection of ligands that are compatible with capsid assembly, target cell binding, vector internalization, subsequent translocation to the nucleus and efficient transgene expression. This system has been described and validated for various cell types *in vitro* [165, 174, 194, 226, 276]. Most of the vectors described in these studies, however, have not been validated for *in vivo* use.

Here we show that library-derived vectors selected for optimized transduction *in vitro* do not necessarily transduce the same cell type *in vivo*. As a consequence, we established a system to screen AAV libraries *in vivo* over multiple selection rounds after systemic administration *via* the blood stream, using tumor and lung as target tissues. Several peptide clones were enriched in tumors and a clear-cut peptide sequence motif was recovered from the lung. While we achieved transduction of the target tissue by the selected vectors, we failed to achieve truly tissue-specific transgene expression. Therefore, except for liver transduction, most of our selected vectors have a tropism that is expanded to rather than specific for the tissue of interest compared to wild-type AAV-2. This may be due to several reasons: 1) the lack of tissue-specific receptors; 2) the expression of receptors conferring optimum transduction in several tissues, so capsids targeting receptors that are tissue-specific, but less efficient for transduction are not enriched; 3) superordinate (not receptor-dependent) factors influencing the selection process such as endothelial barriers, blood-derived factors, or extracellular matrix interactions. The first argument can be virtually excluded based on the overwhelming success of *in vivo* tissue targeting using phage display libraries [4-6, 43, 66, 114, 131, 133-135, 141, 156, 191, 201, 206, 255, 299].

Regarding the expression of non-tissue-specific receptors that are compatible with optimized AAV transduction, we think that two factors may play a role. Some of the selected peptides mediated transduction of several tissues with a clone-dependent

transduction pattern, suggesting that the tropism is mediated by the targeting peptide. Especially for the lung-transducing vectors, the broad-spectrum tropism may also be due to the mechanism of library selection. Upon intravenous injection, virus capsids with optimized *in vivo* transduction behavior may have been enriched in the lung irrespective of tissue specificity due to the first-pass effect after intravenous injection. These vectors may well be cell type-specific but not tissue-specific. They may be directed to endothelia in general, which is underlined by the fact that a similar capsid mutant (PRSVTVP) has been previously selected on primary human coronary artery endothelial cells *in vitro* [174]. This emphasizes the importance of the simultaneous negative selection *in vivo* screenings that can be achieved in tissues other than the lung. The second factor influencing the extended but unspecific tropism relates to the remarkable observation that as long as our selected vectors conferred any transgene expression, it invariably also occurred in the heart in addition to the target tissue. Heart expression of these vectors was even stronger than in wild-type AAV vectors. Such increased heart transduction of selected AAV indicates that a capsid region close to the library insert at position R588 may mediate this tropism and that it is therefore independent of the selected peptide sequence as such. Thus, a capsid region close to the AAV capsid region harboring the library peptide insertion may mediate homing to und transduction of this organ. This is congruent with previous studies describing increased heart transduction upon modification of the VP3 region R484E/R588E [124] and peptide insertions at position R588 [174]. Yet, biodistribution studies have not found increased heart homing by peptide insertion in this region [278, 284], which might in part be attributable to, or at least influenced by, a slightly differing insertion site (position N587 instead of R588). In addition, it re-emphasizes that gene expression of a vector is not necessarily reflected by its biodistribution due to factors influenced by intracellular processing, promoter activity and vector clearance mechanisms. Our results in conjunction with the previously published data suggest that cardiac transduction may be mediated by a redistribution effect resulting from ablation of the endogenous tropism of the vector [124, 174], but clearly is also mediated by the design of the peptide insert as it varied from clone to clone.

Nevertheless, *in vivo* screening of AAV libraries allows selection for vectors with an extended tropism for the tissue of interest. Vectors targeting the endothelial cell layer *in vivo* might be used to deliver anti-angiogenic genes such as endostatin in order to

block neovascularization and tumor growth [142]. Furthermore, vectors transducing various organs might be useful when expression in tissues other than the primary target is desirable or uncritical as it has been performed by expression of the SOD gene delivered by adenovirus to protect lung tissue against radiation-induced fibrosis [64].

Finally, the tumor specific vectors displaying the ESGLSQS peptide that mediates AAV transduction of breast cancer tissue *in vivo* and AAV de-targeting from the liver may further be optimized by using tumor specific expression systems such as the hTERT promoter [271, 293], with the aim to develop a breast cancer-targeted gene therapy approach in the PymT mouse model.

In previous work on AAV libraries, internalized virus particles were amplified by adenoviral delivery of helper proteins [165, 174, 194, 276]. However, the pathogenicity of adenovirus impedes the use of this strategy for *in vivo* selections and in addition it cannot be used for AAV library screenings on cells or tissues not susceptible to adenovirus. Here we introduce a novel, nested PCR-based selection approach allowing for adenovirus-free biopanning of AAV libraries over multiple selection rounds *in vitro* and *in vivo*. We distinguished between three alternative selection pathways, all of which are based on such library amplification by PCR. Pathway A is a cell-based *in vitro* selection approach in which genomes of internalized library viruses are amplified while non-internalized viral particles are eliminated. In a proof of concept screening, we demonstrated the functionality of this technical approach in that clones sharing a common peptide motif (RGDXXXX) were recovered by screening on primary murine breast cancer cells and conferred efficient transduction of these cells. Similar peptide motifs have been selected on PC3 prostate carcinoma cells [276] and M07e human leukemic megakaryocytic cells [194] by adenovirus-based selection. Incorporation of the RGD sequence into the viral capsid retargets the vector to integrins, which are widely expressed on several cell types [81, 228, 231] suggesting that these clones might target *via* the integrin class of receptors [215].

Pathways B and C aimed at selection of viruses after systemic administration of AAV libraries *in vivo*. These pathways have the advantage that tissue homing particles with weak or unspecific binding capacities toward their targets are eliminated by blood clearance mechanisms or by homing to other tissues. Upon using selection pathways A, B, or C for tumor targeting, the enriched peptide sequences varied

depending on the respective selection pathway, indicating that the most suitable screening conditions may have to be evaluated for each individual target tissue.

Compared to the previous *in vitro* work of our group and others, these results are a significant step forward and expand our knowledge on the mechanisms involved in vector targeting profoundly. In addition, they overcome some of the limitations observed in a recent report by Grimm et al. [90]. In this pivotal work, AAV libraries were selected *in vivo* based on topical application to the airways. However, the diversity of recovered AAV after two rounds of selection was restricted to one clone, presumably due to inefficient amplification of clones. Such outcome might change upon applying our novel amplification protocol. Furthermore, novel AAV library principles like sequence evolution by error-prone PCR [158], DNA shuffling [150] might enhance specificity and efficiency if used for *in vivo* selection.

This is the first report of a successful *in vivo* biopanning with a systemically administered random AAV peptide library over multiple selection rounds. We show that vectors displaying *in vivo*-selected peptides have a significantly improved transduction profile in breast cancer or lung tissue. These findings demonstrate the superiority of AAV clones selected *in vivo* over clones selected *in vitro,* as long as *in vivo* transduction is required. Unintended cardiac transduction by selected clones remains the major limitation to be addressed in subsequent studies, *e.g.* by mapping the capsid site mediating this tropism. Our findings broaden the understanding of the AAV transduction behavior *in vivo*, the functionality of random AAV display peptide libraries and, even beyond the specific targets tumor and lung, are an important step in the development of targeted AAV gene vectors *in vivo* in general.

5.6 Acknowledgements

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6 Drug delivery in acute myeloid leukemia

Johannes Kohlschütter[1*], Stefan Michelfelder[2*], Martin Trepel[1,2]

[1] University Medical Center Hamburg-Eppendorf, Department of Oncology and Hematology, Martinistrasse 52, D-20246 Hamburg, Germany

[2] University of Freiburg Medical Center, Department of Hematology and Oncology, Hugstetter Strasse 55, D-79106 Freiburg, Germany

* these authors contributed equally to this work

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6.1 Abstract

Background: Acute myeloid leukemia was among the first malignancies to be cured by drug therapy alone, but overall survival rates remain unsatisfactory and have changed little over the past twenty years. Conventional chemotherapeutic regimens, which almost invariably include cytarabine and anthracyclines, are untargeted, and more specific therapies are needed.

Objective: We have chosen acute myeloid leukemia as a prototype of disease to review established as well as novel, targeted approaches in leukemia treatment.

Methods: Our selection of literature was focused on drug delivery aspects.

Conclusion: While the toxicity profile of chemotherapeutics has been improved by liposomal formulations and antibody-conjugation for leukemia-directed uptake, their efficacy has probably not changed significantly. Drugs with an alternative mode of action, including kinase inhibitors, hold great promise. Further improvements may result from the characterization of novel AML cell receptors and of leukemic stem cells, and from the design of leukemia-targeted gene therapy vectors.

6.2 Introduction

The term leukemia comprises a heterogeneous group of diseases characterized by the malignant clonal proliferation of blood progenitor cells. These cells primarily grow and expand in the bone marrow, and from there spread to the entire body via the blood circulation. Thus, there is an accumulation of abnormal, often immature leukemic cells in the bone marrow, peripheral blood, and other tissues. The expansion of the malignant clone within the bone marrow results in a reduced number of normal red blood cells, platelets, and neutrophils. This causes a variety of systemic symptoms and signs, the most important of which are anemia, bleeding, and an increased risk of life-threatening infections. The latter is the most frequent cause of death in leukemia.

Based on the kinetics of disease onset and course as well as the differentiation of the malignant clone, leukemias are divided in acute and chronic as well as in myeloid and lymphocytic, respectively. While acute leukemias usually have a rapid course and, if untreated, invariably have a fatal outcome within weeks or months after initial presentation, chronic leukemias tend to have a longer course of years or even decades.

In terms of drug delivery, leukemia has unique features. Most importantly, leukemia is by definition a systemic disease, and therefore drug delivery will always have to use a systemic route. Most of the currently available therapeutic agents, both established and experimental ones, are applied intravenously, but an increasing amount of newer drugs are applicable orally or subcutaneously.

This review focuses on acute myeloid leukemia (AML). Drug therapy and delivery have been studied most extensively in this form of leukemia and we can only summarize some of the many new aspects in drug therapy that have evolved for this disease during the last decade. The term AML comprises several subgroups of leukemias which share the acute course and the myeloid marker profile, but vary in differentiation, genetic aberrations, response to treatment, and prognosis. Yet, except for acute promyelocytic leukemia, the therapeutic approach for most AMLs has been similar, which may change over the next decade based on the availability of targeted drugs and tailored treatment strategies.

AML evolves based on a series of genetic changes in a hematopoietic precursor cell, altering normal hematopoietic growth and differentiation, and finally resulting in expansion of the malignant clone in the bone marrow and peripheral blood. These cells apparently have unlimited proliferation potential but usually do not maturate into regular blood cells such as erythrocytes, platelets, or neutrophils. Like in other malignancies, the genetic alterations in AML result in both the activation of oncogenes and the dysfunction of tumor suppressor genes. Unlike most solid tumors, however, many hematologic malignancies including AML are associated with a single characteristic cytogenetic abnormality such as the translocation t(15;17) in acute promyelocytic leukemia.

Current treatment strategies are mainly based on high dose chemotherapy regimens using anthracyclines and cytarabine as backbone drugs [67]. After having achieved complete remission, allogeneic stem cell transplantation plays an increasing role especially in high risk patients with unfavourable cytogenetic profiles, other risk factors, or relapsed disease. The toxicity of these current treatment regimens is considerable, preventing their use especially in elderly patients [67].

A number of factors predicting poor outcome have been described for AML, including poor performance status, advanced age, karyotype and other molecular changes [185]. Overall, the prognosis of patients suffering from AML is still poor, despite significant therapeutic advances over the last two decades. Less than 40% of the AML patients

younger than 60 years of age can be cured [3, 67, 242, 248]. In older adults, accounting for the majority of AML patients, long term disease-free survival is rare and the available treatment options are limited [3, 19, 242]. These discouraging facts have spurred major efforts in the development of novel targeted therapies in the treatment of AML. Many of such new therapies targeted to specific molecular features of AML are currently under clinical evaluation and some of them are discussed below.

This review focuses on which drugs are available to be delivered to AML cells, which are their delivery routes, and which are their potentials and their limitations. Delivery has to take into account the general approach, which is always systemic in leukemia, the route of administration, the interaction of the drug with the cell membrane (active internalization, passive diffusion), and intracellular trafficking.

6.3 Drugs and Drug Delivery for Acute Myeloid Leukemia

6.3.1 Classical Cytostatic Drugs

Standard chemotherapeutic regimens for AML treatment are based on a combination of an anthracycline and cytarabine.

6.3.1.1 Anthracyclines

Anthracycline development began in the 1960s [56]. Most of these agents have to be administered intravenously, except for idarubicin for which an oral formulation is available. Anthracyclines are taken up by the target cell via passive diffusion and, once inside the nucleus, intercalate with DNA. Furthermore, they inhibit strand re-ligation by topoisomerase II, causing DNA double-strand breaks [46]. After hepatic metabolization, anthracyclines are eliminated by biliary excretion. Daunorubicin is the anthracycline most often used for AML treatment. Its lipophilic analogue idarubicin and its active metabolite 13-hydroxyidarubicin have a longer half-life than daunorubicin. Despite preclinical evidence suggesting otherwise, clinical trials have failed to prove a substantial advantage of idarubicin over daunorubicin in terms of efficacy and toxicity [267]. Mitoxantrone is a synthetic anthracycline analogue used in combination with cytarabine for AML with at least comparable, maybe superior efficacy in upfront and re-induction regimens [8, 37].

6.3.1.2 Cytarabine

Cytarabine was approved by the FDA almost 40 years ago. The drug is administered parenterally, for induction regimens usually intravenously, and has a short half-life requiring high-dosed short time or medium-dosed continuous infusions [197, 222]. Inside the cell, the phosphorylated drug enters the nucleus and is incorporated into DNA in place of cytosine, blocking DNA replication. Cytarabine is metabolized by cytidine deaminases and is eliminated by renal clearance. Like other chemotherapeutics, its action is cell cycle-dependent, and therefore its therapeutic effects are focused on rapidly dividing cells like cancer cells despite its unspecific biodistribution.

6.3.1.3 Standard treatment for patients in good physical condition

The most common chemotherapy regimen to induce remission in AML is daunorubicin as a 15 minute intravenous injection daily for three days plus cytarabine given by continuous intravenous infusion for seven days (so-called "3 + 7" regimen). With this regimen, 60-80% of patients, depending on age and other risk factors, achieve a complete remission [27, 67]. This response rate has not been improved to a clinically relevant extent by changing the dose of any of the two agents or by adding an additional drug. The cytostatic agents used for remission induction confer substantial toxicity including myelosuppression, mucositis, diarrhea, and cardiotoxicity.

6.3.2 Novel Therapeutic Agents

In view of the high remission rates achieved in AML patients using the standard chemotherapeutic regimens, novel agents would have to meet high standards of efficacy to replace these regimens [44]. However, relapse rates and toxicity as well as the limited treatment options in elderly patients highlight the urgent need for novel agents that improve disease-free survival and do not add substantial toxicity. While conventional chemotherapy may remain the backbone of treatment, novel agents could be added to improve outcome. Within the last years, many such novel agents have been introduced. Some of them have started to gain the status of a standard treatment option in certain settings, such as liposomal or antibody-conjugated chemotherapy. Others are currently at a more experimental stage, including farnesyltransferase inhibitors [117], histone deacetylase inhibitors [136], proteasome

inhibitors [297], or antiangiogenic agents such as bevacizumab [116]. Yet, many challenges remain, which are addressed at the end of this article.

6.3.2.1 Liposomal Delivery of Chemotherapeutic Drugs

Anthracyclines are one of the two standard chemotherapeutic drugs in AML. However, their toxicity is of concern. Above all, cardiotoxicity is dose-limiting and cumulative dose-dependent, which often prevents anthracycline re-treatment in relapsed AML or even upfront treatment in patients with cardiac disease.

To increase the therapeutic index, liposomal formulations have been proposed as carriers for cancer therapeutics several decades ago [85]. Liposomes encapsulate an aquaeous solution containing the drug inside a hydrophobic membrane. Liposomal encapsulation results in reduced anthracycline uptake by normal, non-neoplastic tissues. In contrast, delivery to tumor tissue and to the bone marrow is enhanced due to the passage of liposomes through fenestrations of the vascular endothelium which are characteristic for these but not other tissues [77, 192]. Liposomes are believed to be taken up by membrane fusion rather than endocytosis unless they are modified specifically to trigger this event[252]. Liposomal formulations are characterized by slower pharmacokinetics compared to non-encapsulated administration of a given drug. They may therefore be the agents of choice when the objective is to maintain a defined plasma concentration with little change over time, rather than high, but quickly decaying, peak levels.

Liposomal formulations of doxorubicin and daunorubicin are currently available for clinical use. The application of liposomal daunorubicin in AML has been extensively reviewed elsewhere [68]. Briefly, compared to conventional daunorubicin application, liposomal daunorubicin results in reduced conversion into its toxic metabolite daunorubicinol and reduction in toxic side effects such as cardiotoxicity, alopecia, nausea, or myelosuppression. In addition, various *in vitro* studies suggest that liposomes may help to overcome P-glycoprotein-mediated efflux of anthracyclines, a mechanism believed to contribute substantially to anthracycline resistance in AML and other tumor cells [166, 250]. Liposomal daunorubicin combined with cytarabine or alone yielded a complete remission rate of approximately 30% - 45% in patients with refractory or recurrent AML [53, 69].

Liposomes can be targeted by incorporation of homing molecules into their hydrophobic surface. For instance, attachment of folate molecules to liposomes [301]

via a PEG anchor was used to target cells expressing the folate receptor, a common property of malignant cells in general [146], and of AML cells in particular [156, 214]. The efficiency of such targeting approaches could possibly be increased if the expression of a receptor of interest can be stimulated such as it is possible with all-trans retinoic acid that induces an upregulation of the folate receptor in AML cells *in vitro* [269].

Efficient liposomal delivery may require sophisticated strategies depending on the drug of interest. For arsenic trioxide, a procedure for the formation of nickel (II) arsenite complexes in liposomes that release the active drug under acidic pH conditions as present in lysosomes has recently been suggested [49]. Increasing particle stability is an important issue in improving liposomal therapy, but it may be achieved at the cost of impaired drug release. A recently described approach using lipase may overcome this problem [51].

6.3.2.2 Novel Drugs Interacting with Intracellular Targets

The tremendous success of the BCR-ABL tyrosine kinase inhibitor imatinib mesylate in chronic myeloid leukemia has stimulated the exploration of novel agents targeting various pathways in cancer. For AML, our increasing knowledge about intracellular signaling cascades involved in this disease has revealed a number of promising targets for inhibitory therapy by small molecules. They are usually applied orally and do not depend on receptors for cellular uptake.

One therapeutic approach is directed towards the RAS protein, which is frequently mutated and therefore dysregulated in AML and other malignancies [180]. Attachment of RAS and other regulatory molecules to the plasma membrane is crucial for their functionality. Small molecule farnesyl transferase inhibitors such as tipifarnib and lonafarnib [112], after passively diffusing into the cell, inhibit RAS membrane anchoring. Tipifarnib has achieved clinical responses in patients with refractory and relapsed poor-risk AML [117] and is currently being evaluated in phase III trials [9, 240].

Another novel therapeutic approach targets the FMS-like tyrosine kinase 3 (FLT3). Mutations in the *FLT3* gene producing internal transmembrane duplications (FLT3/ITD) are common in AML and result in constitutive FLT3 activation [138, 179]. A number of small molecule inhibitors of FLT3 have been evaluated in clinical trials lately, including tandutinib (MLN518), lestaurtinib (CEP-701) [235], and PKC412, and evidence of antileukemic activity has been seen [130, 235, 241]. Like other kinase

inhibitors, these agents are orally applicable and their delivery to AML cells is receptor-independent.

While the oral application of small inhibitory molecules simplifies their use in an outpatient setting, this may not always be the preferred way of administration given the poor oral intake and nausea experienced by many cancer patients under treatment [300]. In addition, target specificity remains an issue in kinase inhibitor therapy. Under some conditions, inhibitors with multiple targets may have beneficial effects, as shown recently for the multi-kinase inhibitor sorafenib in a xenograft model of FLT-driven leukemia [13]. Yet, the lack of specificity of some kinase inhibitors may account for limited anti-leukemic activity and side effects. The latter are usually considered mild compared to those associated with conventional cytostatic drugs, but can occasionally be quite severe, *e.g.* in heart tissue, as described for imatinib and other agents [76].

In terms of specificity, agents such as monoclonal antibodies or peptides targeting cell surface molecules may therefore be superior to small molecules.

6.3.3 Receptor-targeted Drug Delivery in AML

Targeting cell surface molecules in cancer is a paramount issue in drug delivery both affecting efficacy and specificity (and therefore toxicity) of an antineoplastic drug. By specific homing after systemic administration, compounds are directed to the cell type or tissue of interest. This prevents their action in non-target tissues, thereby increasing therapeutic efficiency while decreasing adverse effects. Thus, as for other malignancies, drug-conjugated ligands targeting unique surface receptors have been developed for AML treatment.

6.3.3.1 Anti-CD33 monoclonal antibodies

During the last decade, targeted monoclonal antibodies have revolutionized cancer therapy. In AML, the CD33 antigen is a promising target since it is ubiquitously expressed on myeloid blasts in most patients, but neither on healthy pluripotent hematopoietic stem cells nor most non-hematopoietic cell types. CD33 is a member of the sialic-acid binding Ig-like lectin (Siglec) family and has two cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). CD33 is involved in cell-cell interactions and signaling in the hematopoietic system and may have regulatory

functions in the immune system and in cell proliferation [143, 188]. The first targeted compound successfully used in AML treatment was Gemtuzumab ozogamicin (GO), a monoclonal anti-CD33 antibody linked to the cytotoxic agent calicheamicin. The conjugate is usually given as a two-hour intravenous infusion. Following systemic administration, GO is efficiently and specifically directed to CD33-positive cells. Upon binding to CD33, the GO-CD33 complex is rapidly internalized. The uptake is boosted by new CD33 molecules replacing the internalized ones [258]. Lysosomal release of calicheamicin and translocation to the nucleus cause DNA double-strand breaks and cell death. The efficacy of the drug is influenced both by CD33 expression level and P-glycoprotein activity [268]. Consequently, therapeutic efficacy of GO may be potentiated by *in vivo* stimulation of CD33 surface expression on AML blasts in patients with G-CSF [147], or by reducing the calicheamicin efflux of malignant cells by P-glycoprotein inhibitors [178].

GO treatment in patients with relapsed AML can result in remission rates as high as almost 30% [45, 188, 232, 240, 248, 281]. As CD33 is also expressed by benign myeloid precursor cells, Kupffer and sinusoidal liver cells, myelosuppression and hepatotoxicity are common GO-side effects [188]. In addition, anaphylactic reactions and veno-occlusive disease have been described as life-threatening side effects in a low but significant number of patients. Other toxicities of GO include fever, hypotension, and abnormal liver function tests, all of which are usually transient [239].

Anti-CD33 antibodies have shown effects against leukemic cells *in vitro* even without the attachment of a cytotoxic drug [266]. However, the unconjugated humanized anti-CD33 monoclonal antibody lintuzumab failed to elicit anti-leukemic effects when added to conventional chemotherapy in a phase III trial [70]. Nevertheless, the promising studies using GO reveal the potential of targeted drug delivery in AML treatment.

Since FMS-like tyrosine kinase 3 (FLT3) is expressed on approximately 90% of AML cells and plays a major role in survival and proliferation signaling in leukemia blasts, several FLT3 small inhibitor molecules have been demonstrated to show anti-leukemic activity, as outlined above. Nevertheless, the lack of specificity of these kinase inhibitors remains a significant problem as they also interact with several other cellular kinases [248]. Furthermore, cellular targets of most chemotherapeutic agents are located in the nucleus, therefore rapid internalization of drug-ligand conjugates is critical to maximize therapeutic efficacy while minimizing side effects. Towards this

end, several FLT3-directed antibodies were isolated using a cell-based phage library screening protocol and two fully human antibodies with the capability to trigger efficient receptor internalization upon binding to FLT3 were generated [280]. Such anti-FLT3 antibodies may be promising therapeutic agents in FLT3-expressing AML for receptor blocking or for antibody-guided cytotoxic drug therapy.

For further development of receptor-targeted cancer therapy, a comprehensive understanding of differential receptor expression is needed. So far, very little is known about receptors specifically expressed on AML cells and their interaction during disease development and progression. Some knowledge about unique receptor profiles of AML cells may be gained from microarray gene expression profiling [38, 257]. Among the limitations of such approaches is the fact that the protein expression patterns do not necessarily correlate with the functional state and extracellular accessibility of the potential target molecule. Protein-based techniques may be of advantage here, as discussed in the following section.

6.3.3.2 Novel Cell Surface Markers as Potential Therapeutic Targets in AML

Phage display is a powerful tool to select for novel ligands targeting cell-type specific surface molecules even if only the cell type of interest rather than an exact target receptor is known *a priori*. The receptors bound by such ligands can be subsequently identified in the majority of cases. Screening phage displayed human antibody libraries on primary AML blasts, Bakker *et al.* enriched a single chain Fv fragment strongly binding to myeloid cells. The antigen was identified to be the transmembrane glycoprotein C-type lectin-like molecule 1 (CLL-1). CLL-1 acts as a signaling receptor and is expressed in >90% of AML samples. CLL-1 appears to be restricted to hematopoietic, particularly myeloid, cells. It is also weakly expressed in CD34+/CD38+ or CD34+/CD33+ progenitor cells. Of note, CCL-1 expression is absent in the CD34+/CD38- or CD34+/CD33- stem cell compartment [15] but may be found in CD34+/CD38- leukemic stem cells [259]. Almost 70% of CD33-negative AMLs expressed CLL-1, indicating that CLL-1 complements CD33 as a therapeutic cell surface target for AML. Anti-CLL-1 antibodies may therefore have great potential for AML therapy and for the detection of AML stem cells. This may improve efficacy of current therapeutics, especially when combined with CD33-directed therapy [15].

A non-biased approach to the identification of high-affinity binding ligands is the screening of phage libraries displaying small random peptides. This strategy has

been successful for a variety of cell types and tissues *in vitro* and *in vivo* [135, 254]. Linked to cytotoxic agents, such peptide ligands can be exploited for targeting cytotoxic drugs or other therapeutic agents to the cell type of interest [4, 7, 63, 134, 255]. Furthermore, screening phage peptide libraries allows for the exploration of epitopes recognized by known antibodies or even the identification of novel molecular markers by fingerprinting of circulating antibodies in cancer patients [25, 26, 167, 264].

In a recent study, we selected phage libraries on AML cell lines. We identified a peptide with the amino acid sequence CPLDIDFYC which strongly and specifically binds to AML cells [109]. Binding correlated with the expression of the *AML1/ETO* fusion gene which is a result of the the chromosomal translocation t(8;21), the most frequent karyotype aberration in AML. We identified VLA-4 (α4ß1) integrin as a potential receptor for the leukemia cell-binding CPLDIDFYC peptide [109]. VLA-4 is involved in cell-cell and cell-extracellular matrix adhesion by interaction with the vascular cell adhesion molecule VCAM-1 and the extracellular matrix protein fibronectin. Attachment to fibronectin within the bone marrow stroma appears to mediate resistance to chemotherapeutic drugs in leukemia cells [160]. CPLDIDFYC and other VLA-4 antagonists such as the monoclonal anti-VLA-4 antibody natalizumab may therefore serve as future therapeutic agents in AML for receptor blocking or for cytotoxic drug delivery.

6.3.3.3 Leukemic Stem Cells as Potential Therapeutic Targets in AML

Acute leukemia most likely develops from a single transformed hematopoietic progenitor cell. A substantial amount of evidence suggests that, once this cancer has evolved, a subpopulation of leukemia cells with the stem-cell-like characteristics of asymmetric division and self-renewal capacity drives the course of the disease. The characterization of these leukemic stem cells (LSCs) has therefore gained tremendous interest during the last decade. LSCs may withstand cytotoxic chemotherapy as they are often in a quiescent state, unlike their rapidly proliferating progeny [104]. LSCs are therefore considered to be responsible for recurrence of leukemia even after initial treatment success. LSCs have been characterized by the presence or the absence of various sets of surface markers, but are widely recognized to be part of the CD34+/CD38- cell compartment [34, 144].

LSCs may be distinguished from non-malignant hematopoietic cells by the presence of the interleukin-3 receptor α chain (CD123) [113]. This finding has made CD123 a

potential therapeutic target. A diphtheria toxin-interleukin-3 fusion protein has shown toxicity against LCSs while sparing normal progenitors *in vitro* [72, 102], and such treatment prolonged survival in a mouse model [28]. The compound was recently evaluated in a phase I study [78].

While markers exclusively expressed on LSCs appear particularly attractive for the purpose of targeting LSCs, there is evidence that certain receptors can be promising therapeutic targets even if they are expressed on other cell types as well. The adhesion molecule CD44 – although expressed ubiquitously – is thought to be crucial to the malignant properties of AML LSCs, and an activating anti-CD44 antibody reduced engraftment of AML cells in a mouse model [111].

6.3.3.4 Gene Delivery

Despite many hurdles, gene therapy might be a future option for AML treatment. The spectrum of therapeutic transgenes mediating killing of malignant cells comprises genes encoding toxic, pro-apoptotic, antiproliferative proteins or classical suicide genes such as the herpes simplex virus thymidine kinase gene. Alternatively, immune system-mediated cancer cell elimination may be achieved by delivery of genes encoding costimulatory molecules, *e.g.* interleukin-2 (IL-2), IL-7, IL-12 [62 73, 223], or immunomodulatory molecules such as CD40, CD80 [119, 184, 243], or interferon β [35].

One of the major unsolved issues in gene therapy is vector application and delivery to the cells or tissue of interest. Development of efficient and specific vectors for gene transfer is just as crucial to therapeutic success as is the choice of the transgene itself. Currently, viral vectors remain the most effective means for therapeutic gene delivery, although substantial progress in non-viral transduction of hematopoietic cells has been achieved, including electroporation, nucleofection, and particle bombardement techniques [221]. Initial *in vitro* experiments have suggested lentiviral [243], retroviral, or adenoviral vectors as suitable delivery vehicles for leukemic cells [211]. However, unintended integration of retroviral vectors into the genome or adverse immune reactions elicited by adenovirus administration are serious safety issues to be considered in choosing vectors for clinical application. Over the past years, vectors derived from adeno-associated virus (AAV) have emerged as efficient tools to achieve long-term gene expression in a wide range of cell types. The low frequency of random integration into the genome [47], as well as the absence of a

substantial cellular immune response make AAV vectors promising tools in terms of biological safety [54] [24].

Various approaches have been taken to make the binding of therapeutic vectors to target cells more efficient and specific. Bispecific conjugates such as antibodies that bind to both a vector and a target cell are one strategy [256]. However, such complexes may be unstable or immunogenic, compromising efficiency and safety. This issue may be overcome by covalent vector modifications. Towards this end, AAV offers various opportunities for targeting. The natural tropism of AAV capsids may be changed by exploiting the diversity of natural serotypes [287]. Alternatively or in addition, peptides mediating binding to the cell type of interest can be identified by random phage display library screening and subsequently be introduced into an AAV capsid region critical for receptor binding [88, 155, 182, 209, 228, 278]. However, the success rate of this approach is variable. Our own experience has been that only a minority of selected peptide ligands function equally well in targeted phage particles as they do in modified vector capsids such as adenovirus or AAV. This may be attributable to the fact that the phage-derived peptides were selected only for cell or receptor binding but not for subsequent post-targeting cell entry which is required for gene transfer. Furthermore, the structural context is probably crucial. The binding property of a ligand peptide may change unpredictably when it is incorporated into a virus capsid protein subjecting it to structural constraints not present in the phage capsid that was used for selection of the ligand from the random library. Taking these limitations into account, we and others have developed random peptide-display libraries based on the gene therapy vector capsid itself for AAV [174, 194] and later for retroviruses [42, 95, 125, 126]. Thus, peptide ligands binding to a cell type of interest within the specific viral capsid protein context can be selected. Using this approach, vectors were isolated that specifically and efficiently transduce the cell types they have been selected on [174, 194, 276].

We have recently screened random AAV-displayed peptide libraries on several AML cell lines, enriching the leukemia targeting peptide motif NQVGSWS [165]. Vectors displaying such peptides transduced several hematopoietic cancer cell lines, but not a panel of control cells. Consequently, such targeted AAV mutants can be used for therapeutic suicide gene transfer, achieving cell type specific killing in AML cells [165].

6.4 Conclusion

Despite all efforts to optimize drug therapy during the last two decades, acute myeloid leukemia remains a devastating disease with a dismal prognosis especially in the elderly. Chemotherapy based on anthracyclines and cytarabine, in some cases combined with stem cell transplantation offers the only chance of cure. However, such therapy and curative outcome is usually limited to the minority of all patients, *i.e.* the young, fit patients with few or no risk factors.

Drug delivery to leukemia has to take into account the need for systemic drug administration and the need for prevention of collateral damage caused by the toxicity of current therapy regimens. In terms of drugs and drug delivery, recent progress comprises the liposomal formulation and antibody-guided application of classical chemotherapeutic agents, and the identification of novel drug targets for intracellular kinase inhibitors. These concepts have begun to prove their value in clinical studies and some of them will likely gain status as established leukemia therapeutics within the near future. More experimental approaches that are likely to translate into therapeutic concepts within the next 10 years are the targeting of leukemic stem cells and the design of gene therapy vectors specifically and efficiently targeting leukemia cells.

6.5 Expert Opinion

Acute myeloid leukemia is a systemic disease. As such, it used to be the hallmark of success of modern cancer drug therapy. When the classical cytostatics were introduced in the treatment of cancer several decades ago, acute leukemias were among the few malignancies in which consequent improvement of cytostatic drug development and treatment protocols actually resulted in cure of some of these patients who, before that, invariably died of their disease. Ever since, however, progress has been slow and the gain in survival rates has been slim. Most of this progress has been unrelated to drug development or drug delivery but rather to advances in supportive care (including anti-infectants and optimized transfusion indications) and allogeneic stem cell transplantation which is now associated with significantly less toxicity and is amenable even to the elderly beyond 70 years of age.

But what are the advances in terms of novel drugs or novel drug delivery mechanisms? In fact, substantial progress has been made here, even though it may

not yet have translated into improved survival rates. The development and optimization of liposomal packaging of key drugs in AML treatment like daunorubicin reduce toxicity and therefore improve therapeutic indices. Whether their theoretical advantage in efficacy translates into a clinically meaningful one has yet to be proven. We believe that if there is one, it is probably small, for the reasons discussed below. Along a similar line, the conjugation of cytostatic drugs to antibodies that target AML cell surface receptors such as CD33 must be considered as a significant advancement even though the most significant toxicity profile of classical unconjugated cytostatics, the suppression of hematopoiesis, occurs with anti-CD33 conjugates as well. This is because CD33 is not a truly AML-*specific* antigen. Other side effects, however, are less severe than in conventional chemotherapy and therefore both liposomal as well as antibody-conjugated targeted drugs may replace conventional drug formulations within the next ten years.

While such advances in drug delivery reduce or change the profile of side effects, they seem not to have an impact, or at least not a major one, on relapse rates, compared to conventional drugs. Thus it seems that the issue of leukemia cell resistance to therapy is an issue of the molecular mechanism of drug action rather than an issue of drug delivery. It is therefore mandatory to identify novel therapeutic targets both inside and outside of the leukemia cells to develop drugs with no cross resistance to the ones that are already available. In this regard, like in cancer therapy in general, enormous efforts have been dedicated both by academic research as well as the industry, to translate our ever increasing knowledge in cancer biology into therapeutic strategies.

For AML, the most relevant drug developments have been kinase inhibitors blocking RAS membrane anchoring or FLT3 activity, which both play a major role in AML pathobiology. Many more such small molecule drugs are currently being tested in clinical trials and we consider it very likely that some of them will have an enduring place in the arsenal of weaponry for the combat against AML. Interestingly, unlike for solid tumors, antibody therapies (other than for targeted drug delivery) have played a small, if any, role in the new generation AML drugs so far. This may change within the next years. In fact, early studies suggest that the anti-angiogenic antibody bevacizumab may have antileukemic activity [116]. Beyond the understanding that unconjugated antibodies may be of therapeutic value in AML, such findings bring our

attention to the microenvironment of AML cells rather than the cancer cells as such as a promising therapeutic target in the future.

Further progress in antibody therapy will likely depend on the discovery of novel AML cell surface markers such as it has been achieved with CCL-1, VLA-4 or FLT3. Selected ligands may be suitable to target cytotoxic drugs to AML cells as long as the ligands are internalized upon binding. Moreover, receptor-targeted peptides or antibodies might have the capability to induce further biological features in malignant cells as inhibition of cell proliferation or induction of cell death by blocking natural receptor ligand interactions or activation of complement-mediated cytotoxicity. Further, the combination of ligands covering multiple AML specific receptors could be useful to increase specificity and efficacy of targeted therapies and we consider it mandatory to explore such concepts in the clinic with the newly developed agents within the years to come.

A pertinent question is whether the characterization of leukemic stem cells (LSC) may result in novel treatment options for AML. We consider this to be very likely even though this is still a very novel concept. One explanation for treatment failure in AML might be the resistance of leukemia stem cells to currently used chemotherapeutic agents. Therefore, ligand directed delivery of conventional drugs to leukemic stem cells may not solve all the therapeutic challenges associated with the functional LSC concept. We will need both, further validation of LSC-specific markers allowing for LSC-directed drug delivery as well as drugs that interfere with LSC activity and viability. Such drugs could enforce quiescence in LSC as long as they are applied. This would make AML a chronic disease requiring long term drug treatment like with imatinib in chronic myeloid leukemia. Preferably, however, drugs are needed that efficiently kill LSC much more efficiently than the ones we currently use.

To date, there are established treatment protocols curing some and inducing remission in most AML patients. This may be perceived as an impediment to the clinical evaluation of novel candidate drugs which is therefore mostly done in patients that are not eligible for standard therapy because of their age or frailty or because they relapsed after a preceding treatment. These patients possibly constitute a subgroup of AML cases that is particularly resistant to treatment, which may bias clinical results obtained for novel drugs. Viewed from a different angle, however, this may be a good thing because this patient population is the one most urgently requiring novel drugs with improved efficacy and less toxicity.

While evaluation of novel drugs as single agents in young AML patients without prior conventional therapy is currently not ethically feasible, it is promising to evaluate the effects of upfront combined application of standard antiproliferative therapy and target-specific novel agents. In this setting, beneficial effects could possibly be detected even for candidate substances that have not shown considerable efficacy in previous studies. One problem is that AML may be considered as an “orphan disease” since it is much less frequent than many solid tumors, more difficult to treat and therefore a “market” not perceived as attractive as other cancers by the pharmaceutical industry.

How may the future of AML treatment look like ten years from now? AML therapy will, on the one hand, likely be determined by the introduction of additional targeted drugs. On the other hand, the next significant step following this one will be the characterization of each individual patient as to which cocktail of conventional or novel, targeted drugs he or she will benefit from. This is commonly referred to as “tailored” rather than (but not substitutive to) “targeted” therapy such as it has been done for karyotypic profiling in AML during the last decade. While targeted drugs are in the process of implementation as standard therapies for AML, the molecular profiles allowing for tailored therapy remain to be determined in future trials once the novel generation of drugs is evaluated in larger patient cohorts.

7 Summarizing Discussion

The development of safe and efficient gene vectors has high priority in gene therapy. Vectors engineered to specifically target a specific cell type and/or tissue should improve therapeutic efficiency while negative side effects are diminshed. Moreover, vectors designed to target malignant cells could reach disseminated cancer disease and target specific biological features of cancer cells refractory to conventional therapies.

Gene vectors derived from AAV-2 are promising means for gene therapy since they combine unique features in concerns of biologic safety and efficiency, but their tropism is unspecific [24, 47, 54]. Random peptide libraries displayed on AAV are a promising tool to select viral capsids for improved gene transfer in the cell type they were selected on. The advantage of this over other combinatorial approaches is that it explicitely enriches for peptide ligands that 1) take the unique protein context of the capsid surrounding the targeting ligand into account during selection, 2) allow for sufficient assembly and production of the vector 3) are selected by their ability to allow for capsid mediated internalization and expression of viral genes within the target cells [174, 194, 276].

7.1 Vectors for AML-targeted gene transfer

In the first part of our studies we screened random AAV peptide libraries with the aim to isolate AAV capsid mutants targeting acute myeloid leukemia (AML) cells. AML is a clonal malignancy of hematopoietic precursor cells resulting in expansion of the malignant clone in the bone marrow and peripheral blood with an aggressive clinical course in most patients. Vectors that sufficiently and specifically transduce this cell type are not available [16, 194, 199, 200, 210].

In previous studies, AAV vectors were successfully retargeted to leukemia cells by the use of monoclonal antibodies or ligands binding to receptors expressed on these cells [16, 199, 210]. But their lack of stability, potential immunogenicity and increased particle size limits the use of these conjugated vector systems [174]. These concerns can be addressed by insertion of targeting ligands directly into the viral capsid. Insertion of targeting ligands at positions adjacent to R588 of the capsid protein retarget the vector to alternative cellular receptors [81, 88, 182, 228], while binding to its natural receptor is abrogated. This allows for a detargeting of AAV-2 from to the liver

[124, 174, 195] *in vivo* and furthermore may allow the vectors to evade pre-existing immunity against AAV [106].

In our studies, screening random AAV peptide libraries on the AML cell lines Kasumi-1 and HL60 resulted in enrichment of a distinct peptide motif ($NS^{V}/_{T}LLXS$) displayed on the selected capsid mutants. One clone, displaying the motif variant NQVGSWS transduced up to 90% of Kasumi-1 AML cells. A large screening approach demonstrated that NQVGSWS-AAV was able to overcome transduction resistance especially in various AML and lymphoma (*i.e.* hematopoietic cancer) cell lines while several solid tumor cell lines and normal hematopoietic cells were not permissive for NQVGSWS-mediated transduction. Transduction experiments on co-cultivated cells further verified targeting of Kasumi-1 leukemia cells by NQVGSWS-AAV while CD34+ precursor cells mainly remained untransduced. Therefore, we concluded that the receptor targeted by NQVGSWS is upregulated in a variety of hematopoietic cancer cells, although we cannot rule out that its expression may be a general feature of stable hematopoietic cell lines. Our findings match those of a recent report on a library screening performed by Sellner *et al.*. In this study, comparable peptide motifs were isolated after biopanning on CD34+ hematopoietic progenitor cells. In line with our findings, transduction experiments revealed a superior transduction rate of leukemic cells compared to CD 34+ cells [226].

We showed that AAV-NQVGSWS transduces its target cells *via* an attachment receptor distinct from the primary AAV-2 receptor HSPG. Consequently, we could demonstrate that targeted NQVGSWS-AAV vectors harboring a suicide gene confer selective killing to Kasumi-1 AML-cells but not to SiHa cervical cancer control cells. It would be interesting to investigate whether such cytotoxic effects on AML cells can be potentiated by the use of pro-apoptotic peptides or cytotoxic genes delivered by these vectors. We recently introduced and validated a technical approach that allows the production of AAV vectors harbouring a panel of cytotoxic and pro-apoptotic gene variants (J. Kohlschütter – diploma thesis University of Freiburg 2007 - , S. Michelfelder, M. Trepel, unpublished data). These could be packaged and tested in the leukemia-targeted capsids selected in our experiments with the aim to further improve their antileukemic action.

Taken together, these results distinguish our selected capsid mutant from previously described targeted vectors in regard to specificity and efficiency [81, 194, 228] and make it a potential tool to deliver therapeutic genes to AML cells. Further studies have to be

performed to investigate whether the NQVGSWS clone also transduces primary leukemia cells or even leukemic stem cells. Alternatively or in addition, AAV libraries could and should be directly screened on primary AML cells based on the methodological arsenal presented in this thesis.

7.2 Tissue-directed vector capsids selected by *in vivo* screening of AAV display peptide libraries

Attempts to target AAV vectors to certain tissues *in vivo* are often based on the exploration of different serotypes [30, 187, 246, 273, 305] or by the combination of their different capsid domains (reviewed in [287]), but such approaches are limited by the number of vector serotypes that are available.

Alternatively, peptide ligands identified by screening phage display libraries on a certain target tissue *in vivo* have been introduced into AAV capsid regions critical for receptor binding [277, 278, 284]. However, the receptor affinity of such ligands may deteriorate and their receptor tropism may change when incorporated into the AAV capsid. Furthermore, such ligands are commonly selected only for cell binding and not for cellular internalization and subsequent gene transfer.

We intended to overcome these limitations by *in vivo* biopannings of AAV libraries after systemic administration using tumor and lung as target tissues. We recovered distinct peptide motifs that varied depending on the respective selection pathway and target tissues. Selected capsid clones indeed conferred gene expression in the target tissue which was not detectable in animals injected with vectors displaying a random insert control capsid. However, selected clones failed to achieve tissue-specific transgene expression and even extended the spectrum of tissues they transduce, compared to wild-type AAV-2. The selected AAV capsid variant displaying the ESGLSQS peptide remains the most promising clone as it has the capacity to transduce PymT-induced tumor tissue *in vivo* while detargeting the vectors from the liver.

For the clones selected in lung tissue, our finding of increased but after all unspecific transduction may be explained by the first-pass effect upon intravenous injection performed in our screenings, leading to enrichment of virus capsids with optimized *in vivo* transduction behavior irrespective of tissue specificity. Such vectors might generally target endothelial cells, which is underlined by the fact that a similar capsid

mutant (PRSVTVP) has been previously selected on primary human coronary artery endothelial cells *in vitro* [174]. Further experiments should be performed to specify the transduced cell types. Another characteristic property of the vectors is their propensity to invariably confer gene transduction to the heart in addition to the target tissue. This observation deserves further consideration and follow up in future experiments.

There may be various superordinate reasons for the enrichment of vectors with this kind of “extended” tropism: First, the lack of tissue-specific receptors that allow for vector internalization, functional intracellular processing and gene delivery provided by the respective target tissue. Second, some receptors conferring optimal viral transduction are ubiquitously expressed, so capsids targeting tissue-specific receptors that are less efficient for transduction are not enriched. Third, receptor independent factors such as endothelial barriers, blood-derived factors, or extracellular matrix interactions may influence the selection process.

Taken together, *in vivo* screening of AAV libraries allows selection for vectors with an extended tropism for the tissue of interest. Vectors targeting the endothelial cell layer might be used to deliver anti-angiogenic genes such as endostatin in order to block neovascularization and tumor growth [142]. Furthermore, vectors transducing various organs might be useful when expression in tissues other than the primary target is desirable or uncritical as it has been performed by expression of the SOD gene delivered by adenovirus to protect lung tissue against radiation-induced fibrosis [64].

The tumor specificity of vectors displaying the ESGLSQS peptide may further be optimized by using tumor specific expression systems such as the hTERT promoter [271, 293] with the aim to develop a breast cancer-targeted gene therapy approach in the PymT mouse model.

An alternative approach to enhance specificity and efficiency of AAV vectors could be the use of novel AAV library principles like sequence evolution of capsid encoding regions in the genome by error-prone PCR [158], DNA shuffling [150] and multispecies libraries [90] if used for *in vivo* selection.

7.3 Adenovirus amplification *vs.* PCR-based amplification of library clones

Screening AAV peptide libraries *in vivo* provides much more difficult but also much more realistic conditions than cell culture-based procedures since it allows the selection for viral capsids that are 1) able to overcome anatomical and physical barriers such as the hemodynamics within the blood stream, endothelial cell layers, extracellular matrix, and host immunogenicity and 2) are simultaneously negative selected by host clearance mechanisms and resident non-target tissues.

So far, such *in vivo* selections have been hampered by insufficient technical knowledge regarding the screening conditions that have to be chosen for a particular organ or tissue and the lack of a suitable system to selectively amplify viral library particles that home to the tissue of interest after systemic injection. For cell-based selection approaches, internalized virus particles have been amplified by adenoviral delivery of helper genes [165, 174, 194, 276]. Such helper-dependent approaches would require high doses of adenoviral helper virus *in vivo* that may not be tolerated by the animal and, in addition, confines the selection to tissues and cell types that are susceptible to adenoviral infection. Grimm *et al.* [90] used this approach to select for lung targeted vectors after topical application in the airways. However, the diversity of recovered AAV after two rounds of selection was restricted to one clone, presumably due to an inefficient amplification during selection. While the recovered vectors mediated lung gene transfer after topic application to the airways, they exhibited almost unchanged tropism compared to unselected vectors after systemic application *via* the blood stream [90].

In our studies this problem has been circumvented by a novel PCR-based amplification approach. After systemic administration of the viral library, relevant parts of the genomes of viruses homing to the tissue of interest are amplified *via* nested PCR and subsequently cloned back into the AAV library backbone plasmid. In further steps, so-called secondary libraries are produced by transfection of the library plasmids like in the initial library production procedure and are used for subsequent rounds of selections. The viral titers and diversity obtained with this approach are sufficient to perform unlimited numbers of selection rounds.

In the work presented here, we demonstrated the feasibility of the PCR amplification-based screening by a cell-based *in vitro* selection on primary breast cancer cells. We obtained specific peptide sequence motifs even after two selection rounds. These

clones conferred selective transduction of cytokeratin-positive (*i.e.* not tumor stroma) breast cancer cells. We assume that adenovirus-based selection indeed forces exclusively the enrichment of capsid mutants that mediate internalization and viral gene expression within the target cells. But this may also carry the risk of loosing target specific clones. Our findings indicate the superiority of the PCR-based compared to the conventional adenovirus-based library selections, which mostly require three to five rounds of selection [165, 174, 194, 276].

7.4 Ligands selected within AAV capsids and their potential target receptors

In our studies we isolated the peptide motif ($NS^{V}/_{T}LLXS$) targeting a spectrum of hematopoietic cancer cells. Perabo *et al.* recently selected two peptides (GENQARS and QNEGSRA) for optimized transduction of human B-cell chronic lymphatic leukemia (CLL) [194]. In another study, the peptide motif $N^{S}/_{Q}X^{R}/_{L}XXX$ has been selected on primary human venous and coronary artery endothelial cells (HSaVEC, HCaAEC) [174, 276]. Sellner *et al.* isolated NXVXXX on CD34+ precursor cells [226]. Even though the similarity of these peptide motifs isolated on different target cells is vague, such a common motif might target a similar receptor or receptor class on these cell types. This is particularly feasible since it is known that several receptors on endothelial cells are known to be also expressed on cells of the hematopoietic system (*e.g.* CD34).

Screenings on primary murine breast cancer cells revealed the enrichment of the peptide motif RGDXXXX. Similar peptide motifs have been selected on PC3 prostate carcinoma cells [276] and M07e human leukemic megakaryocytic cells [194] by adenovirus-based selection. Incorporation of the RGD sequence into the viral capsid retargets the vector to integrins, which are widely expressed on several cell types [81, 228, 231], suggesting that these clones might target *via* the integrin class of receptors [215].

Two clones we isolated by *in vivo* screenings, DLGSARA (selected on tumor tissue) and PRSTSDP (selected on lung tissue), presumably target receptors that are ubiquitously expressed *in vivo* since both clones showed gene transduction in various tissues. ESGLSQS may target receptors preferentially expressed on tumor endothelial cells. One strategy to identify potential receptors for peptides displayed

on AAV-2 would be the comparison of relative transduction efficiencies of selected vector capsids with familiar gene expression profiles from cDNA microarray analyses, available for a panel of human tumor cell lines (NCI60). Using such approach, the platelet-derived growth factor receptor (PDGFR) has been identified as a co-receptor for AAV-5 transduction [55].

In our studies, we observed that if the selected vector capsids conferred gene expression *in vivo*, it also invariably occurs in cardiac tissue. This remarkable finding affects the interpretation of the specificity of the selected clones considerably. In view of previously published data, we suggest that cardiac transduction is mediated both by a redistribution effect resulting from ablation of the endogenous tropism of the vector [124], as well as by the design of the peptide insert. This indicates that a capsid region close to the library insert at position R588 has significant influence on cardiac tropism. In contrast, biodistribution studies have not found increased heart homing by peptide insertion in this region [278, 284] which might in part be attributable to, or at least influenced by, the different insertion site (position N587 instead of R588). Therefore there is urgent need to investigate the mechanisms of heart transduction in further detail and to identify capsid domains that interact with heart receptors. Such knowledge would help to design novel AAV library systems improving this innovative display technology in general.

AAV-2 transduction is initiated by binding of the capsid to the primary attachment receptor HSPG [245], followed by interaction with further independent receptors that subsequently trigger cell entry [1, 11, 118, 202, 244]. The heparin-binding site of AAV-2 is ablated by insertion of peptides at position 588 [124, 174]. This is in line with our results. Molecular modeling studies recently identified an NGR residue at position 513-515 in the viral capsid that forms a surface loop close to the three-fold axis of symmetry adjacent to the HSPG binding site, which may act as a binding site for the co-receptor integrin α5β1 [11]. Further capsid protein domains that are involved in co-receptor binding still remain unknown, but it seems possible that displayed peptide ligands of the library act in combination with such domains. Since peptides displayed within the stringent protein context of the capsid may have different conformations and binding characteristics than the same peptide sequence occurring in a natural context, a data base search for homologies based on the sequence is unlikely to be

very informative. Future research may illuminate to what extent peptide insertions at the R588 site influence AAV capsid interaction with the various secondary receptors.
In summary, the results of this thesis emphasize the utility of AAV libraries to select for improved gene delivery vector capsids *in vitro* and *in vivo,* but also demonstrate that successful targeting of AAV *in vivo* would require more than the capsid modifications used in the current AAV library approach. The data serve as a basis for further improvement of random AAV display peptide libraries to generate targeted AAV gene vectors. Therefore, the next step should include the investigation of the the relation between capsid structures and putative target receptors to improve the vector's target specificity to a well-defined cell population under *in vivo* conditions.

8 Abbreviations

aa	amino acid
AAV	adeno-associated virus
Ad5	adenovirus type 5
AML	acute myeloid leukemia
BR	basic region
BSA	bovine serum albumin
bp	base pairs
cap	capsid gene of AAV
CMV	human cytomegalovirus promotor
CLL	Chronic lymphatic leukemia
DMEM	Dulbecco`s Modified Eagle Medium
DNA	desoxyribo nucleiod acid
DNase	desoxyribonuclease
E.coli	*Escherichia coli*
eGFP	enhanced green fluorescent protein
ELISA	Enzyme Linked Immunoabsorbent Assay
FCS	fetal calf serum
GFP	green fluorescent protein
GCV	gancyclovir
HSPG	heparan sulphate proteoglycan
h	hour
kb	kilo bases
kDa	kilo dalton
Luc	luciferase
M	mole
min	minute
MOI	multiplicity of infection
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
N-	amino-
NLS	nuclear localization sequence
OD	optical density
ORF	open reading frame

p	plasmid
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PLA2	phospholipase A2
PymT	polyoma middle T
rAAV	recombinant adeno-associated virus
rep	nonstructural genes of AAV
RLB	reagent lysis buffer
RLU	relative light units
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
scAAV	self-complementary AAV
SD	standart deviation
SDS	sodium dodecyl sulfate
SEM	standart error of the mean
SOD	superoxide dismutase
ss	single stranded
vg	vector genomes
µ (g, l, m)	micro-(gram, liter, meter)
m (g, l, m)	milli-(gram, liter, meter)
n (g, l, m)	nano-(gram, liter, meter)

amino acids:

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

9 List of Figures

10 List of Tables

11 Record of achievement/ Eigenabgrenzung

1. Vectors selected from adeno-associated viral display peptide libraries for leukemia cell-targeted cytotoxic gene therapy.

All results were performed by myself, except the results shown in Figures 6, 7A, 8, 9A, 10 and tables 1, 3, and parts of table 2. These experiments were performed by Mi-Kyung Lee and Felix Kaul and are presented as parts of their Inaugural Dissertations (Zielgerichtete Gentherapievektoren für akute myeloische Leukämiezellen aus einer randomisierten adeno-assoziiert-viralen-Peptidbank, Mi-Kyung Lee, März 2007; Design, Herstellungsoptimierung, Charakterisierung und Anwendung einer randomisierten, auf adenoassoziierten Viren exprimierten Peptidbank, Felix Kaul, Dezember 2003).

2. Tissue-directed gene delivery by vectors selected from AAV peptide libraries *in vivo*.

All results were achieved by myself. Immunflourescence images shown in figure15B were performed by Sabine Jaegle as part of a practical course under my supervision.

3. Drug delivery in acute myeloid leukemia.

The chapter 6.3.3 Receptor targeted drug delivery in AML (parts of 6.3.3.3 were executed by Johannes Kohlschütter) and parts of the chapter 6.5 Experts opinion were executed by myself. Final revision was performed by Prof. Dr. MartinTrepel.

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