Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors

Hildegard Büning1,2 and Arun Srivastava3

1Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany; 2REBIRTH Cluster of Excellence, Hannover Medical School, Hannover, Germany; 3Division of Cellular and Molecular Therapy, Departments of Pediatrics and Molecular Genetics & Microbiology, Powell Gene Therapy Center, University of Florida College of Medicine, Gainesville, FL, USA

In the past decade, recombinant vectors based on a non-pathogenic parvovirus, the adeno-associated virus (AAV), have taken center stage as a gene delivery vehicle for the potential gene therapy for a number of human diseases. To date, the safety of AAV vectors in 176 phase I, II, and III clinical trials and their efficacy in at least eight human diseases are now firmly documented. Despite these remarkable achievements, it has also become abundantly clear that the full potential of first-generation AAV vectors composed of naturally occurring capsids is not likely to be realized, since the wild-type AAV did not evolve for the purpose of therapeutic gene delivery. In this article, we provide a brief historical account of the progress that has been made in the development of capsid-modified, next-generation AAV vectors to ensure both the safety and efficacy of these vectors in targeting a wide variety of human diseases.

Adeno-associated virus (AAV) is a small, single-stranded DNA-containing, non-pathogenic parvovirus with a non-enveloped protein capsid that has gained significant attention as an efficient and safe vector for gene transfer. Recombinant AAV vectors have been or are currently being used in 176 phase I, II, and III clinical trials (https://clinicaltrials.gov). AAV serotype 2 (AAV2) vectors have shown clinical efficacy in three human diseases: Leber’s congenital amaurosis (LCA),8–10 aromatic L-amino acid decarboxylase deficiency (AADC),11 and choroideremia.12 In the past decade, at least 12 additional AAV serotype vectors, some derived from non-human primates, have also become available.13–21 AAV1 vectors have been successfully used in gene therapy for lipoprotein lipase deficiency,22 and AAV8 vectors have shown clinical efficacy in potential gene therapy for hemophilia B.23–25 More recently, AAV5 vectors have been reported as being effective in hemophilia A.26,27 AAV9 vectors have been successfully used in gene therapy for Pompe disease28 and showed impressive efficacy in gene therapy for spinal muscular atrophy.29 The AAV1-LPL vector was approved as a drug designated aliparvovec and marketed under the trade name Glybera in Europe in 2012. In 2017, an AAV2 vector expressing retinal pigment epithelium-specific 65 kDa protein (RPE65) was approved by the Food and Drug Administration as the drug voretigene neparvovec (Luxturna), in the United States. A number of additional phase I and II clinical trials have been or are currently being pursued with AAV1, AAV2, AAV3, AAV5, AAV6, AAV8, AAV9, and AAV10 vectors for potential gene therapy for a wide variety of human diseases.29

Despite these remarkable achievements, it has become increasingly clear that the full potential of this vector system will only be realized after AAV vectors have been modified for improved cell transduction and to evade the host immune response.31

Capsid Modifications
The AAV wild-type (WT) genome contains at least three genes: rep, cap, and X (Figure 1). The X gene, first described in 1999, is located at the 3′ end of the genome (nucleotides 3929–4393 in AAV2) and seems to code for a protein with supportive function in genome replication.32 Significantly more information is available for rep and cap. The rep gene is located in the first half of the AAV WT genome and codes for a family of non-structural proteins (Rep proteins) required for viral transcription control and replication as well as packaging of viral genomes into the newly produced, pre-assembled capsids. For WT AAV2, a Rep-mediated, site-specific integration of the viral genome in AAV integration site 1 (AAVS1) was reported,33 a unique feature of dependoparvoviruses that might have evolved to ensure virus survival in the absence of helper virus co-infection. While being initially discussed as a promising feature to be maintained in AAV vectors, size (half of the coding capacity of AAV vectors) as well as safety (integration of a viral endonuclease) concerns argued for the development of gutless AAV vectors, i.e., for replacing all known viral open reading frames (ORFs) with the (trans)gene cassette to be delivered.34 The second half of the AAV genome contains the cap gene, which codes for the viral proteins (VPs) VP1, VP2, and VP3, and the assembly-activating protein (AAP). Transcription of all VPs, which are the capsid monomers, is controlled by a single promoter (p40 in case of AAV2) resulting in a single mRNA. Splicing (VP1) and an unusual translational start codon (VP2) are responsible for an approximately 10 times lower presence of VP1 and VP2 compared with VP3.34 As expected, when encoded by a single gene, AAV VPs share most of their amino acids. Specifically, the entire VP3

https://doi.org/10.1016/j.omtm.2019.01.008.

Correspondence: Hildegard Büning, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany.
E-mail: buening.hildegard@mh-hannover.de

Correspondence: Arun Srivastava, Division of Cellular and Molecular Therapy, Departments of Pediatrics and Molecular Genetics & Microbiology, Powell Gene Therapy Center, University of Florida College of Medicine, Gainesville, FL, USA.
E-mail: aruns@peds.ufl.edu
sequence is also contained within VP2 and VP1 ("common VP3 region"), and also VP2 and VP1 share approximately 65 amino acids ("common VP1/VP2 region"). Only VP1 contains a unique sequence at its N terminus (approximately 138 amino acids, VP1 unique). AAP was identified in 2010 as a 23 kD protein encoded in an alternative cap ORF.35 It is required for stabilizing and transporting newly produced VP proteins from the cytoplasm into the cell nucleus.36 Interestingly, while AAV serotypes 1-3, 6-9, and rh10 failed to produce capsids in the absence of AAP, a low but detectable capsid production was reported for AAV4 and AAV5.36

The capsids of all AAV serotypes are icosahedra, assembled from 60 VP monomers with approximately 50 copies of VP3, 5 copies of VP2, and 5 copies of VP1.15 Topological prominent capsid surface structures are pores or "channel-like-structures" at each fivefold, depressions at each twofold, and three protrusions surrounding each threefold axis of symmetry.17 The pores allow exchange between the capsid interior and the outside. Rep proteins shuttle the viral genomes into the capsids in the absence of AAP, a low but detectable capsid production was reported for AAV4 and AAV5.36

Availability of cell surface receptors for cell attachment (primary receptor binding) and internalization (secondary receptor binding) determines virus and thus viral vector tropism. Receptor binding not only enables cell entry, but also primes the capsid for the later release of its genetic payload (uncoating)46 and prepares the cell for viral particle transport and processing.46 Since AAV serotypes differ in receptors used for cell attachment and internalization, serotypes other than AAV2, the prototype AAV vector, were developed as alternatives.34 Although serotypes can be produced as isotypes (i.e., vector genomes flanked by serotype-specific ITRs packaged into their own capsid aided by serotype-specific Rep proteins), cross-packaging has become the production strategy of choice.34 In that case, Rep proteins from AAV2 are used to package vector genomes flanked by AAV2-specific ITRs into desired serotype capsids.47

Because of their exposed positions and their function in receptor binding, VRs forming loops of the protrusions are ideal positions for capsid modifications aiming to re-direct or expand AAV tropism (cell surface targeting). While a re-directed tropism (vector re-targeting) combines ablation of natural receptor binding, for example by site-directed mutagenesis, with insertion of a ligand that mediates
transduction through a novel non-natural AAV receptor, AAV vectors with tropism expansion gain the ability to transduce cells through an extra receptor while maintaining their natural receptor binding abilities. Besides the protrusions, additional positions have been identified that allow for genetic modification without affecting viral vector tropism. As discussed below, some of these positions have also been harnessed for cell surface targeting approaches, while others improved AAV vector efficacy by avoiding proteasomal degradation.

**Cell Surface Targeting of AAV Vectors by Genetic Modification of the Capsid**

**Insertion of Peptides into the Common VP3 Region**

When Girod et al.\(^4^8\) reported the first successful capsid modification for re-targeting in 1999, no crystal structure for AAV capsids was available, and possible insertion sites were predicted based upon the crystal structure of canine parvovirus (CPV). Of the six candidate insertion sites (I) for AAV2 (I-261, I-381, I-447, I-534, I-573, and I-587; VP1 numbering), three sites turned out to be suitable for surface display of the β1 integrin-binding model ligand L14 (QAGTFALRGDNPQG).\(^4^8,4^9\) Finally, however, only L14 peptide insertion at I-587, i.e., insertion between the amino acid residue asparagine (N) 587 and arginine (R) 588, allowed for target receptor-mediated cell transduction\(^4^8\) (Figure 2). Target cells were transduced, even in the presence of heparin, the soluble analog of AAV2’s primary receptor heparan sulfate proteoglycan (HSPG).\(^4^8\) This finding was explained upon identification of the HSPG binding motif of AAV2 by the groups of Kleinschmidt\(^5^0\) and Muzyczka\(^5^1\) in 2003. Their work showed that insertion of peptides at I-587 changes the spacing between R585 and R588 and thereby destroys the natural HSPG binding ability of AAV2. Insertion at I-453 requires simultaneous mutations at R585 and R588 for re-targeting and efficient presentation of targeting ligands. ITR, inverted terminal repeat; REP, rep gene; CAP, cap gene; VP1, VP2, VP3, viral capsid proteins; R, arginine residue. Numbers represent amino acid position according to VP1 numbering.

**Figure 2. Capsid Engineering for Re-targeting or Expansion of AAV2 Vector Tropism**

The N terminus of VP2 accepts large-peptide insertions as well as proteins. When combined with natural receptor-binding motif knock-out mutations, insertion of targeting ligands at VP2 re-targets vector tropism; otherwise, vector tropism is expanded. The N’ terminus of VP2 can also be used to label AAV vector particles for ex vivo and in vivo infection biology analyses. For that purpose, EGFP and luciferase were used as fusion partners, while the remaining capsid was left unmodified. Frequently used sites for insertions of targeting ligands within the common VP3 regions are I-453, I-587, and I-588 (not shown). In case of peptide insertions at I-587, R585 and R588 are separated, thereby destroying the natural HSPG binding ability of AAV2. Insertion at I-453 requires simultaneous mutations at R585 and R588 for re-targeting and efficient presentation of targeting ligands. ITR, inverted terminal repeat; REP, rep gene; CAP, cap gene; VP1, VP2, VP3, viral capsid proteins; R, arginine residue. Numbers represent amino acid position according to VP1 numbering.
of this insertion site for capsid engineering, including studies defining peptide features for vector re-targeting.53,54 Thus, the AAV2 I-587 insertion site allows genetic modifications of the capsid without interfering with capsid assembly or genome packaging, accepts peptides of up to a size of 34 amino acids, displays the foreign sequence in such a way that peptides can interact with target cell surface molecules, and enables vector re-targeting in a single step because the AAV2 primary receptor binding motif is modified upon insertion of the novel targeting ligand.55 These advantageous features are the likely reason why I-587 and the neighboring position I-588 have become the most frequently used positions for genetic cell surface targeting approaches employing AAV2 vectors (Tables 1 and 2).

Table 1. Peptide Insertion into the Common VP3 Region of AAV2 at I-587

| Name            | Target Cell Type                          | Insert                  | Comment                                      | Reference |
|-----------------|-------------------------------------------|-------------------------|----------------------------------------------|-----------|
| AAV-I-587       | β1-integrin positive tumor cells          | QAGTFALRGERPNQG         | first 587 targeting vector                   | 48        |
| AAV-588NGR      | CD13-positive tumor cells                 | NGRAHA                  |                                              | 85        |
| AAV-MO7A        | tumor cells                               | RGDAYGV                 | AAV2 peptide display                        | 85        |
| AAV-MO7T        | tumor cells                               | RGDTIPTS                | AAV2 peptide display                        | 85        |
| AAV-MecA        | tumor cells                               | GENQARS                 | AAV2 peptide display                        | 85        |
| AAV-MecB        | tumor cells                               | RSVANVVP                | AAV2 peptide display                        | 85        |
| rRGDS87         | γv integrin positive tumor cells          | CDRGRCDFC               | phage display                               | 50        |
| AAV-C4          | tumor cells                               | PRGTNGP                 | AAV2 peptide display; library pre-clearing on off-target cell type | 116       |
| AAV-D10         | tumor cells                               | SRGATTT                 | AAV2 peptide display; library pre-clearing on off-target cell type | 116       |
| AAV-SIG         | endothelial cells                         | SIGYPLP                 | identified by phage display                 | 117       |
| AAV-MTP         | endothelial cells                         | MTPFFPSNEANL            | phage display                               | 118       |
| AAV-QPE         | endothelial cells                         | QPEHSST                 | phage display                               | 119       |
| AAV-VNT         | endothelial cells                         | VNTANST                 | phage display                               | 119       |
| AAV-CNH         | endothelial cells                         | CNHRYMQMC               | identified by in vivo phage display          | 120       |
| AAV-CAP         | endothelial cells                         | CAPGPKSG                | identified by in vivo phage display          | 120       |
| AAV-EYH         | smooth muscle cells                       | EYHNYK                  | phage display                               | 121       |
| AAVvasMTP       | skeleton muscle cells                     | ASLHIA                  | phage display                               | 122       |
| AAV-r3.45       | neuronal stem cells                       | TQVQGKT                 | AAV2 peptide display                        | 123       |
| AAV2-SSS        | CNS                                       | LPSSLQK                 | in vivo AAV2 peptide display                | 124       |
| AAV2-PFG        | CNS                                       | WPFFYGTP                | in vivo AAV2 peptide display                | 124       |
| AAV2-PPS        | CNS                                       | DSPHPHS                 | in vivo AAV2 peptide display                | 124       |
| AAV2-THL        | CNS                                       | GWTLHVK                 | in vivo AAV2 peptide display                | 124       |
| AAV2-GMN        | CNS                                       | GMNAFRA                 | in vivo AAV2 peptide display in disease model; identification of target receptor | 124       |
| AAV2-7m8        | retinal cell types                        | LGETTRP                 | in vivo AAV2 peptide display; further change in backbone: V708I | 60        |
| AAV-Kera1       | keratinocytes                             | RGDATATL                | AAV2 peptide display after pre-clearing step | 85        |
| AAV-Kera2       | keratinocytes                             | PRGDLAP                 | AAV2 peptide display after pre-clearing step; identification of target receptor | 85        |
| AAV-Kera3       | keratinocytes                             | RGDQVSLS                | AAV2 peptide display after pre-clearing step; identification of target receptor | 85        |
| AAV-588Myc      | none                                      | EQLSISEEDL              | AAV2 peptide display after pre-clearing step; identification of target receptor | 85        |
| AAV2-Z34C       | adaptor                                   | Z34C                    | antibody binding via protein A domain        | 127       |
| AAV2.N587,RS588insBAP | adaptor                             | GLNDJIFEAQKIEWHE       | biotin acceptor peptide (BAP) for purification or targeting via avidin containing ligands | 87        |
| AAV2.4013       | adaptor                                   | LCTPSRAALLTGR           | chemical coupling of ligands                | 128       |
| DMD4            | vaccine                                   | QVSHWVSGLAEGSFG         | AAV2 15-mer peptide display                | 129       |
| DMD6            | vaccine                                   | LSHTSGRVEGVSISL         | AAV2 15-mer peptide display                | 130       |

I-587, peptide insertion between N587 and R588. For details on linker sequences flanking the peptide insert, please see references.
Table 2. Peptide Insertion into the Common VP3 Region of AAV2 at I-588 for Cell Surface Targeting

| Name            | Target Cell Type                  | Insert          | Comment                                                                 | Reference |
|-----------------|-----------------------------------|-----------------|-------------------------------------------------------------------------|-----------|
| A588-RGD4C      | αv integrin-positive tumor cells  | CDCRGDCFC       | test of linker sequences                                                | 130       |
| A588-RGD4CGLS   | αv-integrin positive tumor cells  | CDCRGDCFC       |                                                                         | 130       |
| AAV-VTAGRAP     | tumor cells                       | VTAGRAP         | AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling | 131       |
| AAV-APVTRPA     | tumor cells                       | APVTRPA         | AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling | 131       |
| AAV-DLSLTR      | tumor cells                       | DLSLTR          | AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling | 131       |
| AAV-NQVGWS      | tumor cells                       | NQVGWS          | AAV2 peptide display                                                    | 132       |
| AAV-EARVRPP     | tumor cells                       | EARVRPP         | AAV2 peptide display                                                    | 132       |
| AAV-NSYSLYTT    | tumor cells (CML)                 | NSYSLYTT        | AAV2 peptide display                                                    | 133       |
| AAV-LS1         | tumor cells (CML), CD34⁺ cells    | NDVRSAN⁺        | AAV2 peptide display                                                    | 134       |
| AAV-LS2         | tumor cells (CML), CD34⁺ cells    | NERVLS          | AAV2 peptide display                                                    | 134       |
| AAV-LS3         | tumor cells (CML), CD34⁺ cells    | NRTWEOQQ        | AAV2 peptide display                                                    | 134       |
| AAV-LS4         | tumor cells (CML), CD34⁺ cells    | NSVQSSW         | AAV2 peptide display                                                    | 134       |
| AAV-RGDGLGS     | tumor cells                       | RGDGLGS         | AAV2 peptide display                                                    | 134       |
| AAV-RGDMRSRE    | tumor cells                       | RGDMRSRE        | AAV2 peptide display                                                    | 135       |
| AAV-EGLSQS      | tumor cells                       | ESGLSOS         | in vivo AAV2 peptide display; in vivo tumor targeting; de-targeting from liver, but improved heart transduction | 135       |
| AAV-EYRDSSG     | tumor cells                       | EYRDSSG         | in vivo AAV2 peptide display; in vivo tumor targeting, improved liver (weak) and heart transduction | 135       |
| AAV-DLGSSARA    | tumor cells                       | DLSGASA         | in vivo AAV2 peptide display; improved liver and heart transduction     | 135       |
| AAV-NDVRSAN     | tumor cells                       | NDVRSAN⁺        | AAV2 peptide display                                                    | 136       |
| AAV-GPOQKNS     | tumor cells                       | GPOQKNS         | AAV2 peptide display                                                    | 136       |
| AAV-NSRDLG      | endothelial cells                 | NSRDLG          | AAV2 peptide display; first AAV peptide display selected variant tested in vivo | 82        |
| AAV-NDVRAVS     | endothelial cells                 | NDVRAVS⁺        | AAV2 peptide display                                                    | 82        |
| AAV-NDVRSAN     | endothelial cells                 | NDVRSAN⁺        | AAV2 peptide display                                                    | 82        |
| AAV-NDVRAVS     | endothelial cells                 | NDVRAVS⁺        | AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling | 135       |
| AAV-PRSTSDP     | lung (maybe endothelial cells)    | PRSTSDP         | in vivo AAV2 peptide display; improved lung transduction, but also other organs | 135       |
| AAV-DILRA       | endothelial cells                 | DILRA           | AAV2-5-mer peptide display library                                      | 86        |
| AAV-SYENV       | endothelial cells                 | SYENVASRRPEG    | AAV2-12-mer peptide display library                                     | 86        |
| AAV-PENSV       | endothelial cells                 | PENSVRYGLEG     | AAV2-12-mer peptide display library                                     | 86        |
| AAV-LSLAS       | endothelial cells                 | LSASRNPTATS     | AAV2-12-mer peptide display library                                     | 86        |
| AAV-NDVWN       | endothelial cells                 | NVVWNRDNNSSKRGGTTEAS | AAV2-19-mer peptide display library                                   | 86        |
| AAV-NRTYS       | endothelial cells                 | NRTYSSTSNSTSRSEWDNS | AAV2-19-mer peptide display library                                   | 86        |
| AAV-ESGHGYF     | pulmonary endothelial cells       | ESGHGYF         | in vivo AAV peptide display                                             | 137       |
| AAV-GQHPFG      | cardiomyoblasts                   | GQHPFG⁺         | AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling | 135       |
| AAV-PSVSRP      | cardiomyoblasts                   | PSVSRP          | in vivo AAV2 peptide display; improved tropism for heart                | 138       |

(Continued on next page)
In their seminal work, Girod et al.48 also reported on the successful insertion and surface display of L14 at I-447. However, an L14-mediated cell transduction was not observed. In contrast to CPV, I-447 in AAV2 is part of a β-barrel (GH2) forming the highest protrusion around the threefold axis, but is not part of the loop region at the top (VR-IV).45,52 In an effort to assay whether the VR-IV is suited for cell surface targeting, Boucas et al.56 inserted the model ligand RGD-4C into I-453 (between glycine [G] 453 and threonine [T] 454) (Figure 2). For comparison, they inserted the same ligand into I-587, simultaneously into I-453 and I-587, and combined peptide insertions with R to alanine (A) substitution for R585 and R588 to destroy AAV’s natural HSPG binding ability.56 None of the modifications interfered with vector packaging, revealing that the AAV2 capsid tolerates simultaneous peptide insertions at the highest and the second highest protrusions.56 Interestingly, combining R-to-A substitutions with peptide insertions improved RGD-4C accessibility for both of the single- and the double-insertion mutants. Successful cell surface targeting, i.e., ligand-mediated cell transduction, for I-453 capsid variants was observed in cell culture and in vivo for the variant carrying in addition the R-to-A substitutions revealing that also the highest protrusions of the AAV capsid can be used for cell surface targeting. Interestingly, however, while demonstrating a comparable in vivo biodistribution (24 h after vector application), transgene expression levels 2 weeks after vector application was remarkably lower for the capsid variant displaying RGD-4C in I-453 (combined with R-to-A substitution) in liver, spleen, and lung, but comparable to I-587 for heart, muscle, and kidney. When analyzing this unexpected finding exemplary for the lung, the tissue with the most pronounced difference between the two targeting vectors, Boucas et al.56 observed comparable amounts of vector genomes for both variants, indicating a post-entry barrier for the I-453 capsid variant in some tissues.

Besides I-453, I-587, and I-588, I-520 (combined with I-584), I-584, and I-585 have been successfully explored for cell surface targeting (Table 3). The homologous residues of other serotypes also seem to be well suited for capsid engineering (Table 4).

### Genetic Modification of VP1 and VP2

Yang et al.57 were the first to report a cell surface targeting approach for AAV and the first that focused on the termini of the VPs. As a targeting ligand, they decided on a single-chain antibody against human CD34, a cell surface marker on hematopoietic cells. The single-chain antibody was fused to the N terminus of VP1, VP2, and VP3. Viral capsids were only detected when hybrid vectors consisting of all three WT AAV2 capsid proteins in addition to the single-chain antibody–VP2 fusion protein were produced. However, titers were extremely low with 1.9 × 10^2 infectious units/mL, which might be because AAV particles are assembled in the nucleus, a cell compartment that does not provide the correct conditions for antibody folding. Nevertheless, this study has established the N terminus of VP2 as the position for peptide insertion. Also, the N-terminal region of VP1, more precisely amino acid position 34, accepts peptides, which expanded viral vector tropism when tested for cell surface targeting.58,59 At first sight, results of these studies appear surprising because the N-termini of VP1 and VP2 are buried inside the capsid when AAV viruses or vectors with WT capsids are produced.45,52 Detection of inserted sequences in assembled capsids after vector purification and target receptor binding, however, confirmed surface exposure following genetic modification.58,60,61 Thus, it can be assumed that insertion of foreign sequences interferes with the natural folding and thus prevents masking of the N termini of VP1 and VP2 within the capsid.

The first follow-up studies of VP2 modification placed foreign sequences at the first58,62,63 or second59,65 residue downstream of the N-terminal methionine of the VP2 start codon. Since VP1 is an N-terminal extension of VP2, peptides are not only displayed at the N terminus of VP2, but also at amino acid position 138 or 139 of VP1. Using this strategy, peptide insertions of up to 32 amino acids were tolerated, while larger insertions resulted in a reduced production of VP3, which prevented capsid assembly.63 Providing additional VP3 protein during vector production by co-transfection of a VP3 encoding plasmid restored capsid assembly, but viral vector infectivity remained low.63 To restore infectivity, VP2-fusion proteins had to
be expressed via a non-AAV promoter from a separate plasmid. It can be used for capsid modifications that aim to maintain natural viral infection and vector transduction abilities to expand viral vector tropism, or—if combined with WT receptor knock-out mutagenesis—for vector re-targeting.

Using the above-mentioned strategy of producing VP2 fusion protein containing AAV capsids by separating fusion protein and VP1-VP3 expression allowed for example tracking of AAV vector particles during cell infection or in vivo through incorporation of EGFP or luciferase via fusion to VP2. Furthermore, the AAV vector-based, single-shot, prime-boost vaccine concept as well as switchable AAV vector systems were developed. Specifically, the novel vaccine platform uses antigen-VP2 fusion proteins incorporated into the AAV capsid to prime the antigen-specific humoral immune response, while expression of the same antigen from the vector genome functions as a booster. A proof-of-concept study revealed that this AAV vector—expression of the same antigen from the vector genome—functions as a booster. In addition, vaccination of mice with empty antigen-displaying capsids (no vector genome) was sufficient to induce an antigen-specific memory response. In the case of switchable AAV2 vectors, incorporation of the VP2 fusion protein was combined with an R-to-A substitution of R585 and R588 to blind the vector for primary receptor binding. The human FK-binding protein (FKBP) was chosen as the VP2 fusion partner. Targeting receptor binding of this modified capsid is controlled by an artificial adaptor molecule consisting of a modified FKBP-rapamycin binding (FRB) domain of mammalian target of rapamycin (mTOR), a fluorescent marker protein for visual detection and a designed ankyrin repeat protein (DARPin) with specificity for human epidermal growth factor receptor (EGFR) as the targeting ligand. When AP21967, a rapamycin structural analog, was added, FRB (adaptor site) and FKBP (vector site) formed a heterodimer, thereby equipping the vector with target receptor specificity. Under these conditions, cells expressing EGFR to high or medium level were transduced, while EGFR low or negative cells were not transduced. Likewise, vectors were non-infectious in the absence of AP21967.

As already mentioned, single-chain antibodies cannot be used for cell surface targeting of AAV vectors if a genetic capsid modification strategy is followed. This is not due to the size of the antibody, which is tolerated as a fusion to VP2 (unpublished data), but to reducing conditions in the cell nucleus that interfere with correct folding due to inhibition of cysteine bridge formation. A potent alternative for single-chain antibodies are the already mentioned receptor-specific DARPins. They possess antibody-like affinity and specificity, but do not contain cysteine residues and fold therefore in a correct and functional manner when fused to the N terminus of VP2. Thus, DARPins are not only usable as non-covalently linked adaptors, as described for the switchable AAV targeting vectors, but also as genetic fusion to VP2. In the proof-of-concept study that established DARPins as targeting mediators for AAV, the Her2/neu-specific DARPin 9.29 was incorporated as a VP2 fusion protein into AAV2 capsids with R585A/R588A amino acid substitutions. In mixed-culture experiments, targeting vectors clearly discriminated between target and non-target cells, thus demonstrating target receptor selectivity. Also in vivo, in tumor-bearing mice an impressive target specificity for Her2/neu tumors as well as impressive de-targeting from common off-target organs was observed following vector administration through the tail vein. The sole, albeit weak, off-target activity was detected in the chest region of some animals. Since AAV2 vectors that are blinded for HSPG binding have been reported to transduce heart cells and, to a lower extent, lung cells while being non-infectious in vitro, and since VP2 is not essential for capsid assembly, it was postulated that VP1-VP3 AAV vector particles

Table 3. Peptide Insertion into the Common VP3 Region for Cell Surface Targeting Using Positions Other Than I-587 or I-588

| Position | Name | Target Cell Type | Insert | Comment | Reference |
|----------|------|------------------|--------|---------|-----------|
| I-453    | rRGD45iko | αve integrin-positive tumor cells | CDCRGDCFC | RS85A and RS88A required for targeting | 73 |
| I-453    | AAV-MNVRGDL | endothelial cells | MNVRGDL | AAV2-453-peptide display; multiple further modifications in backbone | 86 |
| I-453    | AAV-ENVRGDL | endothelial cells | ENVRGDL | AAV2-453-peptide display; multiple further modifications in backbone | 86 |
| I-520 and I-584 | A520/N584 (RGD) | αve integrin-positive tumor cells | CDCRGDCFC | first targeting vector with double insertion | 120 |
| I-584    | A584-RGD4C | αve integrin-positive tumor cells | CDCRGDCFC | – | 150 |
| I-584    | A584-RGD4CALS | αve integrin-positive tumor cells | CDCRGDCFC | test of linker sequences | 150 |
| I-585    | AAV-ΔIV-NGR | CD13-positive tumor cells | NGRAHA | peptide insert replaces WT sequence; two further backbone modifications | 115 |
| I-585    | AAV-PTP | plectin-positive tumor cells | KTLIPTP | peptide insert replaces WT sequence at ΔIV; a further backbone modification | 140 |

ΔIV; VR-IV.
(i.e., DARPin-VP2 fusion deficient vectors) contained in the targeting vector preparation were responsible for the observed weak off-target activity. Depletion of vector preparation from DARPin-deficient particles by affinity chromatography not only increased transduction efficiency of the vector preparation, but enabled for the first time a true re-targeting without any detectable off-target activity. Flexibility of

Table 4. Peptide Insertion into the Common VP3 Region of Serotypes Other Than AAV2 for Cell Surface Targeting

| Serotype | Position | Name                                      | Target Cell Type                          | Insert                                      | Reference |
|----------|----------|-------------------------------------------|-------------------------------------------|---------------------------------------------|-----------|
| AAV1     | I-590    | BAP-AAV1                                  | BAP-AAV1                                  | GLNDIFEAQKIEWHE                            | 65        |
| AAV1     | I-590    | BAP-AAV1                                  | tumor cells                               | GLNDIFEAQKIEWHE                            | 141       |
| AAV1     | I-590    | AAV1-RGD                                  | tumor cells, endothelial cells            | CDCRGDCFC                                  | 73        |
| AAV1     | I-590    | AAV1-RGD/BAP (90/10) (mosaic capsid)      | tumor cells, endothelial cells            | CDCRGDCFC and GLNDIFEAQKIEWHE              | 142       |
| AAV1     | I-590    | Tet1c-AAV1 (mosaic capsid)                | tetanus toxin GT1b receptor positive cells | HLNILSTLWKYR                               | 143       |
| AAV1     | I-590    | AAV1-P3-3-SKGRSP                          | fibroblast                                | SKAGRSP                                     | 78        |
| AAV3     | I-586    | BAP-AAV3                                  | tumor cells                               | GLNDIFEAQKIEWHE                            | 88        |
| AAV4     | I-586    | BAP-AAV4                                  | tumor cells                               | GLNDIFEAQKIEWHE                            | 91        |
| AAV5     | I-575    | BAP-AAV4                                  | tumor cells                               | GLNDIFEAQKIEWHE                            | 90        |
| AAV5     | I-575    | AAV5-7m8                                  | tumor cells                               | LGETTRP107                                 | 144       |
| AAV6     | I-585    | AAV6-RGD                                  | tumor cells                               | RGD                                         | 145       |
| AAV6     | I-585    | AAV6-RGD-Y705-731F+T492V                  | tumor cells                               | RGD                                         | 145       |
| AAV8     | I-585    | AAV238-BP2                                | on-bipolar cells                          | PERTAMSLP                                   | 78        |
| AAV8     | I-590    | AAV8-PRSTSDP                              | tumor cells                               | PRSTSDP135                                 | 144       |
| AAV8     | I-590    | AAV8-ESGLSOS                              | tumor cells                               | ESGLSOS135                                  | 144       |
| AAV8     | I-590    | AAV8-VNSTRLP                              | not successful                            | VNSTRLP148                                  | 84        |
| AAV8     | I-590    | AAV8-ASSLNIA                              | heart (weakly improved transduction)      | ASSLNIA122                                  | 84        |
| AAV8     | I-590    | AAV8-PSVSPRP                              | not successful                            | PSVSPRP136                                  | 84        |
| AAV8     | I-590    | AAV8-GQHPRPG                              | heart (weakly improved transduction)      | GQHPRPG144                                  | 84        |
| AAV8     | I-590    | AAV8-SEGKLNL                              | liver                                     | SEGKLNL                                     | 84        |
| AAV8     | I-590    | AAV8-7m8                                  | not successful                            | LGETTRP107                                 | 84        |
| AAV9     | I-589    | AAV-SLRSPPS                               | endothelial cells, smooth muscle cells    | RGDLRVS                                     | 85        |
| AAV9     | I-589    | AAV-RGDLRVS                               | endothelial cells, smooth muscle cells    | RGDLRVS                                     | 85        |
| AAV9     | I-589    | AAV9-NDVRAVS                              | endothelial cells                          | NDVRAVS142                                  | 84        |
| AAV9     | I-589    | AAV9-PRSTSDP                              | tumor cells                               | PRSTSDP135                                  | 144       |
| AAV9     | I-589    | AAV9-ESGLSOS                              | tumor cells (weak targeting)              | ESGLSOS135                                  | 144       |
| AAV9     | I-589    | AAV-VHP.B                                 | CNS                                       | TLAVPFK                                     | 81        |
| AAV9     | I-589    | AAV-VHP.A                                 | CNS                                       | YTLQGGW                                     | 91        |
| AAV9     | I-589    | AAV9-7m8                                  | retinal cells                             | LGETTRP107                                 | 84        |
| AAV9P1   | not disclosed | AAV9P1                                   | neuronal progenitor cells                 | RGD1GLS                                     | 197       |

*AAV1 with amino acids 445–568 of AAV1 replaced by residues from AAV9.

*Targeting peptide does not confer tropism modification in the context of this serotype.

*Peptide selected from a AAV8 peptide display library in which the WT sequence at 585–594 was replaced by the random peptide sequence.

*Additional sequence changes in backbone.
the system was demonstrated by exchanging the Her2/neu-specific DARPin 9.29 for DARPins recognizing human CD4 and human EpCAM, respectively.72

The N’-terminus of VP2 was also used for intracellular targeting. Yu et al.69 inserted the leader sequence for cytochrome oxidase subunit 8 as VP2 fusion into the AAV capsid, which resulted in capsid-engi-

neered AAV vector particles capable of delivering vector genomes to mitochondria after cell transduction.

The most recent development with regard to cell surface targeting for this insertion position is a covalent coupling strategy using protein-

trans-splicing.73 Muik et al.73 introduced complementary split-intein domains to the N terminus of the AAV2 VP2 capsid protein, as well as to the potential targeting ligands. The modified capsid is thereby transformed into a universal acceptor to which ligands can be covalently coupled in a highly flexible manner.

**AAV Peptide Display: High-Throughput Selection Screen to Identify Targeting Ligands**

Identification of capsid positions that can be modified without inter-

fering with capsid assembly or vector genome packaging, and that present peptides/proteins for target receptor binding is half of the bat-

tle. Equally important is knowledge about receptors and respective li-

gands suited for targeted cell transduction. In the initial period of cell surface targeting, peptide ligands identified, for example, by phage display, were tested for their ability to re-direct AAV’s tropism. When receptor binding capabilities were retained following incorpo-

ration into the viral capsid, efficiencies were improved in particular for those cell types where availability of AAV receptors limited trans-

duction (pre-entry barrier). However, a receptor chosen by rational design might not be the best qualified for mediating entry and initi-

ating efficient intracellular processing of the vector. Furthermore, knowledge about barriers to transduction by AAV vectors or on po-

tential target receptors is frequently lacking. In all of these conditions, high-throughput selection screens of AAV peptide libraries offer an elegant and straightforward technical solution (Figure 3). Specifically, oligonucleotides of random sequence are inserted into the cap ORF at sites corresponding to the top of VR-VIII or -IV, thereby generating a library plasmid pool that is subsequently used to produce the AAV peptide display library, commonly in HEK293 cells.78–86 Libraries differ regarding linker sequences flanking the random sequence inser-

tion and whether the random sequence is inserted in addition to the WT sequence or as a replacement.78–86 Furthermore, depending on the cloning strategies, residues neighboring the insertion site are changed or are not.78–86 The capsid variants of the respective library differ only in the peptide sequence displayed at the VRs but are other-

wise identical. To improve efficiency of the selection procedure, the geno-

- and phenotypes of AAV capsid variants need to be coupled, and all 60 subunits of a given variant need to present the same pepti-

de. The library is then screened in cell culture or in vivo by repetitive rounds of selection for those capsid variants that transduce the target cell, in conditions defined by the experimentalist, more efficiently and/or with higher specificity than the rest of the library. The first AAV peptide display library screenings were reported in 2003. Specifi-

cally, Perabo et al.83 developed an AAV2 peptide display library carrying random insertion of seven amino acids at position 587, while Müller et al.82 used the neighboring position. Selections were performed on tumor cell lines and primary cells, respectively, in the presence of adenovirus as a helper virus to induce replication and progeny production for those variants that successfully infected the WT AAV2 refractory or low permissive cell types used as target cells. Candidate capsid variants were then produced as vectors carrying the selected peptide at I-587 or I-588.82 Perabo et al.83 confirmed peptide-mediated target cell transduction in cell culture, and Müller et al.82 further demonstrated target cell transduction following tail vein injection in mice. AAV2-based 7-mer peptide libraries, but also libraries with shorter or larger random peptide insertions as well as libraries with peptide insertions at I-453 have been successfully used to optimize AAV vectors for transduction of various cell types (Tables 1 and 2). In addition, serotypes others than AAV2 are explored as library scaffolds, and frequently, adenovirus co-infection has been replaced by PCR-mediated amplification of viral DNA iso-

lated from the target cells to avoid selection of capsid variants that are dependent on helper virus function for cell transduction.87,89,90,91,92

Although peptide insertions at position I-587 of the AAV2 capsid ablate the natural HSPG binding ability, the peptide itself can restore in the engineered capsid the ability to bind to HSPG.53,54 This feature is beneficial with regard to cell entry efficiency, but not to speci-

ficity.53,54 Depleting capsid variants that bind nonspecifically from the library—for example, by affinity chromatography—should there-

fore be considered to enrich the library for those variants that possess the ability to confer specificity. Sallach et al.55 performed heparin affinity chromatography purification prior to high-throughput screening of primary human keratinocytes. Interestingly, selected capsid vari-

ants carried peptides with an RGD tripartite motif, a hallmark of integri-

rin-binding ligands. The three candidates that were picked for further analyses demonstrated a strong tropism for keratinocytes with an impressive improvement in cell transduction efficiency.77 Also, differentiated keratinocytes in airlifted organotypic 3D cultures were transduced following topical vector application.77 This study was also the first to report about the mapping of the receptor targeted by a capsid variant selected by AAV peptide display demonstrating that comparative gene analysis, a microarray-based bioinformatic approach, is a convenient technology for this purpose.77

A further improvement for AAV peptide display technology is identi-

fication of capsid variants that not only enter the cell efficiently but are also able to express their genetic payload. This issue is of impor-

tance for the PCR-mediated viral library genome amplification where the common protocols cannot distinguish released (uncoated) from capsid-protected viral genomes. To overcome this limitation novel li-

brary designs are tested. Cronin et al.78 for example exchanged the rep ORF for a marker gene expression cassette and used FACS sorting to identify the successfully “infected” target cell population, while Deverman et al.91 used the Cre recombinase system, which, when ex-

pressed in the target cells, modified the released AAV library genomes
to become a template for PCR-mediated viral library genome amplification.

**Rational Design-Based Capsid Engineering for Optimizing Intracellular Processing**

Hansen et al. first described that, following infection, only ~20% of the input AAV2 vectors gain entry into the nucleus, whereas ~80% of the vectors fail to escape the endosome in the cytoplasm. Subsequently, Duan et al. reported that AAV2 capsids become ubiquitinated in the cytoplasm and targeted for degradation by the host cell proteasomal machinery. This negatively impacts the transduction efficiency of first-generation AAV vectors. Thus, one of the major obstacles that limit the transduction efficiency of AAV vectors in general is ubiquitination, followed by proteasome-mediated degradation.
However, the signal for ubiquitination of the incoming AAV particles remains unclear. Mah et al.\(^9\) had previously reported that inhibition of the host cell EGFR protein tyrosine kinase (EGFR-PTK) led to a significant increase in the transduction efficiency of AAV2 vectors. Thus, it was hypothesized that following infection, the AAV2 capsid protein becomes phosphorylated by EGFR-PTK, and that tyrosine phosphorylation is the signal for ubiquitination, followed by proteasomal degradation of AAV2 vectors in the cytoplasm.\(^8,9\) This is illustrated schematically in Figure 4.

**Generation of Tyrosine Mutant AAV2 Vectors**

Zhong et al.\(^9\) provided experimental evidence in 2007 to support the hypothesis that, during trafficking and escape from late endosomes, the AAV capsid indeed becomes phosphorylated at surface-exposed tyrosine residues by EGFR-PTK, and that tyrosine phosphorylation leads to ubiquitination, followed by proteasomal degradation of AAV2 vectors in the cytoplasm.\(^8,9\) As depicted in Figure 4A. These studies led to site-directed mutagenesis of the surface-exposed tyrosine residues in the AAV2 capsid to putatively circumvent this barrier. AAV2 capsid contains seven tyrosine (Y) residues that are surface exposed (Y252, Y272, Y444, Y500, Y700, Y704, and Y730). Zhong et al.\(^9\) mutagenized each of these Y residues to phenylalanine (F) residues because F residues cannot be phosphorylated by cellular tyrosine kinase as F lacks the hydroxyl (OH) group. Seven single mutants (Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, and Y730F) were generated and evaluated for their transduction efficiency in human cells in vitro. The transduction efficiency of three single mutants (Y444F, Y500F, and Y730F) was observed to be significantly higher than that of their WT counterpart.\(^9\) The Y730F single-mutant AAV2 vector was the most efficient in a number of cell types tested. The use of this vector led to the expression of therapeutic levels of human clotting factor 9 (F.IX) in several different strains of mice following intravenous or portal vein administration. This vector was observed to be ~10-fold more efficient than the first generation of AAV2 vector expressing the same therapeutic gene.\(^9\) Markusic et al.\(^9\) combined the three most efficient mutations (Y444F, Y500F, and Y730F) into one capsid and documented that the resulting triple-mutant (Y444+500+730F) vector was ~30-fold more efficient in expressing the F.IX gene in hemophilia B mice. In subsequent studies by various investigators, the tyrosine mutant AAV2 vectors in general, and the triple mutant AAV2 vector in particular, have been shown to be highly efficient in transducing a wide variety of cells and tissues.\(^9\)–\(^9\) Furthermore, the triple mutant AAV2 vector was shown to minimize in vivo targeting of transduced hepatocytes by capsid-specific CD8\(^+\) T cells.\(^9\)

The triple mutant AAV2 vectors has also shown efficacy in phase I and II clinical trials in 13 of 14 patients with Leber hereditary optic neuropathy.\(^9\)–\(^9\)

**Generation of Serine- and Threonine Mutant AAV2 Vectors**

Since in addition to Y residues, two additional amino acids, serine (S) and threonine (T), can also be phosphorylated by cellular serine/threonine kinases\(^9\) and, therefore, can lead to the same ubiquitination- and proteasome-mediated degradation, as shown schematically in Figure 4A, it was reasoned that site-directed mutagenesis of all 15 surface-exposed S residues (S261, S264, S267, S276, S348, S458, S468, S492, S498, S578, S658, S662, S668, S707, and S721) and all 17 surface-exposed T residues (T251, T329, T330, T454, T455, T491, T503, T550, T581, T592, T597, T671, T659, T660, T701, T713, and T716) in AAV2 capsids could also augment the transduction efficiency of these vectors. Thus, each of the 15 surface-exposed serine residues was substituted with valine (V) residues.\(^9,10\) The transduction efficiency of three of these mutants, S458V, S492V, and S662V, was increased by up to ~20-fold in different cell types. The S662V mutant was also found to be efficient in transducing human monocyte-derived dendritic cells (moDCs), a cell type that is not readily amenable to transduction by first-generation AAV2 vectors.
Site-directed mutagenesis of each of the 17 surface-exposed T residues was also performed, and the transduction efficiency of four of these mutants, T455V, T491V, T550V, and T659V, was observed to increase the transduction efficiency of these vectors in human cells in vitro and in murine hepatocytes in vivo following tail vein injection.99

Generation of Lysine Mutant AAV2 Vectors

Since ubiquitination occurs on lysine (K) residues (Figure 4B), Li et al.101 performed site-directed mutagenesis of each of the 10 surface-exposed K residues (K258, K490, K507, K527, K532, K544, K549, K556, K665, and K706) in the AAV2 capsid which were replaced with glutamic acid (E) because of similarity of size and lack of recognition by modifying enzymes. The transduction efficiency of K490E, K544E, K549E, and K556E scAAV2 vectors was increased to ~5-fold in human cells in vitro compared with WT AAV2 vectors, with the K556E mutant being the most efficient. Intravenous delivery of WT and K mutant AAV2 vectors further corroborated these results in murine hepatocytes in vivo.101

Identification of the Most Efficient Next Generation of AAV Serotype Vectors

Interestingly, most, if not all, of the surface-exposed Y, S, T, and K residues are highly conserved among all 10 commonly used AAV serotype vectors, and most of these residues have also been mutagenized in each of the 10 AAV serotype vectors. In addition to AAV2 vectors, the Y, S, T, and K mutants, two representative serotypes, AAV3 and AAV6, are depicted in Figure 5A. As shown in Figure 6B, the most critical Y, S, and T mutations were subsequently combined into one capsid, and the quadruple mutant (Y444+500+730F+T491V) AAV2 vector was identified as the most efficient. This vector increased the transduction efficiency ~24-fold over the WT AAV2 vector, and ~2-3-fold over the triple mutant (Y444+500+730F) vector in a murine hepatocyte cell line in vitro and in murine hepatocytes in vivo.
following tail vein injection in mice. The increase in the transduction efficiency of the quadruple mutant over that of the triple mutant also correlated well with the improved nuclear translocation of these vectors, which exceeded 90%.99

The corresponding Y, S, T, and K mutants of AAV3 and AAV6 serotype vectors are also shown schematically in Figure 5B. A double-mutant (S663V+T492V) for AAV3 and a triple-mutant (Y705+Y731F+T492V) for AAV6 serotypes were identified to be the most efficient. Glushakova et al.102 had previously reported the selective tropism of AAV3 vectors for human liver cells in vitro, since AAV3 utilizes the human hepatocyte growth factor receptor (HGFRI) for cellular entry, as documented by Ling et al.103 The S663V+T492V double-mutant AAV3 vector was also significantly more efficient than the WT AAV3 vector in transducing human liver tumors in a mouse xenograft model in vivo.104 Vercauteren et al.105 reported that the S663V+T492V double-mutant AAV3 vector was ~8 times, and ~80 times more efficient than AAV8 and AAV5 vectors, respectively, in transducing primary human hepatocytes in a “humanized” mouse model in vivo. Furthermore, Li et al.106 also evaluated the safety and efficacy of the WT and the S663V+T492V double-mutant AAV3 vectors in a non-human primate (NHP) model after intravenous delivery and documented efficient and selective liver tropism of both vectors. The transduction efficiency of the S663V+T492V double-mutant AAV3 vector was ~5-fold higher than that of its WT counterpart, with no apparent vector-related toxicity. Thus, S663V+T492V double-mutant AAV3 vector would appear to be an attractive alternative for potential gene therapy in a wide variety of human liver diseases.

Ling et al.107 reported that, among various permutations and combinations of Y, S, T, and K mutants of AAV6 vectors tested, a triple mutant (Y705+Y731F+T492V), shown schematically in Figure 5B, emerged as the most efficient in transducing primary human hematopoietic stem cells (HSCs), yet another human cell type refractory to transduction by all other AAV serotype vectors.108 Three independent groups have corroborated that AAV6 vectors are highly efficient in genome editing in primary human HSCs.109–111 More recently, AAV6 vectors were reported to lead to successful genome editing of sickle mutation in primary human HSCs from patients with sickle cell disease (SCD).112 However, multiplicities of infection (MOI) of 100,000-200,000 viral genomes/cell were required to achieve transduction efficiencies ranging between 45%–55% in those studies. The AAV6 triple-mutant (Y705+Y731F+T492V) vector, with which transduction efficiency exceeding 90%, can be achieved in primary human HSCs at an MOI of 20,000 viral genomes/cell102,107 makes it highly desirable for safe and efficient genome editing in HSCs.

Conclusions

Despite the safe and successful use of first-generation AAV vectors in 176 phase I, II, and III clinical trials to date and the remarkable clinical efficacy achieved in at least eight human diseases thus far, it stands to reason that the safety and efficacy of these vectors could be further enhanced, given that the AAV vectors currently being used are composed of, for the most part, naturally occurring capsids, which are readily targeted by host cell enzymes, thereby impacting their overall performance. In addition, natural AAV serotypes show tissue preferences, but possess, in general, a broad tropism. Therefore, a high number of particles has to be applied to obtain therapeutic transgene expression levels. Besides loosing particles, expression or maybe even just the uptake of viral vector particles in off-target tissues might induce immune response that limit efficacy of AAV vector-based gene therapy. Various strategies on capsid modifications described in this review, have led to the development of next generation of AAV vectors that are likely to overcome some of the limitations associated with the first generation AAV vectors.

In this context, it is important to note that such capsid modifications could potentially alter the host immune response to AAV vectors. The next generation of AAV2 vectors for example has been shown to minimize the capsid-specific CD8⁺ T cell response,95 and the next generation of AAV2 or AAV6 vectors has been shown to be capable of partially evading pre-existing antibodies.113,114

In sum, since the WT AAV did not evolve for the purposes of delivery of therapeutic genes, there is ample basis for optimism that these capsid-modified AAV vectors and future improvements will further add to the safety, efficacy, and specificity of their potential use in gene therapy for a wide variety of human diseases in the foreseeable future.

CONFLICTS OF INTEREST

H.B. is an inventor on patents that have been issued on next-generation AAV vectors. A.S. is a co-founder of, and holds equity in, Lacerta Therapeutics, aaVective, Nirvana Therapeutics, and KASHX Bio, all recently launched AAV gene therapy companies. He is also an inventor on several patents issued on recombinant AAV vectors that have been licensed to various gene therapy companies.

ACKNOWLEDGMENTS

The authors thank their colleagues and collaborators, both past and present, for helpful scientific discussions. The assistance from Mr. Himanshu Rambhai with some of the illustrations and from Dr. Michael Morgan (Hannover Medical School) in proofreading the manuscript is gratefully acknowledged. This work was supported in part by the Federal Ministry of Education and Research (BMBF) and the Ministry for Science and Culture of Lower Saxony (MWK)-funded Professorinnenprogramm Niedersachsen, the German Research Foundation (DFG)-funded REBIRTH Cluster of Excellence (to H.B.), the NIH (grants R01 HL-097088, R41 AI-122735, and R21 EB-015684), a grant from the Children’s Miracle Network, and support from the Kitzman Foundation (to A.S.).

REFERENCES

1. Weinmann, J., and Grimm, D. (2017). Next-generation AAV vectors for clinical use: an ever-accelerating race. Virus Genes 53, 707–713.
7. Berns, K.I., and Muzyczka, N. (2017). AAV: an overview of unanswered questions. Hum. Gene Ther. 28, 308–313.

8. Bainbridge, J.W., Smith, A.J., Barker, S.S., Robbie, S., Henderson, R., Balagkkan, K., Viswanathan, A., Holder, G.E., Stockman, A., Tyler, N., et al. (2008). Effect of gene therapy on visual function in Leber’s congenital amaurosis. N. Engl. J. Med. 358, 2231–2239.

9. Cideciyan, A.V., Aleman, T.S., Boye, S.L., Schwartz, S.B., Kaushal, S., Roman, A.J., Pang, J.J., Sumarako, A., Windsor, E.A., Wilson, J.M., et al. (2006). Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. Proc. Natl. Acad. Sci. USA 105, 15112–15117.

10. Maguire, A.M., Simonelli, F., Pierce, E.A., Pugh, E.N., Jr., Mingozzi, F., Bennicelli, J., Banik, S., Marshall, K.A., Testa, F., Surace, E.M., et al. (2008). Safety and efficacy of gene transfer for Leber’s congenital amaurosis. N. Engl. J. Med. 358, 2240–2248.

11. Hwu, W.L., Muramatsu, S., Tseng, S.H., Tzen, K.Y., Lee, N.C., Chien, Y.H., Snyder, R.O., Byrne, B.J., Tai, C.H., and Wu, R.M. (2012). Gene therapy for aromatic L-amino acid decarboxylase deficiency. Sci. Transl. Med. 4, 134ra61.

12. MacLaren, R.E., Gropp, M., Barnard, A.R., Cottrill, C.L., Tolmachova, T., Seymour, L., Clark, K.R., Durin, M.J., Cremers, F.P., Black, G.C., et al. (2014). Retinal gene therapy in patients with choroideremia: initial findings from a phase 1/2 clinical trial. Lancet 383, 1129–1137.

13. Bantel-Schaal, U., Delius, H., Schmidt, R., and zur Hausen, H. (1999). Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent paroviruses. J. Virol. 73, 938–947.

14. Chiorini, J.A., Yang, L., Liu, Y., Safer, B., and Kotin, R.M. (1997). Cloning of adeno-associated virus type 5 from a personal perspective. Hum. Gene Ther. 8, 358–379.
50. Büning, H., Huber, A., Zhang, L., Meumann, N., and Hacker, U. (2015). Engineering adeno-associated virus type 2 carrying a phospholipase A2 domain required for virus infectivity. J. Gen. Virol. 83, 973–978.

51. Stahinke, S., Lux, K., Uhling, S., Kreppel, F., Hösdl, M., Coutelle, O., Ogris, M., Hallek, M., and Bünig, H. (2011). Intrinsic phospholipase A2 activity of adeno-associated virus is involved in endosomal escape of incoming particles. Virology 409, 77–83.

52. Aumailley, M., Gerl, M., Sonnenberg, A., Deutzmann, R., and Timpl, R. (1990). Heparin binding induces conformational changes in adeno-associated virus capsid protein. J. Virol. 67, 13150–13160.

53. Palma-Wagner, R., Porwal, M., Kann, M., Reuss, M., Weimer, M., Florin, L., and Kleinschmidt, J.A. (2012). Impact of VP1-specific protein sequence motifs on adeno-associated virus type 2 intracellular trafficking and nuclear entry. J. Virol. 86, 9163–9174.

54. Rayaprolu, V., Kruse, S., Kant, R., Venkatakrishnan, B., Movahed, N., Brooke, D., Lins, B., Bennett, A., Potter, T., McKenna, R., et al. (2013). Comparative analysis of adeno-associated virus capsid stability and dynamics. J. Virol. 87, 13150–13160.

55. Halder, S., Van Vliet, K., Smith, J.K., Duong, T.T., McKenna, R., Wilson, J.M., and Hallek, M. (1999). Genetic capsid modifications for their in vivo tropism. J. Virol. 73, 11072–11081.

56. Perabo, L., Godna, D., White, K., Endell, J., Boucas, J., Humme, S., Work, L.M., Janicki, H., Hallek, M., Baker, A.H., and Bünig, H. (2006). Heparan sulfate proteoglycan binding properties of adeno-associated virus retargeting mutants and consequences for their vector tropism. J. Virol. 80, 7265–7269.

57. Uhling, S., Coutelle, O., Wiehe, T., Perabo, L., Hallek, M., and Bünig, H. (2012). Successful target cell transduction of capsid-engineered rAAV vectors requires clathrin-dependent endocytosis. Gene Ther. 19, 210–218.

58. Bünig, H., Huber, A., Zhang, L., Meumann, N., and Hacker, U. (2015). Engineering the AAV capsid to optimize vector-host interactions. Curr. Opin. Pharmacol. 24, 94–104.

59. Boucas, J., Lux, K., Huber, A., Scheivenbusch, S., van Freyend, M.J., Perabo, L., Quadri, Humme, S., Odenthal, M., Hallek, M., and Bünig, H. (2009). Engineering adeno-associated virus serotype 2-based targeting vectors using a new insertion site-position 453 and single point mutations. J. Gene Med. 11, 1103–1113.

60. Yang, Q., Mamounas, M., Yu, G., Kennedy, S., Leaker, B., Merson, J., Wong-Staal, F., Yu, M., and Barber, J.R. (1998). Development of novel cell surface CD34-targeted recombinant adenoassociated virus vectors for gene therapy. Hum. Gene Ther. 9, 1929–1937.

61. Wu, P., Xiao, W., Conlon, T., Hughes, J., Agbandje-McKenna, M., Ferkol, T., Flotte, T., and Muzyczka, N. (2000). Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. J. Virol. 74, 8635–8647.

62. Wang, L.R., McLaughlin, T., Cossette, T., Tang, Q., Foust, K., Campbell-Thompson, M., Martino, A., Cruz, P., Loiler, S., Mueller, C., and Flotte, T.R. (2009). Recombinant AAV serotype and capsid mutant comparison for pulmonary gene transfer of alpha 1 antitrypsin using invasive and noninvasive delivery. Mol. Ther. 17, 81–87.

63. Rabinowitz, J.E., Rolling, F., Li, C., Cheng, S., Xiao, W., Xiao, X., and Samulski, R.J. (2004). Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. J. Virol. 78, 6695–6699.

64. Shi, W., Arnold, G.S., and Bartlett, J.S. (2001). Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. Hum. Gene Ther. 12, 1697–1711.

65. Arnold, G.S., Sasser, A.K., Stachler, M.D., and Bartlett, J.S. (2006). Biochemical thin-layer chromatography provides a unique platform for the purification and targeting of multiple AAV vector serotypes. Mol. Ther. 14, 97–106.

66. Levy, H.C., Bowman, V.D., Govindasamy, L., McKenna, R., Nash, K., Warrington, K., Chen, W., Muzyczka, N., Yan, X., Baker, T.S., and Agbandje-McKenna, M. (2003). Heparan sulfate proteoglycans mediate adeno-associated virus type 2 entry. J. Biol. Chem. 278, 145–146.

67. Aumailley, M., Gerl, M., Sonnenberg, A., Deutzmann, R., and Timpl, R. (1999). Identification of the Arg–Gly–Asp sequence in laminin A chain as a latent cell-binding site being exposed in fragment P1. FEBS Lett. 450, 82–86.

68. Opie, S.R., Warrington, K.H., Jr., Agbandje-McKenna, M., Zolotukhin, S., and Muzyczka, N. (2004). Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. J. Virol. 78, 6695–6699.

69. Shi, W., Arnold, G.S., and Bartlett, J.S. (2001). Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. Hum. Gene Ther. 12, 1697–1711.

70. Arnold, G.S., Sasser, A.K., Stachler, M.D., and Bartlett, J.S. (2006). Biochemical thin-layer chromatography provides a unique platform for the purification and targeting of multiple AAV vector serotypes. Mol. Ther. 14, 97–106.

71. Lux, K., Goerlitz, N., Schlemmering, S., Perabo, L., Godna, D., Endell, J., Leike, K., Koller, D.M., Finke, S., Hallek, M., and Bünig, H. (2005). Green fluorescent protein–tagged adeno-associated virus particles allow the study of cytosolic and nuclear trafficking. J. Virol. 79, 11776–11787.

72. Asokan, A., Johnson, J.S., Li, C., and Samulski, R.J. (2008). Bioluminescent viron shells: new tools for quantitation of AAV vector dynamics in cells and live animals. Gene Ther. 15, 1618–1622.

73. Choudhury, S.R., Harris, A.F., Cabral, D.J., Keeler, A.M., Sapp, E., Ferreira, J.S., Gray-Edwards, H.L., Johnson, J.A., Johnson, A.K., Su, Q., et al. (2016). Widespread central nervous system gene therapy and silencing after systemic delivery of novel AAV-AS vector. Mol. Ther. 24, 726–735.

74. Yu, H., Koikonda, R.D., Chou, T.H., Persia, V., Ordem, S.S., Chiodo, V., Boye, S.L., Boye, S.E., Hauswirth, W.W., Levin, A.S., and Guy, J. (2012). Gene delivery to the mitochondria by targeting modified adenoassociated virus suppresses Leber’s hereditary optic neuropathy in a mouse model. Proc. Natl. Acad. Sci. USA 109, E1238–E1247.

75. Li, H., Zhang, F.L., Shi, W.J., Bai, X.I., Jia, S.Q., Zhang, C.G., and Ding, W. (2015). Immobilization of FLAG-tagged recombinant adeno-associated virus 2 onto tissue engineering scaffolds for the improvement of transgene delivery in cell implants. PLoS ONE 10, e0129013.

76. Hartmann, J., Münch, R.C., Freiling, R.T., Schneider, I.C., Dreier, B., Samuenge, W., Koch, J., Seeger, M.A., Plückthun, A., and Buchholz, C.J. (2018). A library-based screening strategy for the identification of DARPinAs ligands for receptor-targeted AAV and lentiviral vectors. Mol. Ther. Methods Clin. Dev. 10, 128–143.

77. Münch, R.C., Muth, A., Muck, A., Friedel, T., Schmata, I., Dreier, B., Trlaska, A., Plückthun, A., Büning, H., and Buchholz, C.J. (2015). Off-target-free gene delivery by affinity-purified receptor-targeted viral vectors. Nat. Commun. 6, 2642.

78. Muck, A., Reul, J., Friedel, T., Muth, A., Hartmann, K.P., Schneider, I.C., Münch, R.C., and Buchholz, C.J. (2017). Covalent coupling of high-affinity ligands to the surface of viral vector particles by protein trans-splicing mediates cell type-specific gene transfer. Biomaterials 144, 84–94.

79. Hörner, M., Kaufmann, B., Cottugno, G., Wiedke, E., Büning, H., Grimm, D., and Weber, W. (2014). A chemical switch for controlling viral infectivity. Chem. Commun. (Camb.) 50, 10319–10322.
Review

www.moleculartherapy.org

75. Hagen, S., Baumann, T., Wagner, H.J., Morath, V., Kaufmann, B., Fischer, A., Bergmann, S., Schindler, P., Arndt, K.M., and Müller, K.M. (2014). Modular adeno-associated virus (rAAV) vectors used for cellular virus-directed enzyme prodrug therapy. Sci. Rep. 4, 3759.

76. Boersma, Y.L., and Plackthun, A. (2011). DARPins and other repeat protein scaffolds: advances in engineering and applications. Curr. Opin. Biotechnol. 22, 849–857.

77. Müller, O.J., Leuchs, B., Pleger, S.T., Grumm, D., Franz, W.M., Katus, H.A., and Kleinschmidt, J.A. (2006). Improved cardiac gene transfer by transcriptional and transducatinal targeting of adeno-associated viral vectors. Cardiovasc. Res. 70, 70–78.

78. Adachi, K., and Nakai, H. (2010). A new recombinant adeno-associated virus (AAV)-based random peptide display library system: Infection-defective Aav1.9-3 as a novel detoxified platform for vector evolution. Gene Ther. Regul. 5, 31–55.

79. Cronin, T., Vandemerche, L.H., Hantz, P., Juttner, J., Reimann, A., Kacso, A.E., Huckfeldt, R.M., Busskamp, V., Kohler, H., Lagali, P.S., et al. (2014). Efficient transduction and optogenetic stimulation of retinal bipolar cells by a synthetic adeno-associated virus capsid and promoter. EMBO Mol. Med. 6, 1175–1190.

80. Dalkara, D., Byrne, L.C., Jayandharan, G.R., Rivers, A.E., Aslanidi, G.V., Ling, C., Zolotukhin, I., Ma, W., et al. (2013). High-efficiency transduction and correction of murine hemophilia B using AAV2 vectors devoid of multiple surface-exposed tyrosines. Mol. Ther. 18, 2048–2056.

81. Martino, A.T., Bresler-Tschakarjan, E., Markus, D.M., Finn, J.D., Hinderer, C., Zhou, S., Ostrov, D.A., Srivastava, A., Erfl, H.C., Terhorst, C., et al. (2013). Engineered AAV vector minimizes in vivo targeting of transduced hepatocytes by capsid-specific CD8+ T cells. Blood 122, 2224–2233.

82. Feuer, W.J., Davis, J.L., Porciatti, V., Gonzalez, P.J., Koikliouda, R.D., Yuan, H., Hauswirth, W.W., and Lam, B.L. (2017). Gene therapy for Leber hereditary optic neuropathy: low- and medium-dose visual results. Ophthalmology 124, 1621–1634.

83. Feuer, W.J., Schifman, J.C., Davis, J.L., Porciatti, V., Gonzalez, P.J., Koikliouda, R.D., Yuan, H., Lalwani, A., Lam, B.L., and Guy, J. (2016). Gene therapy for Leber hereditary optic neuropathy: initial results. Ophthalmology 123, 558–570.

84. Edelman, A.M., Blumenthal, D.K., and Krebs, E.G. (1987). Protein serine/threonine kinases. Annu. Rev. Biochem. 56, 567–613.

85. Aslanidi, G.V., Rivers, A.E., Ortiz, L., Song, L., Ling, C., Govindasamy, L., Van Vliet, K., Tan, M., Agbandje-McKenna, M., and Srivastava, A. (2013). Optimization of the capsid of recombinant adeno-associated virus 2 (AAV2) vectors: the final threshold? PLoS ONE 8, e59142.

86. Aslanidi, G.V., Rivers, A.E., Ortiz, L., Govindasamy, L., Ling, C., Jayandharan, G.R., Zolotukhin, S., Agbandje-McKenna, M., and Srivastava, A. (2012). High-efficiency transduction of human monocye-derived dendritic cells by capsid-modified recombinant AAV2 vectors. Vaccine 30, 3908–3917.

87. Li, B., Ma, W., Wang, L., Vliet, K., Huang, L.Y., Agbandje-McKenna, M., Srivastava, A., and Aslanidi, G.V. (2015). Site-directed mutagenesis of surface-exposed tyrosine residues leads to improved transduction by AAV2, but not AAV8, vectors in murine hepatocytes in vivo. Hum. Gene Ther. Methods 26, 211–220.

88. Liu, C., Srivastava, A., and Stacpoole, P.W. (2009). AAV3-mediated transfer and transduction of human monocyte-derived dendritic cells by capsid-modified AAV2 vectors in vivo. Hum. Gene Ther. 20, 1741–1747.

89. Liu, C., Yu, Y., Kalsi, J.K., Jayandharan, G.R., Li, B., Ma, W., Cheng, B., Gee, S.W., McGoogan, K.E., Govindasamy, L., et al. (2010). Human hepatocyte growth factor receptor is a cellular coreceptor for adeno-associated virus serotype 3. Hum. Gene Ther. 21, 1741–1747.

90. Ling, C., Wang, Y., Zhang, Y., Ejigjini, A., Yin, Z., Yu, L., Wang, L., Wang, M., Li, J., Hu, Z., et al. (2014). Selective in vivo targeting of human liver tumors by optimized AAV3 vectors in a murine xenograft model. Hum. Gene Ther. 25, 1023–1034.

91. Vercauteren, K., Hoffman, B.E., Zolotukhin, I., Keeler, G.D., Xiao, J.W., Bresler-Tschakarjan, E., High, K.A., Erfl, H.C., Rice, C.M., Srivastava, A., et al. (2016). Superior in vivo transduction of human hepatocytes using engineered AAV3 capsid. Mol. Ther. 24, 1042–1049.

92. Li, S., Ling, C., Zhong, L., Li, M., Su, Q., He, R., Tang, Q., Greiner, D.L., Shultz, L.D., Brehm, M.A., et al. (2015). Efficient and targeted transduction of nonhuman primate liver with systemically delivered optimized AAV3B vectors. Mol. Ther. 23, 1867–1876.

93. Zhong, L., Han, C., Govindasamy, L., Agbandje-McKenna, M., Herzig, R.W., Weigel-Val Aken, K.A., Hobbs, J.A., Zolotukhin, S., et al. (2008). Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intraacellular trafficking and transgene expression. Virology 381, 194–202.

94. Song, L., Kauss, M.A., Kopin, E., Chandra, M., Ul-Hasan, T., Miller, E., Jayandharan, G.R., Rivers, A.E., Aslanidi, G.V., Ling, C., et al. (2013). Optimizing the transduction efficiency of capsid-modified AAV6 serotype vectors

signaling in ubiquitination of AAV2 capsids and viral second-strand DNA synthe-

Mol. Ther. 15, 1323–1330.

Molecular Therapy: Methods & Clinical Development Vol. 12 March 2019 263
in primary human hematopoietic stem cells in vitro and in a xenograft mouse model in vivo. Cytotherapy 15, 986–998.

109. Wang, J., Exline, C.M., DeClercq, J.J., Llewellyn, G.N., Hayward, S.B., Li, P.W., Shivak, D.A., Surosky, R.T., Gregory, P.D., Holmes, M.C., and Cannon, P.M. (2015). Homology-driven genome editing in hematopoietic stem progenitor cells using ZFN mRNA and AAV6 donors. Nat. Biotechnol. 33, 1256–1263.

110. Sather, B.D., Romano Ibarra, G.S., Sommer, K., Curinga, G., Hale, M., Khan, I.F., Sorensen, D.L., Medina, M.A., Surosky, R.T., Gregory, P.D., Holmes, M.C., and Cannon, P.M. (2015). Homology-driven genome editing in hematopoietic stem progenitor cells using ZFN mRNA and AAV6 donors. Nat. Biotechnol. 33, 1256–1263.

111. De Ravin, S.S., Reik, A., Liu, P.Q., Li, L., Wu, X., Su, L., Railey, C., Theobald, N., Choi, U., Song, A.H., et al. (2016). Targeted gene addition in human CD34(+) hematopoietic cells for correction of X-linked chronic granulomatous disease. Nat. Biotechnol. 34, 424–429.

112. Dewer, D.P., Bak, R.O., Reinsch, A., Camarena, J., Washington, G., Nicolas, C.E., Pavol-Dinu, M., Saxena, N., Wilkens, A.B., Mantri, S., et al. (2016). CRISPR/Cas9 8-β-globin gene targeting in human haematopoietic stem cells. Nature 539, 384–389.

113. van Lieshout, L.P., Domm, J.M., Rindler, T.N., Frost, K.L., Llewellyn, G.N., Hayward, S.B., Li, P.W., van Lieshout, L.P., Drebber, U., Hallek, M., and Baker, A.H. (2006). Vascular bed-targeted recombinant adeno-associated viral vectors by applying an AAV random peptide library platform. Oncol. Immunol. 5, e171446.

114. Shi, W., and Bartlett, J.S. (2003). RGD inclusion in VP3 provides adeno-associated virus type 2 (AAV2)-based vectors with a heparan sulfate-independent cell entry mechanism. Mol. Ther. 7, 515–525.

115. Waterkamp, D.A., Müller, O.J., Ying, Y., Trepel, M., and Kleinschmidt, J.A. (2006). Isolation of targeted AAV2 vectors from novel virus display libraries. J. Gene Med. 8, 1307–1319.

116. Michelfelder, S., Lee, M.K., deLima-Hahn, E., Wilmes, T., Kaul, F., Müller, O., Kleinschmidt, J.A., and Trepel, M. (2007). Vectors selected from adeno-associated viral display peptide libraries for leukemia cell-targeted cytotoxic gene therapy. Exp. Hematol. 35, 1766–1776.

117. Stiefelhagen, M., Sellner, L., Kleinschmidt, J.A., Jauch, A., Raues, W., Zeller, W.J., Fuchs, T., and Veldwijk, M.R. (2008). Application of a haematopoietic progenitor cell-targeted adeno-associated viral (AAV) vector established by selection of an AAV random peptide library on a leukemia cell line. Genet. Vaccines Ther. 6, 12.

118. Sellner, L., Stiefelhagen, M., Kleinschmidt, J.A., Raues, W., Zeller, W.J., and Veldwijk, M.R. (2008). Generation of efficient human blood progenitor-targeted recombinant adeno-associated viral vectors (AAV) by selecting an AAV random peptide library on primary human hematopoietic progenitor cells. Exp. Hematol. 36, 957–964.

119. Michelfelder, S., Kohlschütter, J., Skorupa, A., Pflünges, S., Müller, O., Kleinschmidt, J.A., and Trepel, M. (2009). Successful expansion but not complete restriction of tropism of adeno-associated virus by in vivo biopanning of random virus display peptide libraries. PLoS ONE 4, e5122.

120. Naumer, M., Popa-Wagner, R., and Kleinschmidt, J.A. (2012). Impact of capsid modifications by selected peptide ligands on recombinant adeno-associated virus serotype 2-mediated gene transduction. J. Gen. Virol. 93, 2131–2141.

121. Körbelin, J., Sieber, T., Michelfelder, S., Lunding, L., Spies, E., Hunger, A., Alawi, M., Rapti, K., Indenbrink, D., Müller, O.J., et al. (2016). Pulmonary targeting of adeno-associated viral vectors by next-generation sequencing-guided screening of random capsid displayed peptide libraries. Mol. Ther. 24, 1050–1061.

122. Ying, Y., Müller, O.J., Goebinger, C., Lehms, B., Trepel, M., Katus, H.A., and Kleinschmidt, J.A. (2010). Heart-targeted adeno-associated viral vectors selected by in vivo biopanning of a random viral display peptide library. Gene Ther. 17, 980–990.

123. Shi, X., Fang, G., Shi, W., and Bartlett, J.S. (2006). Insertional mutagenesis at positions 520 and 584 of adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors with eliminated heparin-binding ability and introduced novel tropism. Hum. Gene Ther. 17, 353–361.

124. Konkalmatt, P.R., Deng, D., Thomas, S.W., Wu, M.T., Logsdon, C.D., French, B.A., and Kelly, K.A. (2013). Plectin-1 targeted AAV vector for the molecular imaging of pancreatic cancer. Front. Oncol. 3, 84.

125. Stachel, M.D., Chen, I., Ting, A.Y., and Bartlett, J.S. (2008). Site-specific modification of AAV vector particles with biophysical probes and targeting ligands using biotin ligase. Mol. Ther. 16, 1467–1473.

126. Stachel, M.D., and Bartlett, J.S. (2006). Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells. Gene Ther. 13, 926–931.

127. Davis, A.S., Federici, T., Ray, W.C., Bouli, N.M., O’Connor, D., Clark, K.R., and Bartlett, J.S. (2015). Rational design and engineering of a modified adeno-associated virus (AAV1)-based vector system for enhanced retrograde gene delivery. Neurosurgery 76, 216–225; discussion 225.
144. Khabou, H., Desrosiers, M., Winckler, C., Fouquet, S., Auregan, G., Bemelmans, A.P., Sahel, J.A., and Dalkara, D. (2016). Insight into the mechanisms of enhanced retinal transduction by the engineered AAV2 capsid variant -7m8. Biotechnol. Bioeng. 113, 2712–2724.

145. Sayroo, R., Nolasco, D., Yin, Z., Colon-Cortes, Y., Pandya, M., Ling, C., and Aslanidi, G. (2016). Development of novel AAV serotype 6 based vectors with selective tropism for human cancer cells. Gene Ther. 23, 18–25.

146. Michelfelder, S., Varadi, K., Raupp, C., Hunger, A., Körbelin, J., Pahrmann, C., Schreper, S., Müller, O.J., Kleinschmidt, J.A., and Trepel, M. (2011). Peptide ligands incorporated into the threefold spike capsid domain to re-direct gene transduction of AAV8 and AAV9 in vivo. PLoS ONE 6, e23101.

147. Kunze, C., Börner, K., Kienle, E., Orschmann, T., Rusha, E., Schneider, M., Radivojkov-Blagojevic, M., Drucker, M., Desbordes, S., Grimm, D., and Brack-Werner, R. (2018). Synthetic AAV/CRISSPR vectors for blocking HIV-1 expression in persistently infected astrocytes. Glia 66, 413–427.