Chapter 86
Advances in AAV Vector Development for Gene Therapy in the Retina

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Abstract Adeno-associated virus (AAV) is a small, non-pathogenic dependovirus that has shown great potential for safe and long-term expression of a genetic payload in the retina. AAV has been used to treat a growing number of animal models of inherited retinal degeneration, though drawbacks—including a limited carrying capacity, slow onset of expression, and a limited ability to transduce some retinal cell types from the vitreous—restrict the utility of AAV for treating some forms of inherited eye disease. Next generation AAV vectors are being created to address these needs, through rational design efforts such as the creation of self-complementary AAV vectors for faster onset of expression and specific mutations of surface-exposed residues to increase transduction of viral particles. Furthermore, directed evolution has been used to create, through an iterative process of selection, novel variants of AAV with newly acquired, advantageous characteristics. These novel AAV variants have been shown to improve the therapeutic potential of AAV vectors, and further improvements may be achieved through rational design, directed evolution, or a combination of these approaches, leading to broader applicability of AAV and improved treatments for inherited retinal degeneration.

Keywords Adeno-associated virus · Gene therapy · Mutagenesis · Directed evolution · Retinal degeneration

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J. D. Ash et al. (eds.), *Retinal Degenerative Diseases,* Advances in Experimental Medicine and Biology 801, DOI 10.1007/978-1-4614-3209-8_86,

Abbreviations

AAV	Adeno-associated virus
ITR	Inverted terminal repeats
RPE	Retinal pigment epithelium
LCA2	Leber's congenital amaurosis type 2
scAAV	Self-complementary adeno-associated virus

86.1 Introduction

Adeno-associated virus (AAV) is a dependovirus that has not been associated with human disease, and in the absence of co-infection with a helper virus such as adenovirus or herpes simplex virus, AAV is unable to replicate. AAV virions, which are non-enveloped and measure 25 nm in diameter, have a genome of 4.9 kB [7]. The AAV genome, which is single-stranded DNA, consists of three open reading frames (ORFs) flanked by two inverted terminal repeats (ITRs), which are 145 bp palendromic sequences that form elaborate hairpin structures and are essential for viral packaging. The first ORF is *rep,* which encodes 4 proteins involved in viral replication (Rep40, Rep52, Rep68, and Rep72). The second ORF contains *cap*, which encodes the three structural proteins that make up the icosahedral AAV capsid (VP1, VP2, and VP3). A third ORF, which exists as a nested alternative reading frame in the *cap* gene, encodes the assembly-activating protein, which localizes AAV capsid proteins to the nucleolus and participates in the process of capsid assembly (Sonntag, Schmidt, & Kleinschmidt, 2010). AAV has proven to be a safe and efficient vehicle for delivering therapeutic DNA to numerous tissue targets, in particular retinal neurons, and numerous studies have shown the potential of AAV-mediated delivery of genetic material for the treatment of inherited forms of retinal degeneration [4, 6].

86.2 Naturally Occurring AAV Viruses

AAV was initially discovered in 1965 as a contaminant of an adenovirus preparation, but it was not until the 1980s that AAV was first examined as a potential vector for gene therapy [8, 28]. Gene delivery vehicles or vectors based on AAV offer many advantages over other viruses as a vector for the retina. AAV vectors have the ability to infect quiescent cells and give rise to long-term expression of transgenes, and various serotypes exhibit tropisms for different subsets of retinal cells. The delivery efficacy or tropism for different retinal cells implicated in retinal degenerations—including photoreceptors, the retinal pigment epithelium (RPE), Müller glia, and ganglion cells—depends on a combination of the capsid and the route of administration, which can be either subretinal to expose virus to photoreceptors and

RPE or intravitreal to expose virus primarily to retinal ganglion and Müller cells. Over 100 different AAV capsid sequences have been isolated from both humans and primates, and canonically there are nine AAV serotypes. The first AAV serotypes, except for AAV5 which was directly obtained from a human clinical sample, were isolated as contaminants of adenovirus samples [9]. A search for new AAV serotypes with novel traits soon ensued, leading to an expansion of known variants [29]. AAV 2, 5, and 7–9 are capable of infecting photoreceptors, the most prominent cell type for retinal degenerations. Virtually every serotype is capable of infecting the RPE, and this permissiveness could be due to either the presence of AAV receptors on the cell surface or to the inherent phagocytic property of the RPE [28]. AAV2, the best characterized AAV serotype, was used in seminal clinical trials for Leber's congenital amaurosis type 2 (LCA2), an autosomal recessive retinal dystrophy caused by a mutation in *RPE65*, by three independent groups in 2008 [2, 10, 18]. In the LCA2 trials, subretinal administration of the vector was well tolerated and led to marked improvement in vision, especially in younger patients [26]. There are two additional clinical trials underway using a subretinally adminstered AAV2. The first supplies a modified soluble Flt1 receptor for the treatment of age-related macular degeneration, and the second trial seeks to treat choroideremia using an anti-VEGF molecule [16, 17].

86.3 Next Generation AAV Vectors

Although the safety, efficiency, and long-term expression achieved by naturally occurring AAVs make these vectors excellent tools for gene delivery, they suffer from several drawbacks that limit their utility for gene therapy in the retina. The onset of gene expression is limited by both the rate of internalization and breakdown of virions, and the time required for synthesis of the complementary strand from the single-stranded DNA genome. Transgene expression can thereby be delayed by weeks after injection of the vector. This delay in onset of expression has been reduced by self-complementary vectors (scAAV) [20], whose genomes contain both a sense copy of the transgene and a reverse complement, separated by a linker. These two copies are able to anneal and serve as a double stranded template that can be transcribed without the need for generation of any complementary strand by the host cell. scAAV2 [14], scAAV5 [24] and scAAV8 [21] have been shown to have faster onset of expression in retinal cells, with a similar pattern of expression as the single-stranded vectors. A number of rodent studies have used scAAVs to deliver a therapeutic transgene, leading to rescue in models of early onset retinal degeneration [15, 22]. However, the carrying capacity of scAAVs are further limited, roughly by one half, as two copies of the transgene must be included [30].

Another limitation to onset and extent of gene expression is AAV vector degradation, which can occur through phosphorylation of surface-exposed tyrosine residues that targets particles for ubiquitination and proteosome-mediated degradation. It has been demonstrated that mutation of these tyrosine residues to phenylalanine

(another aromatic residue that differs from tyrosine only by the lack of a para-hydroxyl group that serves as the substrate for tyrosine phosphorylation) enables vectors to partially circumvent this pathway and thereby allows highly efficient AAV transduction [31].

Several AAV serotypes have been shown to be amenable to tyrosine-to-phenylalanine mutations, leading to increased transduction after subretinal, intravitreal, and intravenous administration compared to their naturally occurring counterparts [25]. Furthermore, mutation of serine residues to valine on AAV2 has also recently been shown to increase transduction efficiency in vitro and in vivo, further supporting the benefits of a rational approach to engineering of the AAV capsid [1].

86.4 Directed Evolution

Both natural and rationally designed variants of AAV have been successful as gene delivery tools in the retina; however, currently existing AAVs still lack important characteristics that would greatly facility successful translation into the clinic, such as the ability to evade the immune system, the capacity to efficiently cross the physical barrier of the inner limiting membrane, and specific cell tropism. This is a result of the fact that viruses did not evolve as vectors for human gene therapy, and the mechanistic basis for these problems is often so mechanistically complex that rational design is not possible. This has led to the development of directed evolution, a high throughput molecular engineering approach, for the generation of novel AAV variants with enhanced properties for gene therapy (Fig. 86.1) [3]. For directed evolution of AAV, large ($\sim 10^7$) viral genetic capsid libraries based on wildtype AAVs are generated using one of several methods, including error prone PCR, random peptide insertion, and capsid DNA shuffling [12, 13, 23]. It is important to note that each novel capsid particle in the resulting virion library encapsidates the genome encoding that capsid, which can therefore be harnessed as "barcodes" for later identification. The viral libraries are packaged and exposed to a selective pressure such as binding to a surface receptor of a particular cell type, high affinity antibodies against AAV, or crossing a particular biological barrier. The capsid gene sequences of variants that are able to overcome the chosen selective pressure are then recovered, packaged, and subjected to additional rounds of selection. After a number of iterative rounds, a diversification step involving the introduction of new mutations to the selected capsid pool, followed by additional rounds of selection, may be conducted to further evolve the capsid toward a selected trait. The final surviving variants are individually screened and the most successful variant is determined. This method has led to novel AAV variants with enhanced properties, including viruses capable of better infecting embryonic stem cells, crossing the inner limiting membrane to infect Müller glia from the vitreous, and increased resistance to high affinity antibodies [11, 19]. Furthermore, we have recently demonstrated that AAV variants can be evolved for the ability to infect photoreceptors and RPE from the vitreous, a property with potentially strong clinical implications [5].

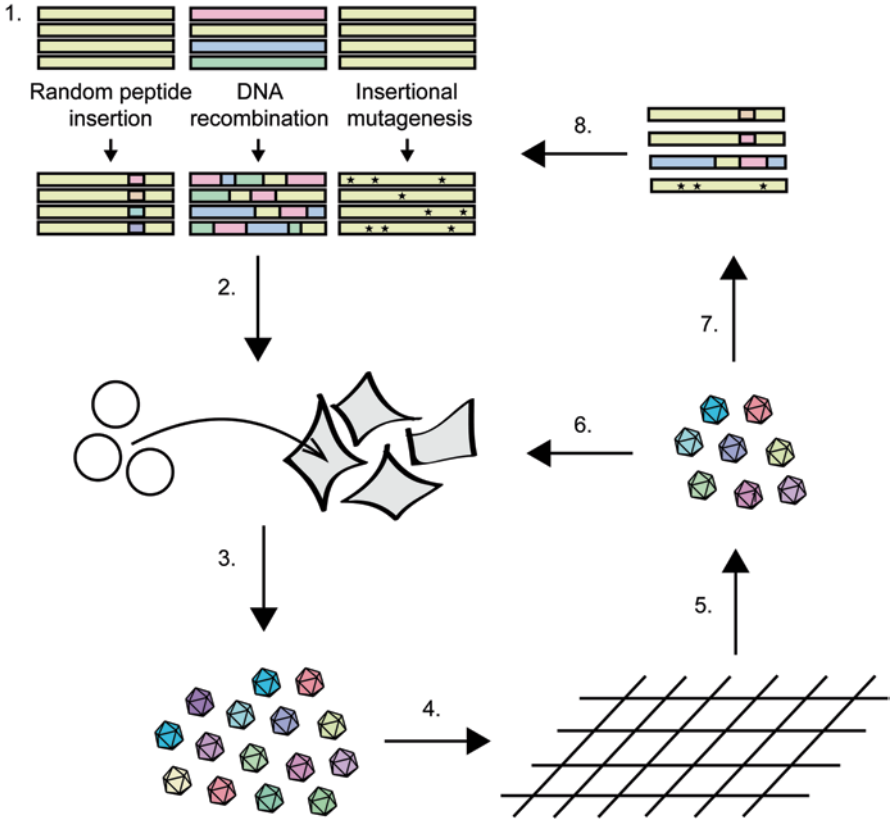

Fig. 86.1 Steps in the directed evolution of AAV. *1* Viral libraries are created through mutation of the *cap* gene. *2* Viruses are packaged, here shown as triple transfection of HEK293 cells, so that each virion contains the *cap* gene encoding that virion's capsid proteins. *3* AAV is harvested and purified. *4* A selective pressure is applied. *5* Successful viruses are isolated. *6 Cap* genes from harvested viruses are amplified through PCR and virus is repackaged. Repeated selection steps are performed to enrich for the most successful clones. *7* Sequencing is used to analyze the sequence of *cap* genes from successful viruses. *8 Cap* genes are mutated again to introduce additional diversity. [3]

86.5 Conclusions

To date, significant progress has been made in the development of next generation AAV vectors. The use of tools such as directed evolution will enable the creation of AAV vectors that are able to overcome remaining formidable challenges for clinical translation. It is important to note that the benefits and efficacy of next-generation vectors must be tested in large animal models, as the size and anatomy of human eyes and the rate of cell death in human disease are significantly different from the rodent models most often used for AAV gene therapy studies. However, AAV has been shown to be flexible and amenable to structural changes that correspond to

improvements in function, indicating that the vector may be tailored to the specific demands of a variety of eyes from different species and forms of retinal degeneration. Both rational design and library selection strategies have been shown to be useful strategies for achieving improved function of AAV, and a combination of these approaches may be used to synergistically improve the function of AAV in the retina.

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