Clinical and Experimental Medicine (2019) 19:289–298
https://doi.org/10.1007/s10238-019-00557-8

REVIEW ARTICLE

Adeno-associated virus as a gene therapy vector: strategies to neutralize the neutralizing antibodies

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Received: 21 November 2018 / Accepted: 23 April 2019 / Published online: 3 May 2019

Abstract
Adeno-associated virus (AAV)-derived vectors are currently the most common type of viral vectors used in gene therapy clinical trials. The presence of neutralizing antibodies (NAbs) against wild-type AAVs in the host body is one of the limitations for the successful use of AAV vectors. AAV capsid manipulation, by which recombinant vectors lose their ability to interact with NAbs, can help overcome this obstacle. Various methods can be used for this purpose, including directed evolution as well as conjugation of certain chemical groups to AAV epitopes. The present review concisely explains the use of AAV vectors in the clinic for gene therapy of some diseases, their limitations, and solutions to these limitations.

Keywords Gene therapy · Adeno-associated vectors · Neutralizing antibodies · Capsid modification

Introduction

Monogenic disorders arise from a mutation in a single gene on one or two chromosomes, which can lead to impaired gene function. There are approximately 5000–8000 monogenic diseases that affect about 6% of the population [1]. Replacing a defective gene with its functional copy, so-called gene therapy, can be a solution for such disorders. Tremendous strides have been recently made in gene therapy for inherited autoimmune disorders, hemophilia, neurodegenerative disorders, and lymphoid cancers, some of which received approval in the USA and Europe [2]. Fundamentally, a gene therapy consists of three components, including (I) a foreign gene that is transferred, (II) a target cell or tissue, and (III) a gene delivery vector. Such a manipulation is generally carried out by viral vectors to deliver a functional copy of the missing or defective gene to appropriate cells. Adeno-associated virus (AAV) vectors have emerged as a safe and efficient gene delivery system for monogenic disorders. However, some limitations of the commonly used AAV vectors often prevent their extensive application in gene therapy. Here, we provide a brief introduction to AAV biology, and comprehensively review the development of AAV vectors, the obstacles to clinical applications of such vectors, as well as circumvent of the obstacles.

The biology of Adeno-associated viruses (AAVs)

AAV, first isolated in 1965 from adenovirus infections [3], is a small (25 nm), nonenveloped virus containing a single-stranded DNA genome (4.7 kb) which belongs to the genus *Dependovirus* within the family *Parvoviridae*. There are currently 12 serotypes of this virus (AAV-1 through AAV-12) and over 100 serotypes in human beings and other primates, respectively. The genomic analysis of these viruses reflected the evolutionary similarity of different serotypes (Fig. 1). The similarity among capsid protein sequences derived from various AAV serotypes is between 55 and 99% [4]. The genome of the virus consists of two inverted repeat sequences known as "Inverted Terminal Repeats (ITRs)" that flank two open reading frames (ORFs), Rep, and Cap

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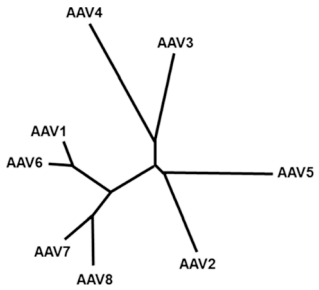
Fig. 1 Evolutionary relationship between different AAV serotypes is shown through an unrooted phylogenetic tree (**a**). Similarity of different AAV serotypes is based on protein capsids (**b**)

A

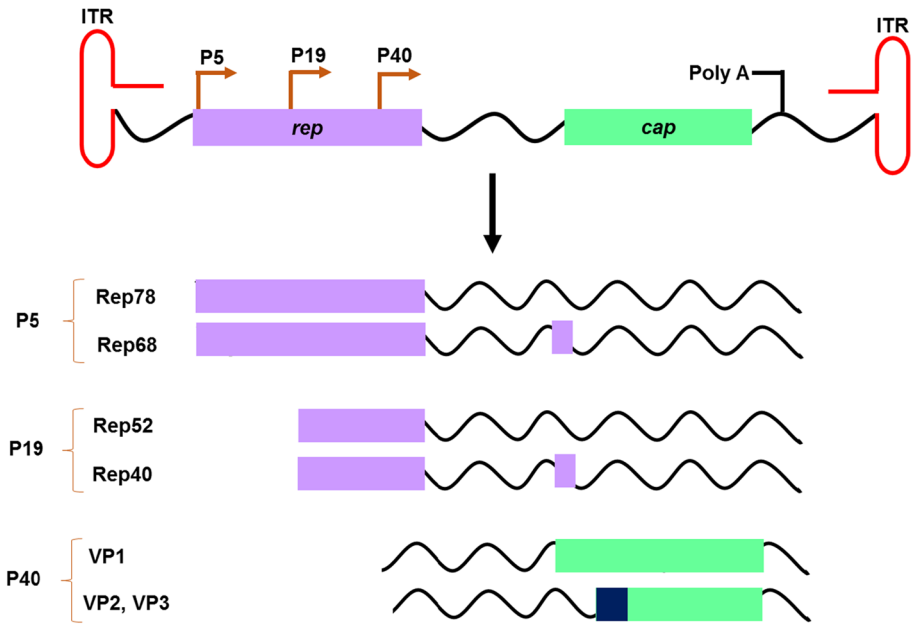


B

AAV	Homology							
	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8
AAV1	100							
AAV2	83	100						
AAV3	87	88	100					
AAV4	63	60	63	100				
AAV5	58	57	58	53	100			
AAV6	99	83	87	63	58	100		
AAV7	85	82	85	63	58	85	100	
AAV8	84	83	86	63	58	84	88	100

(Fig. 2) [5]. The ITR sequence, which folds into a T-shaped structure, is approximately 145 nucleotides and plays a role in DNA replication, genome packaging, gene expression, and site-specific integration in the chromosome 19. The *Rep* ORF encodes four proteins that play a regulatory role throughout the life cycle of AAV. On the other hand, the *Cap* ORF encodes three viral capsid proteins (VP1, VP2, and VP3 with molecular weights of 87, 72, and 62 kDa, respectively). VP1, VP2, and VP3 proteins form a 22-nm-diameter $T=1$ icosahedral symmetrical structure with a 11:10 molar ratio. However, there is also evidence that the VP1:VP2:VP3 ratio can be 1:1:18. One of the viral *Cap* ORF can generate

Fig. 2 Genetic pattern of AAV. The AVV genome contains a variety of ORFs, including rep and cap, that codes functional proteins (*Rep*) and virus structure (*Vp*)



three different protein sequences through different mechanisms [6]. In fact, the alternative splicing creates two different types of RNAs: The first one encodes a VP-1 protein after transcription, while the second one has two start codons that encode VP-2 and VP-3 proteins. Approximately 60 copies (monomers) of various VP proteins participate in the formation of capsids. The protein sequences of these monomers (VP-1, VP-2, and VP-3) vary in the N-terminal regions, while there is no variability in the amino acid residues in the C-terminal.

The viral capsid binds to host cell receptors, such as heparan sulfate (serotype AAV2), and the virus enters cells mainly through clathrin-dependent endocytosis [7]. The virus can replicate intracellularly in the presence of helper viruses such as adenovirus and herpesvirus. Otherwise, AAV can establish a latent infection due to integration into the host chromosome (Fig. 3) [8]. Because of its nonpathogenic nature, AAV did not receive medical attention for decades. However, its valuable characteristics, including persistence within host cells, the presence of various serotypes and nonpathogenicity, make AAV an appropriate vector for gene therapy.

AAV viral vectors

Recent studies have shown that gene therapy has stunning results for some genetic disorders, including lipoprotein lipase deficiency. Importantly, gene therapy for lipoprotein lipase deficiency has received approval in Europe. For use in gene therapy applications, viral vectors should have several features, including the ability to bind and enter the cell, successfully deliver transgenes into the cell nucleus, and express the transgenes in the nucleus for a long time as well as the lack of toxicity. Long-term and sufficient levels of gene expression are two main goals for the gene therapy of genetic disorders. Currently, most gene therapy strategies are genetically engineered using two types of vectors, lentivirus, and AAV (to transfer the gene to stem cells ex vivo and post-mitotic cells in vivo, respectively). Despite the great success achieved in this field for animal models of various diseases, the application of gene therapy in human beings has been faced with several problems such as transgene inactivation, insertional mutagenesis, and immunotoxicity, resulting in limited clinical trials for gene therapy of human diseases. However, AAV-derived vectors have almost all of the above-mentioned features, currently accounting for the most common type of viral vectors used in gene therapy. Highlights from the last meeting of the American Society of Gene Therapy (ASGT) showed that about half of the gene therapy research was carried out using the AAV vectors. Nonpathogenicity is considered as one of the most reasons why the virus is a favorite in gene therapy. So far, various clinical trials have been conducted using AAV vectors for a variety of diseases such as cystic fibrosis, Parkinson's

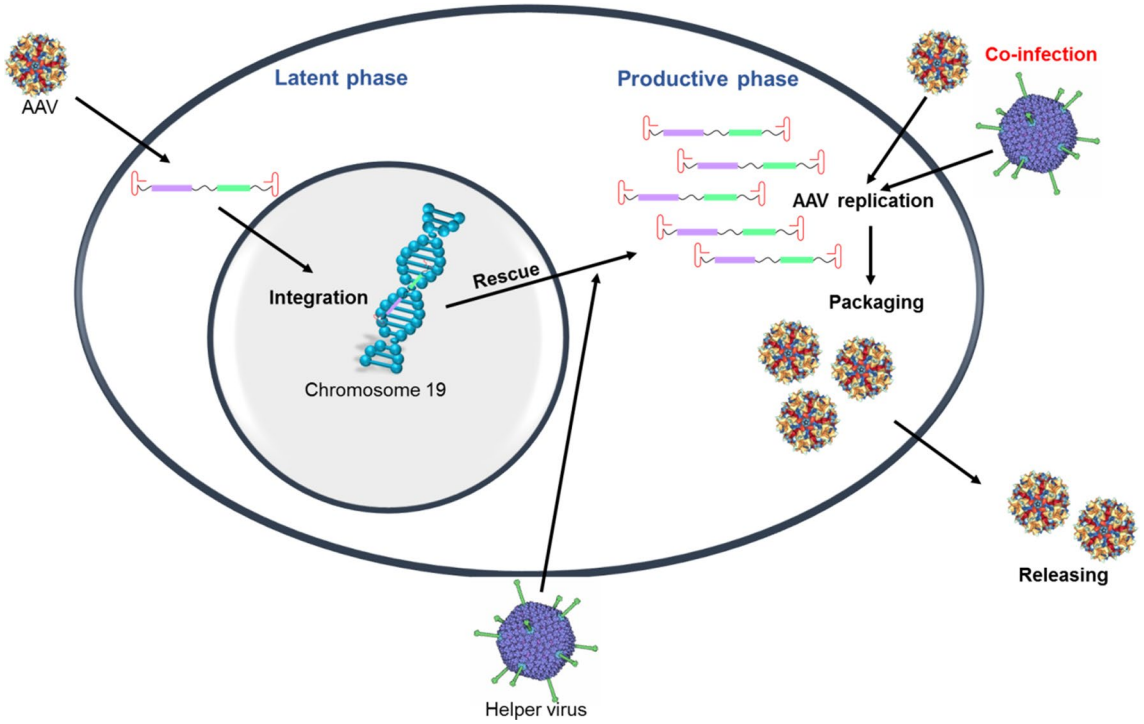


Fig. 3 Life cycle of AAV

disease, Alzheimer's disease, muscular dystrophy, epilepsy, heart failure, joint inflammation, prostate cancer, and hemophilia [9, 10]. Table 1 shows some of the clinical trials using AAV vectors. Two decades following the initiation of gene therapy trials and more than 1000 clinical trials, there is now sufficient information regarding gene therapy applications as

Table 1 Some clinical trials using AAV vectors. (clinicaltrials.gov)

Disease name	Serotype	Route of administration	Clinical trial phase	NCT numbers
Hemophilia B	FLT180a	Intravenous	Phase I	NCT03369444
	AAV8	Intrahepatic	Phase I/II	NCT00076557
	AAV8	Intrahepatic	Phase I/II	NCT02396342
	AAV5	Intravenous	Phase I/II	NCT03489291
	AAV5	Intravenous	Phase II	NCT01620801
	AAV8	Intrahepatic	Phase II	NCT02484092
	SPK-9001	Intravenous	Phase I/II	NCT03307980 (Spark & Pfizer)
	AAV5 (AMT-060)	Intravenous	Phase III	NCT03569891 (UniQure)
LPL deficiency	AAV1 (AMT-011)	Intramuscular	Phase II/III	NCT01109498
	AAV1	Intramuscular	Phase II/III	NCT00891306
Cystic fibrosis	AAV2	Intranasal	Phase I	NCT00004533
α1 antitrypsin deficiency	AAV1	Intramuscular	Phase I	NCT00430768
	AAVrh.10	Intravenous/Intrapleural	Phase I/II	NCT02168686
	AAV1	Intramuscular	Phase II	NCT01054339
Duchenne muscular dystrophy	rAAV2/5	Intramuscular	Phase I	NCT00428935
	rAAVrh74	Intramuscular	Phase I	NCT02376816
	SRP-9001	Intravenous	Phase II/III	NCT03769116 (Sarepta)
Pompe disease	AAV1	Intradiaphragmatic	Phase I/II	NCT00976352
	AAV2/AAV8	Intravenous	Phase I/II	NCT03533673
Heart failure	AAV1	Coronary artery infusion	Phase I/II	NCT00454818
	AAV1	Coronary artery infusion	Phase II	NCT01643330
	AAV1	Percutaneous	Phase II	NCT00534703
Parkinson's disease	AAV2	Intracranial	Phase I	NCT00252850
				NCT00229736
				NCT00195143
				NCT00229736
Rheumatoid arthritis	AAV2	Intra-articular	Phase I	NCT00617032
	AAV2	Intra-articular	Phase I/II	NCT00126724
Neuronal ceroid lipofuscinosis	AAV9	Intrathecal	Phase I/II	NCT03770572
Choroideremia	AAV2	Subretinal	Phase II	NCT03507686
	AAV2	Subretinal	Phase I/II	NCT02341807
	AAV2	Subretinal	Phase II	NCT02553135
	AAV2	Subretinal	Phase III	NCT03496012
Achromatopsia	AAV2/AAV8	Subretinal	Phase I/II	NCT03758404
Inherited retinal dystrophy	AAV2	Subretinal	Phase III	NCT00999609
Leber congenital amaurosis				(Spark)
Hemophilia A	AAV8 (BAX 888)	Intravenous	Phase I/II	NCT03370172
	AAV5	Intravenous	Phase I/II	NCT03520712
	AAV5	Intravenous	Phase III	NCT03392974 (BioMarin)
Spinal muscular atrophy	AAV9 (AVXS-101)	Intravenous	Phase I	NCT02122952
	AAV9 (AVXS-101)	Intravenous	Phase III	NCT03505099 (AveXis)

well as the concern about its safety. Although gene therapy is faced with a variety of setbacks, success stories have begun to steadily appear in this field. The positive recommendation for a gene therapy product (Glybera) by the EMA for approval in the European Union and the positive trials for the treatment of ADA deficiency, SCID-X1, are representative examples. More importantly, the US Food and Drug Administration (FDA) has recently approved gene therapy for some diseases. In July 2016, Spark Therapeutics and Pfizer Inc. received Breakthrough Therapy Designation from FDA, designed to facilitate faster development and approval of investigational drugs, for SPK-9001, an AAV capsid expressing a codon-optimized, high-activity human FIX variant. In addition, in January 2017, uniQure N.V. received Breakthrough Therapy Designation from the FDA, for AMT-060, its proprietary, investigational gene therapy in patients with severe hemophilia B. Furthermore, FDA has approved Spark Therapeutics' LUXTURNA™ (voretigene neparvovec-rzyl), a one-time gene therapy for patients with biallelic RPE65 mutation-associated retinal dystrophy. AveXis, Inc., a Novartis company, has recently received FDA approval for AVXS-101, a gene therapy replacing the survival motor neuron 1 (SMN1) gene, which is missing or mutated in individuals with spinal muscular atrophy (SMA). BioMarin's clinical trial of gene therapy holds promise for patients with hemophilia A. Sarepta Therapeutics, a commercial-stage biopharmaceutical company, focused mainly on the discovery and development of precision genetic medicine to treat rare neuromuscular diseases. The company is focused primarily on rapidly advancing the development of its potentially disease-modifying Duchenne muscular dystrophy (DMD) drug candidates. The FDA has recently lifted the clinical hold for the Company's Duchenne muscular dystrophy (DMD) micro-dystrophin gene therapy program.

Intravenous injection of the recombinant AAV2/AAV8 vector carrying factor IX, which encodes a gene for hemophilia patients, led to improvement of the disease with the lowest side effects in short- [11] and long-term [12] periods. Currently, a variety of vectors have been developed for the treatment of various diseases using different serotypes of this virus. However, the AAV-2 serotype is the most commonly used and well-known serotype for vector construction. Vectors derived from AAV serotypes can infect both proliferating and nonproliferating cells, such as the muscle, brain, retina, lung, and liver. For example, the AAV8 serotype shows increased tropism for hepatic cells. The first report on the successful treatment of hemophilia A in mice was performed using the AAV8 vector [13]. Building upon this success, the AAV8 vector has also been recently used in a clinical trial to transfer the factor IX gene to patients with hemophilia B [12]. Comparison of AAV2, AAV6, and AAV8 vectors has shown that AAV8 transduction into the liver is more effective than other vectors. In addition, the virus replicates faster than other vectors (about 4–10 times more than AAV2), as demonstrated in mice [14]. Several studies have recently examined the transduction rate of AAV8 vectors in large animal models such as monkeys and dogs, demonstrating that the transduction rate in these models is lower than that previously reported in mice [15, 16]. However, the dog models of hemophilia were able to consistently express the transgene at normal levels after transduction with the AAV8 vector [17]. Successful gene therapy using the AAV vectors has also been achieved in the clinic for a variety of diseases. However, the presence of neutralizing antibodies (NAbs) against the wild-type AAV in the host body is considered as one of the limitations to use AAV vectors. It has been shown that more than 70% of the humans were infected with at least one serotype of the virus, resulting in the presence of AAV-NAbs in the previously infected population [18]. These antibodies bind to and prevent viral vectors from entering the target cell, thereby leading to reduced expression of transgenes in the target cells and, ultimately, reduced efficiency of gene therapy. In the primary clinical trial in which the AAV vector was transferred through the bloodstream, a very low level of NAbs (titer 17:1) was found to be able to completely eliminate a large amount of vectors (2×10^{12} vg/kg). Studies in mice and nonhuman primates have shown that the 1:5 titer of NAbs can completely prevent liver transduction. In contrast to circulating anti-AAV2 antibodies, circulating anti-AAV5 and AAV8 antibodies are less common in humans [18]. Subclass analysis of anti-AAV antibodies has shown that most of these antibodies are IgG1-type antibodies. Nonetheless, there are reports finding a small amount of IgG2, IgG3, and IgG4 [19]. The innate immune system is generally the body's first line of defense against viruses, as a result of which gene transfer is difficult using viral vectors such as adenoviral vectors. However, AAV vectors mediate a mild proinflammatory response. Results from clinical trials of more than 300 individuals using the AAV vector showed no inflammatory response, such as changes in vital signs, nausea, and vomiting [20]. Viral capsid manipulation using different methods, in which recombinant viruses or vectors lose their ability to interact with NAbs, can overcome this limitation. Such methods generally include chemical methods (use of polyethylene glycol), targeted evolution (genetic engineering) and in silico methods. It has been recently demonstrated that in silico reconstruction of the viral evolutionary lineage yields a potent vector for gene therapy [21].

The small packaging capacity of AAV is another limitation of AAV-based vectors, so that the vector genome larger than 5 kb is rarely packaged [22]. This significantly limits the application of AAV gene therapy for those diseases requiring a larger therapeutic expression cassette. To bypass this issue, a variety of dual-vector strategies have been introduced to duplicate the AAV packaging capacity [23].

Development of AAV viral vectors

Since the beginning of the first studies on AAV vector design, there was no standard plaque assay to isolate AAV virus clones because of the fact that AAV viruses replicate only in the presence of helper viruses. This problem was solved by Samulski et al. [24] when they realized that the AAV virus was only able to replicate in adenovirus-infected cells. These findings have allowed scientists to remove some parts of the AAV genome, replace these parts with foreign DNA, and replicate these recombinant viruses in the presence of plasmids expressing *rep* and *cap* genes [25]. To generate recombinant AAV vectors, a transfection method using three vectors is used in HEK293 cells expressing E1a and E1b genes (Fig. 4). Naturally, a wild-type AAV produces approximately 10^5 viral particles within a cell, and this rate is lower for AAV recombinant vectors (about 10^3 viral particles per cell). This may be due to poor cotransfection efficiencies and nonoptimal performance of the *rep* and *cap* genes [10].

Immune responses to AAV vectors

AAV vectors are complex biological therapeutics which comprise a viral capsid, DNA genome, and therapeutic transgene, each of which has the ability to interact with both innate and adaptive immune systems at multiple levels. Innate immune responses not only play an important role in defending the host against viral infections but also are considered as the main toxicity feature in the development of gene transfer strategies using adenoviral vectors [26]. AAV vectors have received more attention as in vivo gene transfer vectors because of their highly mild proinflammatory properties. Nevertheless, there is evidence demonstrating that

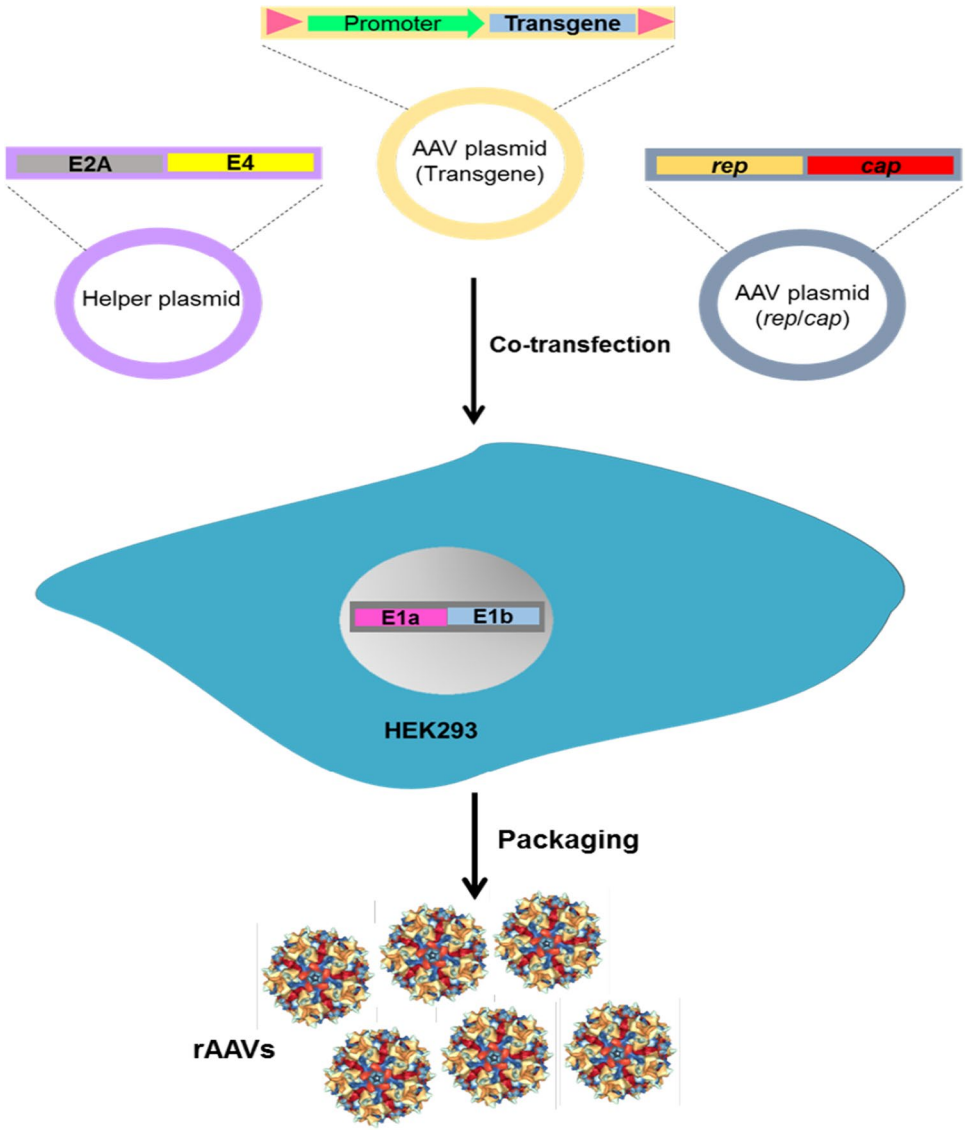


Fig. 4 Production of recombinant AAV viruses using 3-plasmid transfection

the interactions between AAV vector components and the innate immune system determine the fate of gene transfer. The single-stranded DNA genome of AAV vectors interacts with the innate immune system via Toll-like receptor (TLR) 9 [27] as well as type I interferon cascade [28]. The capsid of AAV serotype 2 (AAV2), in addition to the viral genome, may also interact with the innate immune system through TLR2 [29]. While these documents demonstrated the innate immune recognition of AAV in animals, there is little information about the consequences of such interactions in the clinical settings, mainly how innate immunity against AAV impacts adaptive responses against the recombinant vector. Clinical observation of a number of subjects injected with AAV has revealed that there is no evidence of acute clinical responses such as changes in vital signs or nausea and vomiting [20]. Humoral immunity against wild-type AAV is the first defense and one of the most effective barriers to successfully deliver systemic genes with AAV vectors. In the first clinical trial for hemophilia B, AAV vectors were delivered through the bloodstream; despite the presence of NAbs, it did not lead to exclusion of subjects from the trial; the reason was because it was unclear what level of titers would actually block transduction. The study demonstrated that relatively low NAb titers were able to completely neutralize large doses of the vector [30].

Manipulation of AAV vectors to neutralize immune responses

The main problem with the use of AAV vectors is the presence of NAbs (against the wild-type AAV) in the host body. Such antibodies bind to and prevent viral vectors from entering the target cell, thereby reducing the transgene expression in the desired tissues and, ultimately, the gene therapy efficiency [31]. Up to now, researchers have developed a variety of strategies to manipulate AAV capsids for improving the AAV tropism to desired tissue or reducing interaction with NAbs. This limitation can be addressed through viral capsid manipulation using different strategies in which recombinant viruses (vectors) lose their ability to interact with NAbs [32]. To this end, various strategies can be used which generally include directed evolution (using genetic engineering and screening in the presence of NAbs) [33], shielding of the viral capsid by chemical modification [34, 35], generation of novel AAVs by hybridization of various serotypes [36], combined genetic and chemical capsid modifications [37], as well as capsid epitope mapping followed by site-directed of mutagenesis [32, 38].

In order to develop a directed evolution, a variety of randomized mutations are first created in the AAV gene *cap* using a molecular method for mutation generation such as Error prone PCR [39] or DNA shuffling [40]. Subsequently, a library of recombinant viruses containing different capsid sequences is generated using a packaging system (using HEK293 cells). In the next step, this set of viruses is subjected to HeLa cells in the presence of NAbs against capsid proteins of the wild-type virus to carry out transduction and cell entry. This screening test is called transduction inhibition assay [31]. In fact, viruses that can infect HeLa cells and replicate in these cells are those that have not been detected by NAbs. After repeating these steps, recombinant viruses are obtained with capsids that escape NAbs. After these steps, the genomes of such viruses are extracted and sequenced to identify the desired mutation (Fig. 5). In this method, AAV variants are chosen in the presence of monoclonal antibodies or pooled human sera. Such AAV variants have mutations on vital neutralizing epitopes.

Another way to obtain reduced binding of NAbs to AAV vectors is to attach amino acid residues present on the surface of the viral capsids to chemical groups [34, 41, 42]. Chemical modifications to enhance viral vector gene delivery are comprehensively reviewed [42, 43]. By such a method, epitopes present on the surface of viral particle capsids are covered and therefore less exposed to NAbs. Chemical polymers can also be used for this purpose, including polyethylene glycol (PEG), poly-*N* methacrylamide (poly-HPMA), and some polysaccharides [7, 43]. For example, AAV pegylation not only protects viral particles from NAbs but also improves the stability of viral vectors and tissue tropism [41, 44].

The application of hybrid viruses is another strategy to reduce immune responses to AAVs. In a study, Hauck et al. [45] characterized a hybrid vector derived from AAV1 and AAV2 (AAV-221) for its effectiveness to treat hemophilia B in mice. Therefore, combination of different serotypes can be a valuable strategy to develop new recombinant vectors with improved characteristics.

There are two main approaches that have been utilized for epitope mapping on viral capsids; the first one is epitope searching, achieved by peptide scanning [46]. The second one, a structure biology-based approach, utilizes cryo-electron microscopy and image reconstruction of AAV capsids interacted with fragment antibodies, which are generated from monoclonal antibodies, to directly visualize the epitopes [47, 48]. After identification of antigenic epitopes that interacted with NAbs, it is possible to modify antigenic epitopes by site-directed mutagenesis [49]. Recently, *Gurda* et al. conducted a study in which cryo-electron microscopy and image reconstruction (cryo-reconstruction) combined with molecular biology approaches were used to define an antigenic epitope on the AAV8 capsid surface for a NAb. This structure-directed strategy for characterizing the antigenic regions of AAVs can thus generate useful information to help re-engineer vectors that escape host neutralization and are hence more efficacious [38]. While there is a need for further studies to reduce immune responses to

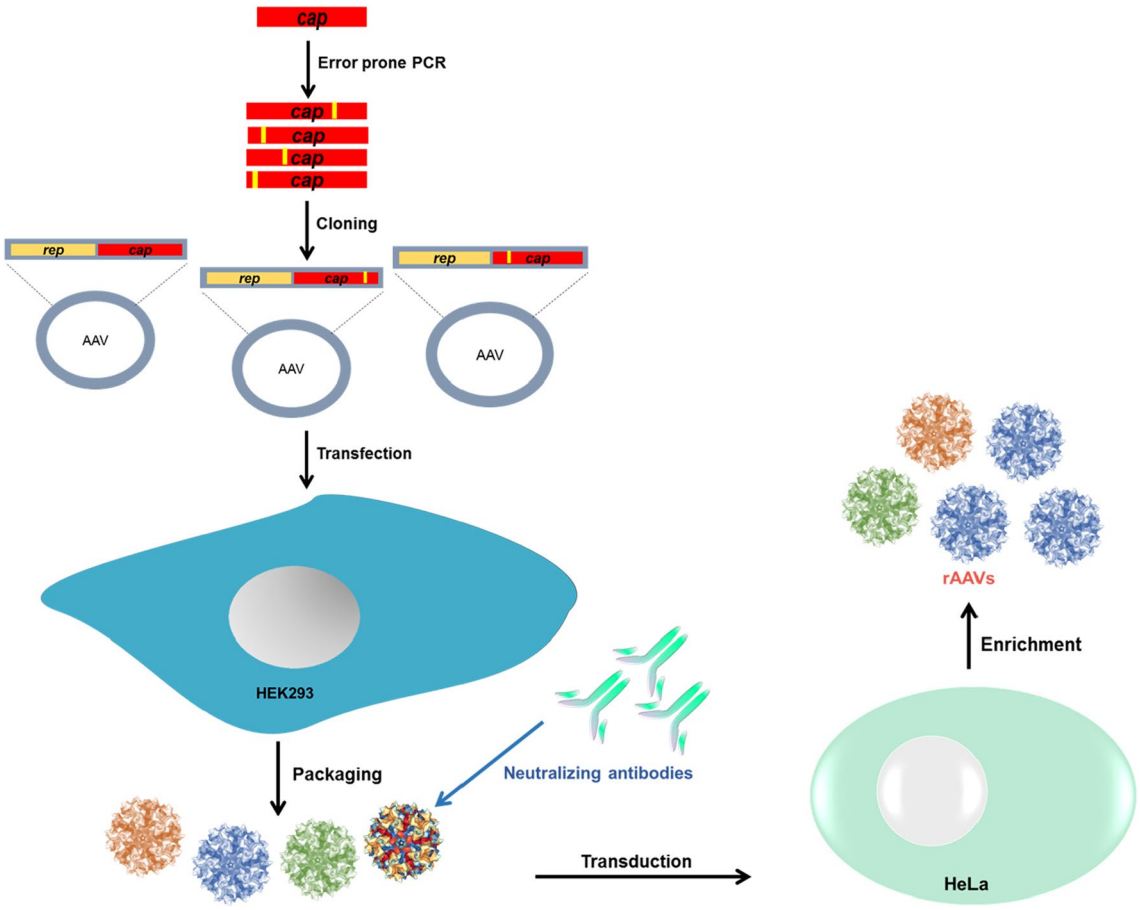


Fig. 5 The schematic representation and summary of the stages of targeted evolution and screening through transduction inhibition assay

AAV vectors, the combination of different molecular, chemical, and structural biology-based approaches, together with depletion of the immune system by some pharmacologicals [50], can be an appropriate strategy to escape the virus from viral NAbs, resulting in increased clinical efficacy of AAV vectors.

Concluding remarks

Over the past several years, there have been studies in humans showing the therapeutic potential of in vivo gene transfer using AAV vectors. Nevertheless, host anti-capsid immune responses serve as a deterrent to therapeutic success when it comes to AAV vectors. Therefore, to generate a more practical and efficacious gene transfer system using the AAV vector system, it is crucial to understand how both neutralizing and nonneutralizing antibodies interact with the AAV capsid, especially to dominantly identified epitopes. Sufficient information about the AAV antigenic structure, capsid determinants of tissue tropism as well as transduction makes it feasible to design a neutralization-escaping vector capable of evading the host antibody immune responses while maintaining optimum tissue tropism and transduction efficiency. Some approaches to map antigenic epitopes on AAV capsids, such as directed evolution, epitope searching, and structure biology-based approaches, can be an effective tool to pave the road for safe and efficient gene therapies. In addition, several studies have recently demonstrated the astonishing capability of CRISPR-Cas9 for gene therapy in several animal models delivered by AAV vectors [51, 52]. Combination of the both recombinant AAV vector and CRISPR/Cas9 technologies can have significant advantages compared with traditional gene therapy and might extensively contribute to the treatment of many diseases in the near future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval statement We confirm that the manuscript has not been submitted or previously published in whole or in part elsewhere

and is not split up into several parts to increase the quantity of submissions ("salami-publishing"). The manuscript is not currently being considered for publication in another journal. In addition, no data have been fabricated or manipulated (including images) to support our conclusions and no data, text, or theories by others are presented as if they were the author's own ("plagiarism"). All authors, whose names appear on the submission, have been personally and actively involved in substantive work leading to the manuscript have, and all agreed to submit the manuscript to this journal.

Human and animal rights The manuscript contains no studies with human participants or animals performed by any of the authors.

Informed consent Formal consent is not required for this type of study.

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