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Review

Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors

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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.[1–7] 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 disease[28] and showed impressive efficacy in gene therapy for spinal muscular atrophy.[29] The AAV1-LPL vector was approved as a drug designated alipogene tiparvovec 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.[30]

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.[33] 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

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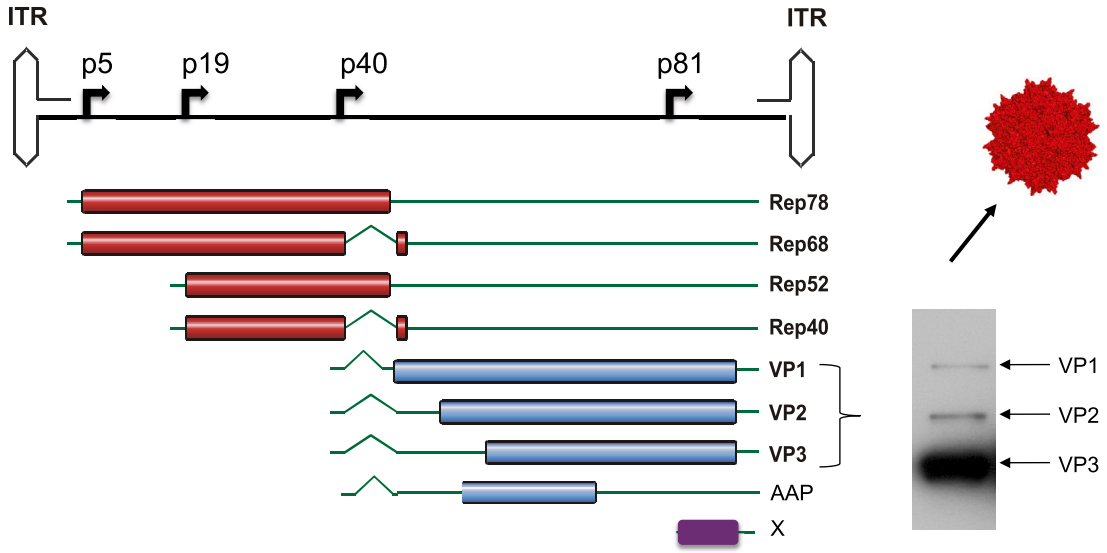

Figure 1. AAV Serotype 2 Vector Genome Conformation

The AAV viral genome is flanked by inverted terminal repeats (ITRs), which serve as packaging signal and origin of replication. The *rep* gene encodes a family of multifunctional proteins (Rep proteins) responsible for controlling viral transcription, replication, packaging, and integration in AAVS1. For AAV2, four Rep proteins are described. Expression of Rep78 and Rep68 is controlled by the AAV2-specific p5 promoter, while p19 controls expression of the smaller Rep proteins (Rep52 and Rep40). Rep68 and Rep40 are splice variants of Rep78 and Rep52, respectively. Numbers indicate the molecular weight. Expression of AAP and the viral capsid proteins VP1 (90 kDa), VP2 (72 kDa), and VP3 (60 kDa), all encoded in the *cap* gene, is controlled by the p40 promoter. The X gene is located at the 3′ end of the genome within a region shared with the *cap* gene and possesses its own promoter (p81). While the X protein seems to enhance viral replication, AAP is essential for capsid assembly. The three different VPs contribute in a 1 (VP1):1 (VP2):10 (VP3) ratio to the icosahedral AAV2 capsid.

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.[34] 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.[37] The pores allow exchange between the capsid interior and the outside. Rep proteins shuttle the viral genomes into the capsids through the pores, and they may also serve as sites of viral genome release.[38] Furthermore, the N termini of VP1 and VP2 are externalized through the pores early in the infection process,[39] exposing domains required for escape from the endosomal compartment prior to lysosomal degradation (phospholipase A2 domain[40,41]) and for trafficking toward and into the nucleus.[39,42] The depressions, more precisely the floor at each twofold axis, are the thinnest part of the viral capsid.[43,44] The protrusions around the threefold axis harbor five of the nine so-called variable regions (VRs).[45] Specifically, VR-IV, -V, and -VIII form loops at the top of the protrusions, while VR-VI and -VII are found at their base.[45] VRs differ between serotypes and are responsible for serotype-specific variations in antibody and receptor binding.[45]

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 (pseudopackaging) 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

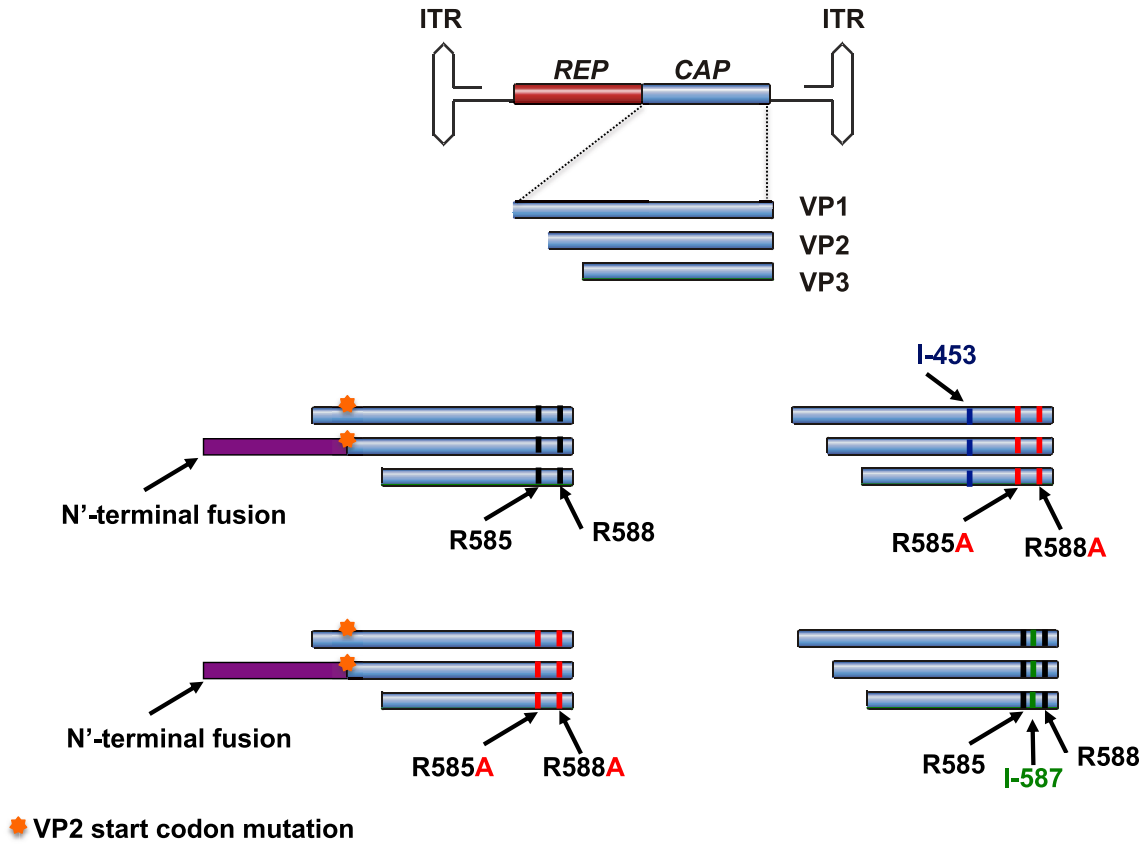

Figure 2. Capsid Engineering for Re-targeting or Expansion of AAV2 Vector Tropism

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.

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.[48] 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) .[48,49] 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[48] (Figure 2). Target cells were transduced, even in the presence of heparin, the soluble analog of AAV2's primary receptor heparan sulfate proteoglycan (HSPG) .[48] This finding was explained upon identification of the HSPG binding motif of AAV2 by the groups of Kleinschmidt[50] and Muzyczka[51] in 2003. Their work showed that insertion of peptides at I-587 changes the spacing between R585 and R588 and thereby destroys the AAV2 HSPG binding motif. Mapping of I-587 to AAV2's crystal structure confirmed that this insertion site is indeed located at the tip of a loop.[52] Specifically, I-587 is part of VR-VIII, which forms the top of the second highest of the three protrusions at the 3-fold axis of symmetry.[45,52] A portfolio of subsequent studies confirmed the potential

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	QAGTFALRGDNPQG	first 587 targeting vector	[48]
AAV-588NGR	CD13-positive tumor cells	NGRAHA		[115]
AAV-MO7A	tumor cells	RGDAVGV	AAV2 peptide display	[83]
AAV-MO7T	tumor cells	RGDTPTS	AAV2 peptide display	[83]
AAV-MecA	tumor cells	GENQARS	AAV2 peptide display	[83]
AAV-MecB	tumor cells	RSNAVVP	AAV2 peptide display	[83]
rRGD587	αv integrin positive tumor cells	CDCRGDCFC	phage display	[56]
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	MTPFPTSNEANL	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	CAPGPSKSG	identified by *in vivo* phage display	[120]
AAV-EYH	smooth muscle cells	EYHHYNK	phage display	[121]
AAV_{587}MTP	skeleton muscle cells	ASSLNIA	phage display	[122]
AAV-r3.45	neuronal stem cells	TQVGQKT	AAV2 peptide display	[123]
AAV2-LSS	CNS	LPSSLQK	*in vivo* AAV2 peptide display	[124]
AAV2-PFG	CNS	WPFYGTP	*in vivo* AAV2 peptide display	[124]
AAV2-PPS	CNS	DSPAHPS	*in vivo* AAV2 peptide display	[124]
AAV2-TLH	CNS	GWTLHNK	*in vivo* AAV2 peptide display	[124]
AAV2-GMN	CNS	GMNAFRA	*in vivo* AAV2 peptide display in disease model; identification of target receptor[125]	[124]
AAV2-7m8	retinal cell types	LGETTRP	*in vivo* AAV2 peptide display; further change in backbone: V708I	[80]
AAV-Kera1	keratinocytes	RGDTATL	AAV2 peptide display after pre-clearing step	[87]
AAV-Kera2	keratinocytes	PRGDLAP	AAV2 peptide display after pre-clearing step; identification of target receptor[87]	[87]
AAV-Kera3	keratinocytes	RGDQQSL	AAV2 peptide display after pre-clearing step	[87]
AAV-588Myc	none	EQLSISEEDL	tag	[115]
AAV2-Z34C	adaptor	Z34C[126]	antibody binding via protein A domain	[127]
AAV2.N587_R588insBAP	adaptor	GLNDIFEAQKIEWHE	biotin acceptor peptide (BAP) for purification or targeting via avidin containing ligands	[65]
$AAV2^{Ald13}$	adaptor	LCTPSRAALLTGR	chemical coupling of ligands	[128]
DMD4	vaccine	QVSHWVSGLAEGSFG	AAV2 15-mer peptide display	[129]
DMD6	vaccine	LSHTSGRVEGSVSLL	AAV2 15-mer peptide display	[129]

I-587, peptide insertion between N587 and R588. For details on linker sequences flanking the peptide insert, please see references.

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 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	–	130
A588-RGD4CGLS	αv-integrin positive tumor cells	CDCRGDCFC	test of linker sequences	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-DLSNLTR	tumor cells	DLSNLTR	AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling	131
AAV-NQVGSWS	tumor cells	NQVGSWS	AAV2 peptide display	132
AAV-EARVRPP	tumor cells	EARVRPP	AAV2 peptide display	133
AAV-NSVSLYT	tumor cells (CML)	NSVSLYT	AAV2 peptide display	133
AAV-LS1	tumor cells (CML), $CD34^+$ cells	NDVRSAN*	AAV2 peptide display	134
AAV-LS2	tumor cells (CML), $CD34^+$ cells	NESRVLS	AAV2 peptide display	134
AAV-LS3	tumor cells (CML), $CD34^+$ cells	NRTWEQQ	AAV2 peptide display	134
AAV-LS4	tumor cells (CML), $CD34^+$ cells	NSVQSSW	AAV2 peptide display	134
AAV-RGDLGLS	tumor cells	RGDLGLS	AAV2 peptide display	135
AAV-RGDMSRE	tumor cells	RGDMSRE	AAV2 peptide display	135
AAV-ESGLSQS	tumor cells	ESGLSQS	*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-DLGSARA	tumor cells	DLGSARA	*in vivo* AAV2 peptide display; improved liver and heart transduction	135
AAV-NDVRSAN	tumor cells	NDVRSAN*	AAV2 peptide display	136
AAV-GPQGKNS	tumor cells	GPQGKNS	AAV2 peptide display	136
AAV-NSSRDLG	endothelial cells	NSSRDLG	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; novel strategy for optimizing phenotype/genotype coupling	131
AAV-NDVRAVS	endothelial cells	NDVRAVS#	AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling	131
AAV-PRSTSDP	lung (maybe endothelial cells)	PRSTSDP	*in vivo* AAV2 peptide display; improved lung transduction, but also other organs	135
AAV-DIIRA	endothelial cells	DIIRA	AAV2-5-mer peptide display library	86
AAV-SYENV	endothelial cells	SYENVASRRPEG	AAV2-12-mer peptide display library	86
AAV-PENSV	endothelial cells	PENSVRRYGLEE	AAV2-12-mer peptide display library	86
AAV-LSLAS	endothelial cells	LSLASNRPTATS	AAV2-12-mer peptide display library	86
AAV-NDVWN	endothelial cells	NDVWNRDNSSKRGGTTEAS	AAV2-19-mer peptide display library	86
AAV-NRTYS	endothelial cells	NRTYSSTSNSTSRSEWDNS	AAV2-19-mer peptide display library	86
rAAV2-ESGHGYF	pulmonary endothelial cells	ESGHGYF	*in vivo* AAV peptide display	137
AAV-GQHPRPG	cardiomyoblasts	GQHPRPG+	AAV2 peptide display; novel strategy for optimizing phenotype/genotype coupling	131
AAV-PSVSPRP	cardiomyoblasts	PSVSPRP	*in vivo* AAV2 peptide display; improved tropism for heart	138

(*Continued on next page*)

Table 2. *Continued*

Name	Target Cell Type	Insert	Comment	Reference
AAV2-VNSTRLP	cardiomyoblasts	VNSTRLP	*in vivo* AAV2 peptide display; Improved tropism for heart	138
AAV-GQHPR	cardiomyoblasts	GQHPRPG+	AAV2-7-mer peptide display library	86
AAV-LSPVR	cardiomyoblasts	LSPVRPG	AAV2-7-mer peptide display library	86
AAV-MSSDP	cardiomyoblasts	MSSDPRRPPRDG	AAV2-12-mer peptide display library	86
AAV-GARPS	cardiomyoblasts	GARPSEVTTRPG	AAV2-12-mer peptide display library	86
AAV-GNEVL	cardiomyoblasts	GNEVLGTKPRAP	AAV2-12-mer peptide display library	86
AAV-KMRPG	cardiomyoblasts	KMRPGAMGTTGEGTRVTRE	AAV2-19-mer peptide display library	86
AAV_{588}MTP	skeleton muscle	ASSLNIA	phage display	122

I-588, peptide insertion C-terminal of R588. All vectors originating from AAV2 peptide display screens contain an N587Q modification. For details on linker sequences flanking the peptide insert, please see the references. Three different sequences (each labeled with its own symbol: #,*, or +) have been selected independently multiple times.

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).[45,48,52] 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 first[58,62,63] or second[64,65] 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

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	rRGD453ko	αv integrin-positive tumor cells	CDCRGDCFC	R585A and R588A required for targeting	56
I-453	AAV-MNVRGDL	endothelial cells	MNVRGDL	AAV2-453-peptide display; multiple further modification 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)	αv integrin-positive tumor cells	CDCRGDCFC	first targeting vector with double insertion	139
I-584	A584-RGD4C	αv integrin-positive tumor cells	CDCRGDCFC	–	130
I-584	A584-RGD4CALS	αv integrin-positive tumor cells	CDCRGDCFC	test of linker sequences	130
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	KTLLPTP	peptide insert replaces WT sequence at ΔIV; a further backbone modification	140

ΔIV, VR-IV.

be expressed via a non-AAV promoter from a separate plasmid[63,66] (Figure 2).

The N terminus of VP2 is particularly advantageous for capsid modification if the foreign fusion partner should be enabled to adopt its native conformation and is suited for capsid engineering across serotypes.[67,68] It can be used for capsid modifications that aim to maintain natural viral infection and vector transduction abilities,[59,61,63,66,67,69,70] to expand viral vector tropism,[59] or—if combined with WT receptor knock-out mutagenesis—for vector re-targeting.[60,68,71–75]

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.[66,67] 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.[61,74] A proof-of-concept study revealed that this AAV vector-based vaccine induced a faster onset of antigen-specific antibodies and, in line with its function as a single-shot prime-boost concept, antigen-specific antibodies with a significantly higher avidity compared with conventional vector constructs.[61] In addition, vaccination of mice with empty antigen-displaying capsids (no vector genome) was sufficient to induce an antigen-specific memory response.[61] 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.[74] 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.[74] 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.[71,76] 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.[60,72] 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.[60,72,74] 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.[60] 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.[60] 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*,[50,77] and since VP2 is not essential for capsid assembly, it was postulated that VP1-VP3 AAV vector particles

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	Scavidin displaying BT4C (rat glioma)	GLNDIFEAQKIEWHE	65
AAV1	I-590	BAP-AAV1	endothelial cells	GLNDIFEAQKIEWHE plus CDCRGDCFC (RGD4C)	141
AAV1	I-590	AAV1-RGD	tumor cells, endothelial cells	CDCRGDCFC	142
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[a]	AAV1.9-3-SKAGRSP	fibroblast	SKAGRSP	78
AAV3	I-586	BAP-AAV3	tumor cells	GLNDIFEAQKIEWHE	65
AAV4	I-586	BAP-AAV4	tumor cells	GLNDIFEAQKIEWHE	65
AAV5	I-575	BAP-AAV4	tumor cells	GLNDIFEAQKIEWHE	65
AAV5	I-575	AAV5-7m8	not successful[b]	LGETTRP[80]	144
AAV6	I-585	AAV6-RGD	tumor cells	RGD	145
AAV6	I-585 plus Y705-731F+T492V	AAV6-RGD-Y705-731F+T492V	tumor cells	RGD	145
AAV6	I-585plus Y705-731F+T492V+K531E	AAV6-RGD-Y705-731F+T492V+K531E	tumor cells	RGD	145
AAV8	I-585[c]	AAV2/8-BP2	on-bipolar cells	PERTAMSLP	79
AAV8	I-590	AAV8-PRSTSDP	not successful[b]	PRSTSDP[135]	146
AAV8	I-590	AAV8-ESGLSOS	tumor cells	ESGLSOS[135]	146
AAV8	I-590	AAV8-VNSTRLP	not successful[b]	VNSTRLP[138]	84
AAV8	I-590	AAV8-ASSLNIA	heart (weakly improved transduction)	ASSLNIA[122]	84
AAV8	I-590[d]	AAV8-PSVSPRP	not successful[b]	PSVSPRP[138]	84
AAV8	I-590[d]	AAV8-GQHPRPG	heart (weakly improved transduction)	GQHPRPG[86]	84
AAV8	I-590[d]	AAV8-SEGLKNL	liver	SEGLKNL	84
AAV8	I-590	AAV8-7m8	not successful[b]	LGETTRP[80]	144
AAV9	I-589	AAV-SLRSPPS	endothelial cells, smooth muscle cells	SLRSPPS	85
AAV9	I-589	AAV-RGDLRVS	endothelial cells, smooth muscle cells	RGDLRVS	85
AAV9	I-589[d]	AAV9-NDVRAVS	endothelial cells	NDVRAVS[82]	85
AAV9	I-589[d]	AAV9-PRSTSDP	not successful[b]	PRSTSDP[135]	146
AAV9	I-589[d]	AAV9-ESGLSOS	tumor cells (weak targeting)	ESGLSOS[135]	146
AAV9	I-588	AAV-PHP.B	CNS	TLAVPFK	81
AAV9	I-588	AAV-PHP.A	CNS	YTLSQGW	81
AAV9	I-588	AAV9-7m8	retinal cells	LGETTRP[80]	144
AAV9P1	not disclosed	AAV9P1	neuronal progenitor cells	RGDLGLS	147

[a]AAV1 with amino acids 445–568 of AAV1 replaced by residues from AAV9.
[b]Targeting peptide does not confer tropism modification in the context of this serotype.
[c]Peptide selected from a AAV8 peptide display library in which the WT sequence at 585–594 was replaced by the random peptide sequence.
[d]Additional sequence changes in backbone.

(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.[72] Flexibility of

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-engineered 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 interfering with capsid assembly or vector genome packaging, and that present peptides/proteins for target receptor binding is half of the battle. Equally important is knowledge about receptors and respective ligands 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 incorporation into the viral capsid, efficiencies were improved in particular for those cell types where availability of AAV receptors limited transduction (pre-entry barrier). However, a receptor chosen by rational design might not be the best qualified for mediating entry and initiating efficient intracellular processing of the vector. Furthermore, knowledge about barriers to transduction by AAV vectors or on potential 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 insertion 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 otherwise 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 peptide. 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. Specifically, 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[83] 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 isolated from the target cells to avoid selection of capsid variants that are dependent on helper virus function for cell transduction,[78,79,81,84,85]

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 specificity.[53,54] Depleting capsid variants that bind nonspecifically from the library—for example, by affinity chromatography—should therefore be considered to enrich the library for those variants that possess the ability to confer specificity. Sallach et al.[87] performed heparin affinity chromatography purification prior to high-throughput screening of primary human keratinocytes. Interestingly, selected capsid variants carried peptides with an RGD tripartite motif, a hallmark of integrin-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.[87] Also, differentiated keratinocytes in airlifted organotypic 3D cultures were transduced following topical vector application.[87] 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.[87]

A further improvement for AAV peptide display technology is identification of capsid variants that not only enter the cell efficiently but are also able to express their genetic payload. This issue is of importance 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 library designs are tested. Cronin et al.[79] 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.[81] used the Cre recombinase system, which, when expressed in the target cells, modified the released AAV library genomes

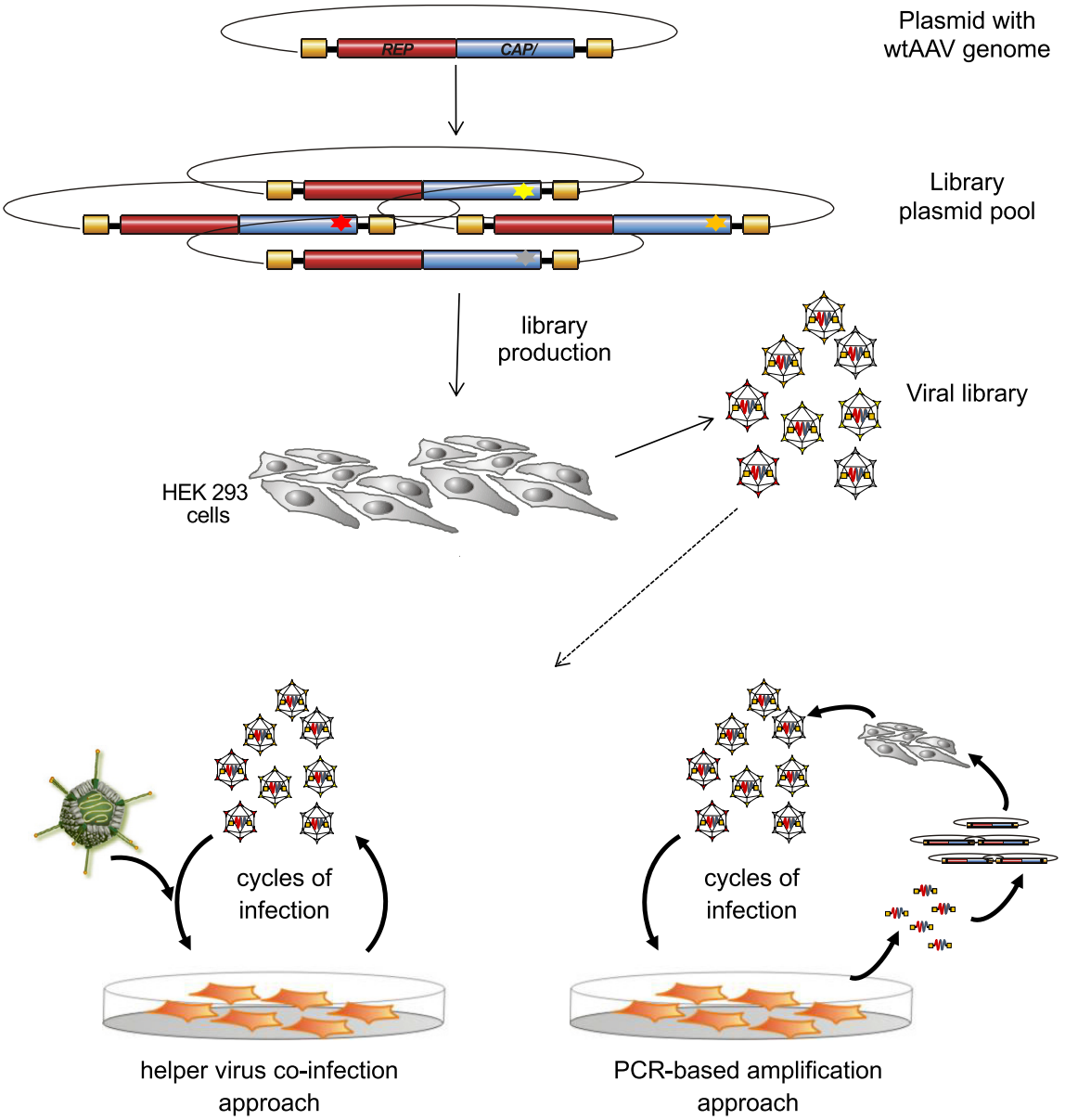

Figure 3. AAV Peptide Display Library Concept

Schematic representation of AAV peptide display library production and selection. A pool of oligonucleotides with random sequence is inserted into the *cap* gene, for example for presentation of peptides with random sequence at I-587. Thereby, a plasmid pool is generated that is used to package the AAV peptide display library. Subsequently, the library is used for high-throughput screenings in cell culture or *in vivo* (not shown). Commonly, multiple rounds of selections are done. Sublibraries either originate from viral library progeny produced by helper virus co-infection (helper virus co-infection approach) or from viral library genomes isolated from target cells or target tissue. The genomes are amplified by PCR, cloned as a sublibrary plasmid pool, and then used for sublibrary production (PCR-based amplification approach). wt, wild-type.

to become a template for PCR-mediated viral library genome amplification.

Rational Design-Based Capsid Engineering for Optimizing Intracellular Processing

Hansen et al.[88] 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.[89] 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.

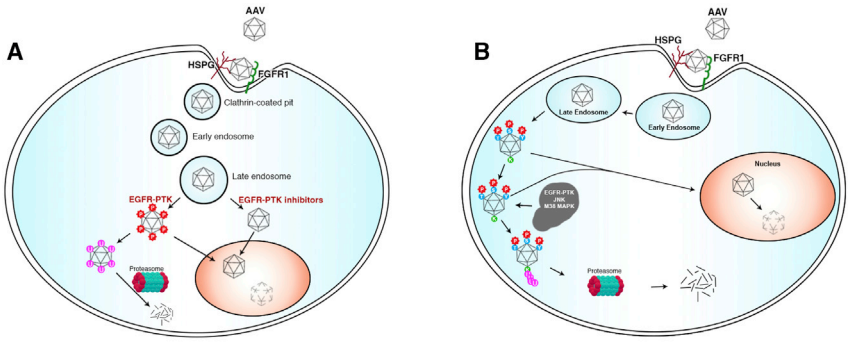

Figure 4. The Life Cycle of AAV Vectors and the Basis of the Development of Tyrosine-, Serine/Threonine-, and Lysine-Modified Next Generation of Recombinant AAV Vectors

(A) Following AAV2 binding to its primary cell surface receptor, heparan sulfate proteoglycan (HSPG), the vector interacts with human fibroblast growth factor receptor 1 (FGFR1) as one of the co-receptors to gain entry into cells via a Clathrin-coated pit, traffics through early and late endosomes and becomes phosphorylated by epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK). Phosphorylation of the capsid is a signal for ubiquitination, following which a large fraction of AAV vectors is degraded by the cellular proteasome machinery, leading to impaired intracellular trafficking and inefficient nuclear entry of AAV2 vectors. Inhibitors of EGFR-PTK prevent phosphorylation of the capsid, bypassing the ubiquitination- and proteasome-mediated degradation pathway and thereby improving intracellular trafficking and nuclear transport of AAV2 vectors. (B) In addition to surface-exposed Y residues, surface-exposed specific serine (S) and threonine (T) residues on AAV capsids can also be phosphorylated, which is a signal for ubiquitination. Surface-exposed specific lysine (K) residues on AAV capsids can be ubiquitinated and subsequently degraded by the host cell proteasome machinery. Site-directed mutagenesis of these residues led to the generation of AAV2 vectors, which were more efficient.

However, the signal for ubiquitination of the incoming AAV particles remains unclear. Mah et al.[90] 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.[89,91] This is illustrated schematically in Figure 4.

Generation of Tyrosine Mutant AAV2 Vectors

Zhong et al.[92] 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,[89,91] 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.[93] 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.[94] 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.[93] Markusic et al.[94] 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.[93–95] Furthermore, the triple mutant AAV2 vector was shown to minimize *in vivo* targeting of transduced hepatocytes by capsid-specific $CD8^+$ T cells.[95]

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.[96,97]

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[98] and, therefore, can lead to the same ubiquitination- and proteasome-meditated 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.[99,100] 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.

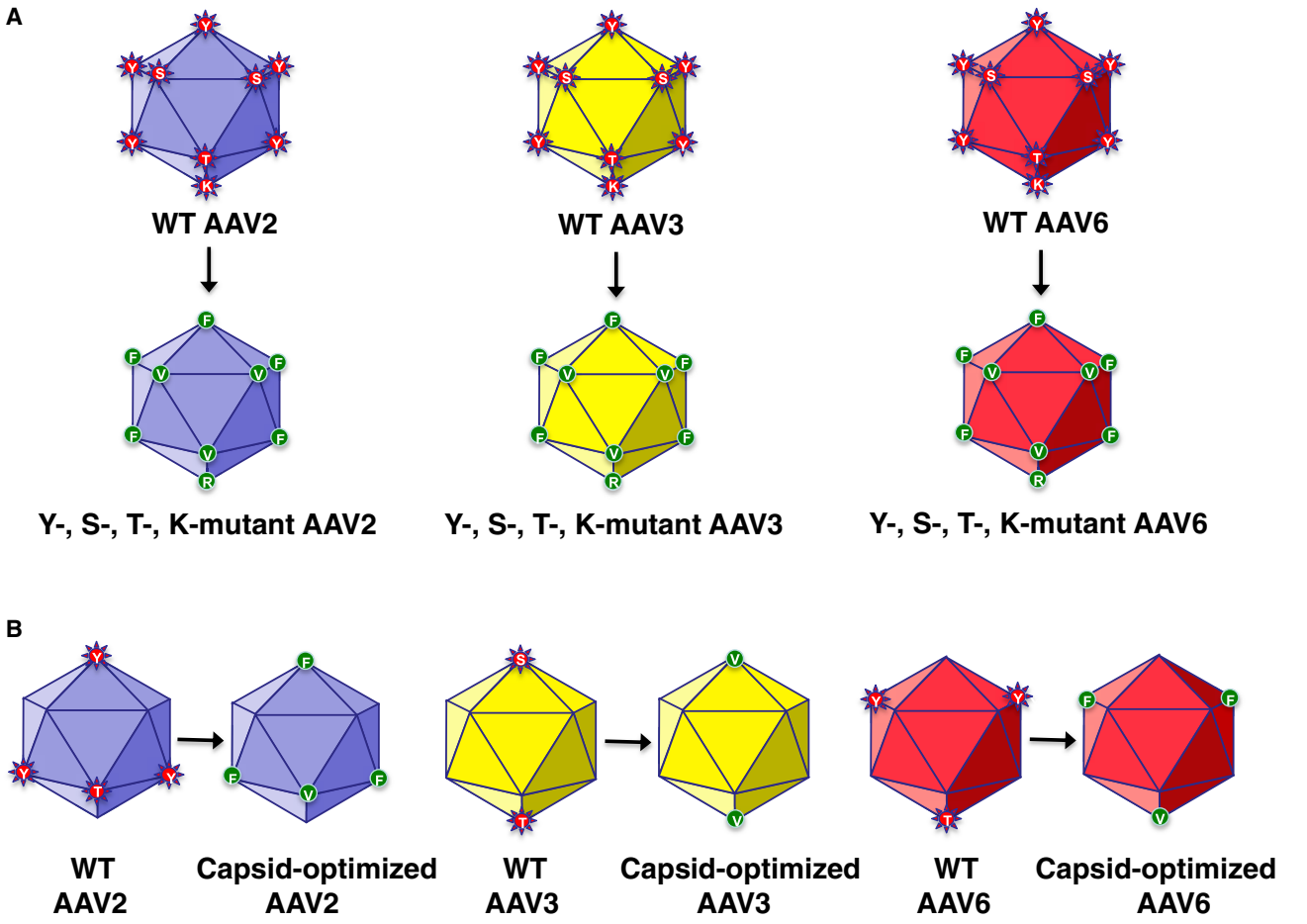

Figure 5. Schematic Representation of the Most Efficient Capsid-Modified Next Generation of Recombinant AAV Serotype Vectors

(A) Various surface-exposed specific Y, S, T, and K residues in AAV2, AAV3, and AAV6 capsids are denoted, the site-directed mutagenesis of which has led to the generation of Y, S, T, and K mutant AAV2, AAV3, and AAV6 vectors. (B) Specific examples of the most efficient AAV2,[99] AAV3,[104] and AAV6[107] serotype vectors containing various permutations and combinations of the Y, S, and T mutations generated thus far, are depicted.

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+731F+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 (HGFR) 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/cell[102,107] 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.

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