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REVIEW

Directed evolution of novel adeno-associated viruses for therapeutic gene delivery

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Gene therapy vectors based on adeno-associated virus (AAV) are currently in clinical trials for numerous disease targets, such as muscular dystrophy, hemophilia, Parkinson's disease, Leber's congenital amaurosis and macular degeneration. Despite its considerable promise and emerging clinical success, several challenges impede the broader implementation of AAV gene therapy, including the prevalence of neutralizing antibodies in the human population, low transduction of a number of therapeutically relevant cell and tissue types, an inability to overcome physical and cellular barriers *in vivo* and a relatively limited carrying capacity. These challenges arise as the demands we place on AAV vectors are often different from or even at odds with the properties nature bestowed on their parent viruses. Viral-directed evolution—the iterative generation of large, diverse libraries of viral mutants and selection for variants with specific properties of interest—offers an approach to address these problems. Here we outline progress in creating novel classes of AAV variant libraries and highlight the successful isolation of variants with novel and advantageous *in vitro* and *in vivo* gene delivery properties.
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INTRODUCTION

AAV biology

Adeno-associated virus (AAV) is a nonpathogenic parvovirus composed of a 4.7-kb single-stranded DNA genome within a non-enveloped, icosahedral capsid.[1] The genome contains three open reading frames (ORFs) flanked by inverted terminal repeats that function as the viral origin of replication and packaging signal.[1] The *rep* ORF encodes four nonstructural proteins that have roles in viral replication, transcriptional regulation, genomic integration and virion assembly.[1] The *cap* ORF encodes three structural proteins (VP1–3) that assemble to form a 60-mer viral capsid.[1] Finally, an ORF present as an alternate reading frame within the *cap* gene produces the assembly activating protein,[2,3] a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly process.[2]

The virus's capsid governs its ability to transduce cells, from initial cell surface receptor binding to gaining entry into the nucleus. Briefly, AAV2—the variant most broadly studied to date—is internalized via receptor-mediated endocytosis, with evidence supporting a role for both the clathrin-coated pit pathway[4,5] and the clathrin-independent carriers/glycosylphosphatidylinositol-anchored-protein-enriched endosomal compartment pathway.[6] Following cellular entry, the virion escapes from early endosomes and traffics to the perinuclear area. There is evidence supporting both AAV trafficking into the nucleus prior to uncoating[4,5] and AAV uncoating prior to viral DNA entry into the nucleus.[4] Upon nuclear entry, second-strand synthesis—that is, conversion of its single-stranded genome into double-stranded, transcriptionally available DNA—must occur for viral gene expression.[7] Finally, in the absence of helper virus coinfection, AAV enters a latent life cycle, in which viral genomes can integrate selectively into the AAV1S locus on human chromosome 19 (for replication competent, wild-type AAV) or persist as extrachromosomal episomes (for both wild-type and recombinant AAV).[8–10]

In recombinant versions of AAV, a gene of interest is inserted between the inverted terminal repeats in place of *rep* and *cap*, and the latter are provided *in trans*, along with helper viral genes, during vector production.[11] The resulting vector can transduce both dividing and nondividing cells with stable transgene expression in the absence of helper virus for years in post-mitotic tissue. There are 11 naturally occurring serotypes and over 100 variants of AAV, each of which differs in amino-acid sequence, particularly within the hypervariable regions of the capsid proteins, and thus in their gene delivery properties.[12,13] Importantly, no AAV has been associated with any human disease, making recombinant AAV attractive for clinical translation.[1]

AAV has yielded promising results in an increasing number of clinical trials. As a prominent example, during Phase I clinical trials for Leber's congenital amaurosis, numerous patients who received a subretinal injection of AAV2 encoding a protein required for the isomerohydrolase activity of retinal pigment epithelium showed sustained improvement in both subjective and objective measurements of vision.[14–16] Furthermore, there were no significant adverse events during either the pre-trial efficacy studies or the trial.[14–16] As a second recent example, AAV8-mediated delivery of cDNA encoding factor IX to the liver of hemophila B patients resulted in sufficient levels of secreted protein to alleviate the patients' bleeding phenotype.[17] AAV vectors are also being clinically explored for muscular dystrophy, Parkinson's disease and Alzheimer's disease.[18] AAV thus has

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considerable promise. Nevertheless, there are impediments that may limit its utility, such as anticapsid immune responses, low transduction of certain tissues, an inability for targeted delivery to specific cell types and a relatively low carrying capacity. Rational design has made progress in creating AAV variants with enhanced properties.[19,20] In many situations, however, there is insufficient mechanistic knowledge to effectively empower rational design with the capacity to improve AAV. As an alternative, directed evolution has been emerging as a strategy to create novel AAV variants that meet specific biomedical needs.

Directed evolution

Directed evolution is a high-throughput molecular engineering approach that has been successfully utilized to generate protein pharmaceuticals with enhanced biological activities, antibodies with enhanced binding affinity and enzymes with new specificities.[21] The method emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic modifications that progressively improve a molecule's function, even without knowledge of the underlying mechanistic basis for the problem. For AAV (Figure 1a), this process has involved mutating wild-type AAV *cap* genes to create large genetic libraries (described below), which can be packaged to generate libraries of viral particles, each of which is composed of a variant capsid surrounding a viral genome encoding that capsid. A selective pressure is then applied—such as high-affinity antibodies against the AAV capsid (Figure 1b), the need to bind new cell surface receptors or circumvent intracellular barriers (Figure 1c), or tissue structures that bar the virus from accessing target cells *in vivo* (Figure 1d)—to promote the emergence of variants able to surmount these barriers. After each such selection step, the successful variants can be recovered (for example, by superinfection with a helper virus or PCR amplification) and used as the starting material for the next cycle of selection (Figure 1a, step 6) to further enrich for improved variants. If the process is halted after a single library diversification and selection step, it is referred to as library selection. However, after several such selection steps, directed evolution can be conducted by subjecting the resulting *cap* gene pool (Figure 1a, step 7) to additional mutagenesis and selection (Figure 1a, step 8). After library selection or directed evolution, the resulting variants can be analyzed clonally for the desired property.

Successful library selection or directed evolution begins with the creation of high quality, high diversity libraries. In the last few years, a variety of library types have been created using several *in vitro* and *in vivo* techniques for viral DNA mutagenesis. The resulting libraries can be used individually or in combination to isolate novel variants. In addition, the techniques used to create the libraries can be used singly or in tandem for additional genetic diversification midway through the evolution process.

Library classes

As the most straightforward library, suboptimal PCR, that is, error-prone PCR, can be used to amplify and introduce random point mutations into the AAV *cap* ORF at a defined and tunable rate (Figure 2a).[22] This approach has been used to introduce mutations into either single[22,23] or multiple[24] AAV serotypes for subsequent selection.

In addition to point mutations, genetic recombination has been used to generate chimeric capsids. The earliest report of random chimeras of AAV *cap* genes was an *in vivo* viral rescue method, in which cellular co-transfection of a defective AAV2 genome with PCR fragments of the *cap* gene of another serotype (AAV3) led to rescued

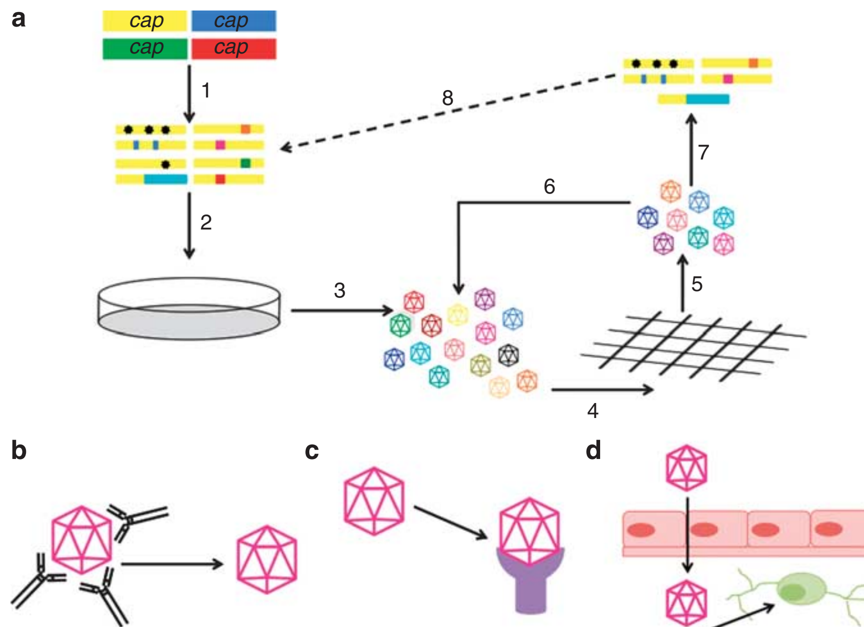

Figure 1 Directed evolution of AAV. (**a**) In a schematic of the process, (1) a viral library is created by mutating the *cap* gene. (2) Viruses are packaged (typically in HEK293T cells using plasmid transfection), such that each particle is composed of a mutant capsid surrounding the *cap* gene encoding that protein capsid. (3) Viruses are harvested and purified. (4) The viral library is placed under selective pressure. (5) Successful viruses are amplified and recovered. (6) Successful clones are enriched through repeated selection steps. (7) Isolated viral DNA reveals selected *cap* genes. (8) Selected *cap* genes are again mutated to serve as a new starting point for further selection steps to iteratively increase viral fitness. (**b**–**d**) Examples of selective pressure for directed evolution. (**b**) Evasion of antibody neutralization. (**c**) Altered receptor binding. (**d**) Cell specificity within complex tissue structures.

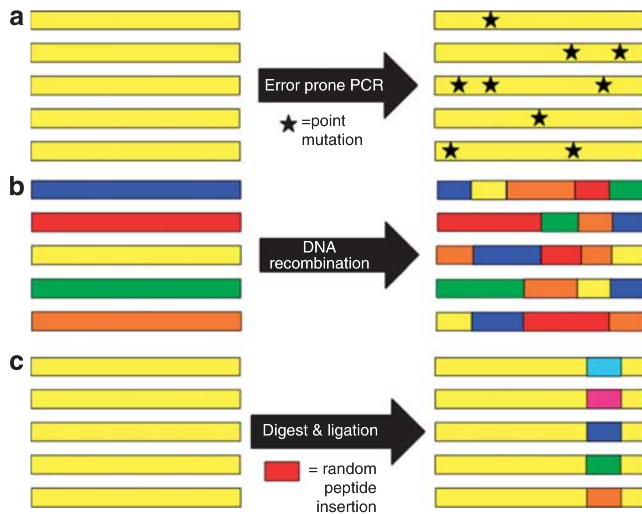

Figure 2 Schematic of library AAV capsid protein engineering strategies. Methods for generation of highly diverse viral libraries include (**a**) random point mutagenesis (error-prone PCR), (**b**) *in vitro* recombination (for example, DNA shuffling) and (**c**) insertion of random peptides. Directed evolution strategies use these approaches as part of an iterative strategy to increase AAV's fitness for various applications.

viral chimeras capable of replication.[25] DNA shuffling, an *in vitro* PCR-based method, has subsequently been implemented to create large chimeric *cap* gene libraries composed of multiple serotypes (Figure 2b).[26–29]

Furthermore, random peptide sequences have been inserted into defined sites of the viral capsid, such as in the heparin-binding domain of the AAV2 capsid (at capsid residue R588), via ligation of degenerate oligonucleotides into the *cap* ORF (Figure 2c).[30] This technique has recently been extended to AAV9.[31] Defined peptide-encoding sequences can also be inserted into random locations of the AAV2 *cap* ORF via transposon mutagenesis.[32] This approach was used to incorporate hexahistadine tags randomly throughout the AAV2 capsid to explore AAV clones capable of immobilized metal affinity chromatography purification.[33]

Finally, diversity can be focused on several hypervariable regions of the AAV capsid, which lie on surface-exposed loops. One of the first studies to conduct DNA shuffling of the AAV capsid noted that many of the functionally selected variants were composed primarily of a single serotype (AAV1 and AAV6) with surface loops exchanged with other serotypes.[27] This motivated the development of a 'loop-swap' library, in which four loops of AAV2 were replaced with a library of peptide sequences designed based on the level of conservation of each amino-acid position among natural AAV serotypes and variants.[34]

IN VITRO SELECTION AND EVOLUTION

Alternate receptor targeting

AAV mutant libraries are being utilized in an increasing number of selection strategies to isolate novel variants, both *in vitro* and *in vivo*. For example, there is a strong biomedical motivation to generate AAV variants capable of transducing previously nonpermissive cell types and/or to target gene delivery to specific, therapeutically relevant cell types. The important finding that insertion of small (seven amino acid) peptides into the heparin-binding domain of the AAV2 capsid could alter viral tropism without disrupting capsid stability lay the groundwork for initial library selection approaches.[30,35] In particular, Perabo *et al.*[35] inserted random peptide sequences into AAV2's heparin-binding domain and selected the resulting library for the capacity to infect a human megakaryocytic cell line and a B-cell lymphocytic leukemia cell line. Variants isolated from the human megakaryocytic cell selections shared an RGD motif and were capable of up to a 100-fold increase in transduction versus AAV2.[35] Müller *et al.*[30] also generated a random peptide insertion library, selected for the capacity to infect primary human coronary artery endothelial cells, and thereby created variants capable of 4- to 40-fold increased gene transfer to endothelial cells *in vitro* compared with AAV2. These mutants also showed increased accumulation in heart tissue and decreased localization to liver *in vivo*, demonstrating that *in vitro* selections can produce variants with improved *in vivo* transduction properties.[30]

AAV2 random peptide library selections have subsequently been applied to improve transduction of primary human venous endothelial cells, lung carcinoma cells, prostate carcinoma cells,[36] acute myeloid leukemia cells (and other hematopoietic cancer cell lines)[37] and primary human hematopoietic progenitor cells.[38] Recent selection of an AAV9 random peptide insertion library for the capacity to transduce human coronary artery endothelial cells yielded a variant with a 200-fold improved infection efficiency compared with AAV9 on postnatal human umbilical vein endothelial cells.[31]

In addition to AAV peptide display, other forms of mutagenesis can alter receptor binding. As a proof of concept, Maheshri *et al.*[23] showed that directed evolution can be applied to modulate the affinity of AAV for its primary receptor. Specifically, they evolved AAV2 variants with both increased and decreased affinity for heparan sulfate proteoglycans by repeatedly selecting an error-prone AAV2 library for elution from a heparin-affinity chromatography column at salt concentrations either higher or lower than those needed to elute wild-type AAV2.[23] In addition, shuffling the *cap* genes of several AAV serotypes can enable a shift in viral tropism, potentially modulating affinity for existing viral receptors, pairing different primary and secondary receptors or creating binding domains for new receptors. Li et al. used a shuffled library of AAV1–9 (excluding AAV7) to isolate a variant capable of melanoma cell transduction. A chimeric variant of AAV1, 2, 8 and 9 was more efficient at infecting hamster, mouse and human melanoma cell lines *in vitro*, as well as hamster melanoma cell-derived tumors *in vivo* following direct injection.[28] Mutational studies to map the melanoma-specific tropism identified residues 705 to 735, contributed by AAV9 as having a critical but not sufficient role in the new tropism, demonstrating that these variants can contribute to knowledge of the structure–function relationships of capsid regions.[28]

Adult and pluripotent stem cells

AAV can potentially be applied as an *in vitro* tool to improve the biomedical utility of stem cells. It is now well-recognized that AAV vector genomes carrying gene-targeting constructs can mediate homologous recombination with target loci in a cellular genome at efficiencies 10^3- to 10^4-fold higher than corresponding plasmid constructs.[39] AAV-mediated gene targeting could thus aid in creating stem cell lines harboring mutations involved in human disease for basic investigation of disease mechanisms or high-throughput *in vitro* small-molecule drug discovery and toxicity studies. Gene targeting can also mediate the safe harbor integration of transgenes that guide differentiation into specific lineages, mediate secretion of therapeutic products or enable cells to better resist the toxic effects of a diseased tissue. Finally, in the long term, gene correction of disease-causing mutations may enhance the therapeutic potential of individualized cell replacement therapies, such as ones based on pluripotent stem cells. However, while AAV-mediated gene targeting has been successfully

applied to cells that AAV can effectively transduce, naturally occurring AAV variants are typically highly inefficient at infecting a number of stem cell types.

Directed evolution was first applied to stem cells by Jang *et al.*[40] to isolate a variant capable of efficient transduction of neural stem cells (NSCs). Selections using an error-prone AAV2 library, an AAV2 with random peptide insert library and an AAV2 pairwise shuffled library on NSCs (from the adult hippocampus) yielded an AAV2 variant containing a peptide insertion that mediated 50-fold increased transduction of rat NSCs, as well as increased transduction of murine NSCs, human fetal NSCs and human embryonic stem cell (hESC)-derived neural progenitor cells.[40] Presumably as a result of the increased transduction, the variant also exhibited a fivefold increased rate of targeted gene correction compared with natural serotypes in NSCs.[40]

The most successful report of gene targeting to human pluripotent stem cells using wild-type AAV showed correct targeting of 1.3% of all colony-forming units, which corresponds to an overall gene targeting frequency for the originally infected hESCs of approximately 0.03%.[41] To build upon this result, Asuri *et al.*[24] applied directed evolution to create an AAV variant capable of enhanced gene delivery and gene targeting in hESCs and human induced pluripotent stem cells. Selecting an AAV2 and AAV6 error-prone library, an AAV2 loop-swap library, a shuffled library (containing AAV 1, 2, 4–6, 8 and 9) and a random peptide insert library for enhanced infection of hESCs yielded a simple AAV2 variant harboring a single R459G mutation. This mutant, which exhibited higher heparin affinity, was capable of increased transduction of the hESCs used in the selection, as well as other hESC and human induced pluripotent stem cell lines.[24] In addition to increased transduction, the variant exhibited a 0.1% gene targeting efficiency, already substantially higher than plasmid-mediated gene targeting, which increased to over 1% in the presence of zinc-finger nuclease-induced double-stranded breaks at the target locus.[24] This increase in gene targeting efficiency was most likely due to an increase in both the total number of cells transduced by the variant, as well as an increase in the number of viral copies that infected each cell.[24]

Antibody evasion

AAV has been successful in clinical studies involving delivery to immune privileged regions,[14–16,42] and immunosuppression is a promising approach for reducing antiviral cellular immune responses in general.[17,43–45] However, humoral immunity—for example, anti-AAV antibodies resulting from childhood exposure to one or more serotypes, or from prior administration of an AAV vector—poses a significant challenge, particularly for intrahepatic and intravascular administration.[46,47] Recent analysis indicated that the prevalence of anti-AAV immunoglobulin-G antibodies in humans was highest for AAV2 (72%) and AAV1 (67%), but AAV9 (47%), AAV6 (46%), AAV5 (40%) and AAV8 (38%) antibodies were also present in a large portion of the population studied.[48] Other studies have shown a lower but significant prevalence of anti-AAV antibodies in the population against multiple AAV serotypes.[49]

Directed evolution can create AAV variants that evade such neutralizing antibodies. Using potent serum from rabbits immunized with AAV2 vector, Maheshri *et al.*[23] evolved an antibody-resistant AAV2 variant using error-prone PCR and staggered extension process mutagenesis over two rounds of evolution (that is, two mutagenesis and six selection steps). An AAV2-based variant containing E12A, K258N, T567S, N5871 and T716A point mutations emerged from the selections. Compared to vector with wild-type AAV2 capsid, a 96-fold higher neutralizing antibody concentration was required to neutralize the variant *in vitro*, and 100- to 1000-fold higher levels of antiserum were required *in vivo*.[23]

Perabo *et al.*[22] selected an error-prone AAV2 library for variants capable of transduction in the presence of human serum. Mutations to amino acids 459 and 551 were dominant among the analyzed clones, and a variant with R459K and N551D substitutions withstood 5.5-fold higher neutralizing antibody levels compared with wild-type AAV2 *in vitro*.[22] Grimm *et al.*[26] selected a library of shuffled *cap* genes from wild-type AAV serotypes of primate (AAV2, AAV4, AAV5, AAV8, AAV9) and nonprimate (caprine, bovine, avian) origin for variants that could more efficiently transduce HepG2 liver cells in the presence of intravenous immunoglobulin, the pooled immunoglobulin-G fraction from over 1000 human donors. A single clone with sequence contributions from AAV2, AAV8 and AAV9 emerged from the selection and showed higher transduction efficiency compared with wild-type AAV1, 2, 3, 4, 5, 6, 8 and 9 in liver, kidney, fibroblast and lung cell lines.[26] In addition, the variant exhibited antibody resistance levels comparable to AAV8 and AAV9, but much higher than AAV2 *in vivo*.[26]

Library selection can be guided by some aspects of rational design to analyze the effects of comprehensive amino-acid substitutions at certain sites of interest. For example, Maersch *et al.*[50] performed saturation mutagenesis at amino acid positions 449, 458, 459, 493 and 551 of the AAV2 capsid, positions previously implicated in key antibody epitopes.[22,23] Though the resulting variants did not reach the antibody resistance levels of some natural serotypes, the mutants isolated from selections in the presence of human serum importantly reduced the antibody susceptibility of AAV2 while conserving tropism.[50]

In addition to selections designed specifically to reduce antibody neutralization, modulating other AAV properties (such as tissue or cell tropism) can serendipitously yield variants that also exhibit anti-AAV antibody resistance. For example, Koerber *et al.*[27] selected functional AAV chimeras from a DNA shuffled library of *cap* genes from AAV serotypes 1, 2, 4–6, 8 and 9. Though the selections did not involve antibodies, four out of the seven clones analyzed withstood higher neutralizing antibody concentrations than their parent serotypes, potentially through the loss of key epitopes at the junctures of capsid regions from different serotypes.[27] As another example, a chimeric variant isolated by Li *et al.*[28] during selections on hamster melanoma cells had no cross-reactivity to antisera of mice immunized with AAV1, AAV8 or AAV9, and low crossreactivity with AAV2. In addition, a chimeric variant isolated by Yang *et al.*[29] from an *in vivo* selection to identify a muscle-targeting variant (described in more detail below) exhibited a similar level of *in vitro* resistance to intravenous immunoglobulin as compared to AAV8, and a higher level of resistance compared to AAV2. Finally, Varadi *et al.*[31] demonstrated that random peptide insertions can alter AAV immunoreactivity, as AAV9-SLRSPPS and AAV9-RDVRAVS vectors exhibited enhanced *in vitro* transduction in the presence of intravenous immunoglobulin compared with wild-type AAV2 and AAV9. Furthermore, the anti-AAV9 antibody ADK9 did not neutralize the variants.[31] These results indicate that it may be possible to simultaneously select for two independent properties: antibody resistance and maintenance of existing or engineering of novel tropism.

***In vitro* models of human disease**

Similar to the selections to alter the receptor binding of AAV, *in vitro* selections can be performed to increase transduction of specific cell types used in culture models of human disease. Excoffon *et al.*[51]

selected several libraries for efficient transduction of human airway epithelial cell cultures from the apical surface, a property that is critical for cystic fibrosis gene therapy and that natural AAV variants lack. A chimera of the VP1-unique domain of AAV2 with the remainder of the AAV5 capsid, along with a key A581T point mutation in the sialic acid-binding domain, emerged from two rounds of evolution. This evolved variant, AAV2.5T, bound to the apical surface of human airway epithelial cells at 100-fold higher levels than AAV5, transduced human airway nearly 100-fold more efficiently than AAV2 and AAV5, and as a result was able to correct the chloride ion transport defect of human cystic fibrosis airway epithelia upon delivery of the *CFTR* gene.[51] Li *et al.*[52] created a library of shuffled *cap* genes from AAV1-6, 8 and 9, and selected for transduction of primary differentiated ciliated airway epithelium. The selections yielded two chimeras composed of AAV1, 6 and/or 9.[52] These variants were able to increase CFTR mRNA 25-fold over endogenous cystic fibrosis levels and rescue up to 31% of the normal CFTR response to forskolin in cystic fibrosis airway epithelia.[52]

Neurodegenerative diseases, in both the central and peripheral nervous systems, are also clinical targets that could significantly benefit from improved vectors. As one example, AAV typically has strong tropism for neurons; however, for a number of reasons it would be beneficial to develop variants that transduce glia. Astrocytes outnumber neurons in some regions of the nervous system, often have natural neuroprotective roles that can be further enhanced, and have even been shown to contribute to disease pathology in Alzheimer's disease and amyotrophic lateral sclerosis.[53,54] Using an AAV2 error-prone library, an AAV2 loop-swap library, a shuffled library containing AAV 1, 2 and 4–9, and AAV2 with random peptide insertions, Koerber *et al.*[34] evolved variants for the ability to infect primary human astrocytes. In addition to enhanced transduction of astrocytes *in vitro*, two AAV2-based variants from the shuffled and loop-swap libraries transduced 5.5- and 3.3-fold more astrocytes than AAV2 within the striatum following intracranial injection in rats.[34] As described below, one of these variants had highly advantageous properties in another neural tissue, the retina. Finally, Maguire *et al.*[55] created a library of shuffled *cap* genes from AAV1, 2, 5, 8, 9 and 10, and selected for infection of glioblastoma multiforme cells. The isolated variant was capable of efficient transduction of these cells *in vitro* and performed as well as or better than AAV2 on a panel of other glioma cells.[55]

IN VIVO SELECTION AND EVOLUTION

Tissue-specific transduction

A number of variants selected *in vitro* have exhibited correspondingly promising properties *in vivo*. However, in numerous situations cell culture models cannot adequately emulate the properties of some complex tissues, such as those with complex physical and cellular barriers that can impede viral gene delivery, delicate cells that cannot be cultured or situations in which targeted delivery to one cell type within a heterogeneous tissue is desired. In one of the first examples of *in vivo* selection, Michelfelder *et al.*[56] isolated variants from an AAV peptide insertion library for the potential to transduce either the lung or a tumor cell graft. The resulting clones mediated much higher gene expression in the target tissue; however, isolated vectors also transduced heart tissue (both tumor and lung variants) and other tissues (lung variants),[56] indicating that targeted *in vivo* delivery can be challenging.

Similarly, Yang *et al.*[29] isolated a chimeric variant composed of AAV1, 6, 7 and 8 through *in vivo* biopanning for variants that infect muscle. Compared with AAV9 (the most efficient muscle-transducing serotype), the variant showed nearly equal cardiac infectivity and significantly decreased localization to the liver. Interestingly, analysis of the variant's *in vitro* transduction of cardiomyocytes and *in vivo* transduction after direct muscle injection showed that its increased infectivity of muscle tissue was due to more efficient crossing of tight endothelial barriers, an example where library selection provided both high infectivity of target cells and the ability to overcome a cellular barrier within the tissue.[29]

Crossing physical and cellular barriers

Cellular and extracellular matrix barriers significantly impede the transport of macromolecules to target sites within tissues,[57] and AAV can be evolved for the ability to overcome these limitations. For example, the majority of retinal diseases afflict photoreceptors (PRs) and retinal pigment epithelia, cells that lie deep within the retina. Numerous AAV serotypes can infect PR and retinal pigment epithelia when administered subretinally (injected underneath the retina), which contributed to success in three recent clinical trials for Leber's congenital amaurosis.[14–16] However, unlike Leber's congenital amaurosis, in the majority of retinal diseases the retina undergoes comparatively rapid cell death and tissue degeneration and can be further damaged by the retinal detachment that accompanies subretinal injection. This concern motivates the need for gene delivery approaches that can transduce or otherwise rescue PRs upon noninvasive administration to the vitreous humor of the eye. One type of retinal cell, the Müller glia, spans the entire length of the retina and contacts all retinal neuronal cell types. Klimczak *et al.*[58] tested whether AAV variants previously selected for the ability to infect central nervous system glia were capable of Müller cell transduction. *In vivo* analysis revealed an AAV6-based mutant (ShH10) capable of highly specific (94%) and efficient infection of Müller cells compared with AAV2 and AAV6.[58] In subsequent work using a rat model of retinitis pigmentosa, infection of Müller glia with ShH10 encoding glial-derived neurotrophic factor slowed the progression of retinal degeneration and enhanced retinal electrophysiological responses for 5 months.[59]

While using Müller cells to secrete factors within the retina is a useful strategy, in other situations—such as dominant disorders directly affecting the outer retina—it would be advantageous to directly transduce PRs. Dalkara *et al.*[60] used a randomly mutagenized AAV2 *cap* library, a library of chimeric AAV *cap* genes from serotypes 1, 2 and 4–9, and a library with randomized peptides inserted near the threefold axis of symmetry on the AAV2 capsid to evolve variants capable of transducing PRs from the vitreous humor of the eye. A resulting variant mediated strong, pan-retinal expression in the PRs with both ubiquitous and PR-specific promoters,[60] work with implications for the treatment of a range of retinal degenerative diseases.

Recent promising clinical studies have investigated central nervous system gene therapy to treat Canavan's disease[42,61] and Parkinson's disease.[62] However, in some situations the target cells are surgically inaccessible, delicate or span large regions of a tissue that would require multiple direct vector injections. Gray *et al.*[63] selected a library of shuffled *cap* genes from wild-type AAV serotypes 1–6, 8, 9 and AAV8 with an E531K mutation for the ability to gain access to regions of the brain in which seizure had compromised the blood–brain barrier. Two clones composed primarily of AAV1, 8 and 9 could transduce either the piriform cortex or both the piriform cortex and ventral hippocampus upon tail vein administration of the vectors after induction of central nervous system seizure,[63] and no transduction occurred in brain areas where the blood–brain barrier was not compromised. Within the targeted brain areas, the variants efficiently

transduced oligodendrocytes and neurons, but not astrocytes or microglia, demonstrating a tropism consistent with that of AAV8.[63] This approach highlights the potential for targeting specific regions of the central nervous system upon systemic administration.

FUTURE DIRECTIONS

Directed evolution is a powerful approach that enables relatively rapid selection and isolation of AAV variants with novel and therapeutically valuable properties. Numerous reports have now demonstrated the utility of this methodology to create AAV mutants capable of utilizing alternate cell-surface receptors for transduction, transducing specific cells and tissues *in vitro* and *in vivo*, and evading neutralizing antibodies. There are considerable additional opportunities to further improve the AAV vector repertoire and in turn, through investigation of the structure–function relationships of the resulting variants, to progressively enhance our understanding of AAV biology. For example, evolution could be used as a forward genetic screen to identify and investigate functionally important regions of the AAV capsid. In addition, the discovery of an alternative reading frame within the *cap* gene encoding the assembly activating protein potentially complicates the analysis of variants isolated from directed evolution.[2,3] Mutations that are silent in the *cap* ORF could constitute non-synonymous, advantageous mutations in the assembly activating protein that affect viral assembly. In addition, while *in vitro* selections have generated vectors with properties that are useful *in vivo*,[23,26,34] it is likely that increasingly complex challenges for AAV vector engineering will require *in vivo* selection.[29,56,59]

Finally, as novel AAV variants enter into the therapeutic pipeline, they will likely progress closer to the clinic. Phase I and Phase I/II clinical trials involving wild-type AAV1, 2, 5, 6 and 8 serotypes have been approved by the US Food and Drug Administration, and they are increasingly yielding promising results.[14–17,64] Recently, the first clinical trial involving an engineered AAV variant established that this rationally designed AAV1/2 chimera was safe and well tolerated for treatment of Duchenne muscular dystrophy, laying the foundation for future trials involving additional AAV variants designed to suit a given clinical objective.[65] Engineered and evolved AAV vectors are therefore highly promising for a range of applications from the lab to the clinic.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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