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Journal

Nature Reviews Genetics, 15(7)

ISSN

1471-0056

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Publication Date

2014-07-01

DOI

10.1038/nrg3742

Peer reviewed

HHS Public Access

Author manuscript

Nat Rev Genet. Author manuscript; available in PMC 2015 April 11.

Published in final edited form as:

Nat Rev Genet. 2014 July ; 15(7): 445–451. doi:10.1038/nrg3742.

Engineering adeno-associated viruses for clinical gene therapy

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Abstract

Clinical gene therapy has been increasingly successful, due both to an enhanced molecular understanding of human disease and to progressively improving gene delivery technologies. Among the latter, delivery vectors based on adeno-associated virus (AAV) have emerged as safe and effective – in one recent case leading to regulatory approval. Although shortcomings in viral vector properties will render extension of such successes to many other human diseases challenging, new approaches to engineer and improve AAV vectors and their genetic cargo are increasingly helping to overcome these barriers.

Introduction

The vast majority of the approximately 7,000 monogenic disorders – which collectively afflict millions worldwide, with often debilitating personal and societal consequences – have no treatment options. Sequencing efforts to date have identified the genes responsible for approximately 50% of these disorders, and with the rapidly progressing advances in next-generation sequencing technologies the remainder will likely be identified within a decade[1]. In parallel, the field of gene therapy has surmounted numerous hurdles for safe and efficient gene delivery, which has led to unprecedented treatments for some monogenic disorders. Furthermore, gene therapy is showing signs of success in several complex disorders, for example, chronic conditions such as heart disease, neurodegenerative disorders, stroke and diabetes mellitus. The prospect of single-administration treatments for monogenic and complex human diseases — developed by integrating knowledge of disease genetics and pathology with effective gene therapy — has the potential to be paradigm shifting for healthcare.

Therapeutic success to date has been enabled by the identification of several viruses that can be engineered into effective gene delivery vectors, including the non-pathogenic parvovirus adeno-associated virus (AAV; Figure 1), among others. In particular, an increasing number of phase I–III clinical trials using AAV vectors have yielded promising results (for an overview of published clinical trials using AAV, their achievements, and associated

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limitations, see Supplementary information S1 (table)). For instance, in trials for familial lipoprotein lipase (LPL) deficiency, an AAV1-based vector encoding the gain-of-function variant *LPL*^{S447X} resulted in persistent gene expression and protein activity, which led to sustained decreases in the incidence of pancreatitis[2–4]. Based on these outcomes and its safety profile, this product — Glybera (alipogene tiparvovec) — received market approval in the European Union in October 2012, albeit under "exceptional circumstances" (see EMEA website [http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002145/WC500135472.pdf]), representing the first approved gene therapy in Western nations. Other monogenic disorders in which AAV vectors have demonstrated safety and efficacy include Leber's congenital amaurosis type 2[5–10], choroideremia[11], and hemophilia B[12], among others (Supplementary information S1 (table)). In parallel to successes with monogenic disorders, AAV has been applied to idiopathic diseases. For example, administration of an AAV1 vector encoding the *SERCA2a* gene resulted in improvements of various key outcomes in patients with advanced heart failure[13,14]. Gene therapy with AAV is thus showing increasing promise for both Mendelian inherited and complex diseases.

That said, the effective delivery of genetic material has been and will continue to be a major challenge in the field (Box 1), **since** in many cases the naturally evolved infectious properties of viral vehicles are mismatched with the delivery needs of many therapeutic indications. A number of novel approaches have been used to overcome some of these barriers. For example, progressive improvements in knowledge of AAV capsid structure[15,16] are facilitating rational design of AAV capsids, and considerable progress in AAV capsid library development[17,18] and screening methodology[19,20] are facilitating directed evolution of AAV capsids. Furthermore, although gene therapy to date has primarily been successful in gene replacement therapies for recessive disorders, advances with therapeutic payloads may soon enable treatment of genetically dominant diseases.

Box 1

Challenges of AAV gene delivery and efficacy

Immune interactions

The immune system is highly effective at preventing the delivery of foreign nucleic acids, thereby posing many challenges to therapeutic gene delivery. Widespread natural exposure to AAV has resulted in a large fraction of the population harboring neutralizing anti-capsid antibodies in blood and other bodily fluids. Furthermore, following cellular transduction, AAV capsid epitopes can become cross-presented on MHC I complexes, leading to the elimination of transduced cells by capsid-specific cytotoxic T lymphocytes and corresponding loss of gene expression, as evident in the decline in Factor IX expression observed in an early clinical trial for hemophilia B[24]. Many human CD4$^+$ and CD8$^+$ T cell epitopes have been identified for AAV2[24,46,47] and AAV8[48], and MHC loci are among the most polymorphic in the human genome, making it difficult to engineer an AAV capsid that could evade recognition by all possible MHC combinations. However, it may be possible to engineer capsids that are not as readily processed by proteasomes or Transporter associated with Antigen Processing (TAP) proteins.

Transport to and tropism for target cells

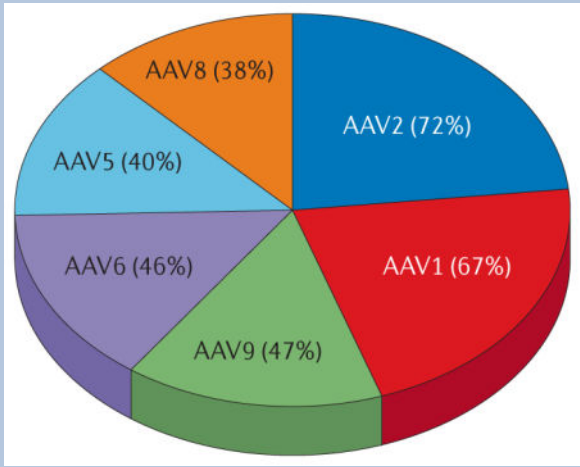
For systemically administered viruses, the liver is often the default destination, which can represent a barrier when other organs are the intended targets. In addition, endothelial cell layers, especially within the blood-brain barrier, pose a physical barrier for entry into a tissue. A vector that gains access to an organ, or is directly administered to that organ, can then encounter numerous transport barriers to efficient transduction of the often large tissue volumes involved in disease, including cell bodies and intervening extracellular matrix to which many AAV variants bind[49] (for example, heparan sulfate[50]).

Cellular barriers

Once vectors have arrived at the surface of a target cell, it may lack the primary and/or secondary receptor necessary for vector binding and internalization. Furthermore, endosomal escape, proteasomal escape, nuclear entry, and vector unpackaging all represent barriers to transduction.

Packaging capacity

Natural adeno-associated viruses have a single-stranded 4.7 kb DNA genome. Gene delivery vectors based on AAV have been shown to be capable of packaging genomes of up to approximately 5 kb at near wild-type titers and infectivity[51,52], beyond which packaging efficiency significantly decreases and genomes with 5' truncations become encapsidated[51,52].



This Progress article focuses on recent innovations in vector engineering, specifically, the rational design and directed evolution of AAV variants, as well as novel approaches for modifying genetic cargo. Successful applications of several vector engineering strategies that have created novel AAV variants to overcome some of the current challenges facing AAV-mediated gene delivery are discussed, particularly recent developments in directed evolution approaches for capsid engineering.

Engineering delivery systems

Challenges with gene delivery using AAV (Box 1) arise from the simple consideration that the properties that constitute success for natural viral infections are distinct from those needed for most medical applications, and viruses did not evolve for the latter. However, vector engineering can release viruses from the constraints of natural evolution and thereby enable them to acquire novel and biomedically valuable phenotypes. Such vector engineering advances can be grouped into rational design and directed evolution efforts.

Rational design of AAV variants

In some cases, knowledge of delivery mechanisms, coupled with AAV structural analyses[15,16], can aid vector improvement. For example, the basic discovery that phosphorylation of capsid tyrosine residues results in ubiquitination and promotes proteasomal degradation of AAV virions[21] led to the development of vehicles in which tyrosines were mutated to phenylalanines via site-directed mutagenesis[21,22]. In one such study, these vectors were capable of 10-fold higher transgene expression *in vitro* and up to 30-fold higher transgene expression *in vivo*[21]. Recently, this approach was used to engineer a novel AAV2(Tyr-Phe) mutant capsid with a potentially reduced risk of cytotoxic T lymphocyte immune responses, which is a key limitation of clinical AAV-mediated gene therapy (Box 1)[23]. Specifically, MHC class I presentation of AAV capsid epitopes is believed to underlie cytotoxic T lymphocyte reactions against AAV-transduced hepatocytes in clinical trials for hemophilia B[12,24]. Since the tyrosine mutations affect proteasomal processing of capsids, they also offer the potential to reduce MHC class I presentation of capsid antigens, a process that in general begins with through proteasomal degradation of cytosolic proteins.

Rational design approaches have also been pursued to address challenges with pre-existing neutralizing antibodies (Box 1). The majority of the human population has been naturally exposed to AAV, and natural AAV variants and serotypes exhibit considerable sequence identity[20]. As a result, a substantial percentage of the human population harbors neutralizing antibodies that markedly limit gene delivery by many natural vectors (AAV2: 72%, AAV1: 67%, AAV9: 47%, AAV6: 46%, AAV5: 40% and AAV8: 38%)[25]. This issue has been circumvented in most clinical studies by excluding patients with neutralizing antibodies, but improvements will have to be developed to broaden the patient pool that can benefit from a therapy. Several strategies have been utilized to discover and mutate the epitopes responsible for anti-capsid antibody binding. Linear and conformational epitopes responsible for neutralizing antibody binding to the AAV capsid have been mapped for several antibodies[26,27]. Lochrie *et al*. subsequently used an *in silico* structural analysis of potential docking sites for a murine IgG2a antibody with the AAV2 surface to determine sterically accessible, candidate positions, which were then subjected to extensive site-directed mutagenesis to develop variants with reduced neutralization by mouse and human antibodies *in vitro*[28]. A more recent, alternate approach by Mingozzi *et al*. involved the generation of empty AAV2-based capsid particles with mutations that ablate primary cell receptor binding[29]. When mixed with recombinant vectors carrying therapeutic transgenes, these empty capsids functioned as decoys to bind low to moderate levels of neutralizing

antibodies and thereby enhance transduction of the co-administered vector in mice and non-human primates to levels equal to or greater than transduction in naïve animals[29].

In another example of rational design, the incorporation of high-affinity ligands into the AAV capsid can confer binding to alternate cell surface receptors and thereby restrict or re-direct viral tropism. In a recent preclinical study, Münch *et al*. inserted designed ankyrin repeat proteins specific to the HER2 receptor at the N terminus of the VP2 region of the AAV2 capsid (Figure 1), thereby increasing the specificity of the vector to tumor cells overexpressing the HER2 receptor by ~30-fold *in vitro* and ~20-fold *in vivo*[30]. In addition, structural alignment and knowledge of regions involved in receptor binding can enable shifts in tropism. For instance, Shen *et al*. used site-directed mutagenesis to incorporate the amino acids responsible for AAV9 binding to galactose residues at the corresponding sites in the AAV2 capsid to design dual glycan-binding AAV vectors that could use both heparan sulfate and galactose to enter cells[31]. By virtue of this dual receptor binding, the vector showed significantly higher infectivity of the liver than AAV2 and greater specificity to the liver than AAV9.

In many situations, however, knowledge of the viral structure–function relationships underlying a given gene delivery problem is insufficient to enable rational design of AAV's complex virion. A vector engineering approach that has emerged in recent years to address this dilemma is directed evolution, which emulates the process of natural evolution.

Directed evolution of AAV variants

Directed evolution strategies harness genetic diversification and selection processes to enable the accumulation of beneficial mutations that progressively improve the function of a biomolecule (Box 2, Figure 2). In this process, wild-type AAV *cap* genes are diversified by several approaches to create large genetic libraries that are packaged to generate libraries of viral particles, and selective pressure is then applied to isolate novel variants that can overcome gene delivery barriers (Box 2)[32,33]. Importantly, the mechanistic basis underlying a gene delivery problem need not be known for directed evolution function, which can thus accelerate the development of enhanced vectors.

Box 2

Elements of directed evolution for capsid engineering

Library generation strategies

Error-prone PCR

The most straightforward library generation approach is low fidelity polymerase chain reaction (PCR) — also known as error-prone PCR — which introduces random point mutations into the AAV *cap* ORF at a predetermined, modifiable rate[34]. This approach has been used to introduce mutations into either single[32,34] or multiple[17] AAV serotypes for subsequent selection. The introduction of point mutations usually results in relatively few changes to the capsid, but error-prone PCR can be combined with other mutagenesis strategies to further optimize variants.

Chimeric capsids

Using an *in vivo* viral recombination method[53] or more commonly via DNA shuffling[38,54–56], random chimeras of AAV *cap* genes can be generated, which yields chimeric *cap* gene libraries that are composed of multiple serotypes. These 'bred' capsids can combine their parental properties in novel ways. However, many of the mutated capsids may be incapable of packaging, which substantially reduces the diversity of the library.

Random peptide insertions

Random peptide sequences can be inserted into defined sites of the viral capsid, such as in the heparin binding domain of the AAV2 capsid[57,58] and in AAV9[18], via ligation of degenerate oligonucleotides into the *cap* ORF. The insertion of random peptides into the capsid can potentially shift the binding of AAV vectors to a new cell surface receptor. Conversely, defined peptide-encoding sequences can be inserted into random locations of the AAV2 *cap* ORF via transposon mutagenesis[59,60].

Randomization of surface loops

Diversity can also be concentrated onto multiple hypervariable regions of the AAV capsid, which lie on surface-exposed loops. Such 'loop swap' libraries were for example generated by replacing four surface loops of AAV2 with libraries of peptide sequences bioinformatically designed based on the level of conservation of each amino acid position among natural AAV serotypes and variants[61]. Similar to the random peptide insertion libraries, only a small area of the capsid is mutated, but this method can be paired with additional mutagenesis strategies to modify the full capsid.

Selection

Binding affinity columns

AAV variants with increased or decreased binding affinity for a cell surface protein can be selected using affinity columns[32], where elution of different fractions yields variants with altered binding. While allowing for rapid selection of AAV variants with novel receptor binding affinity or specificity, this approach does not take into consideration other important aspects of the infection pathway such as extracellular or intracellular trafficking.

In vitro cell culture models

When cell culture models can emulate key aspects of an*in vivo* context, such as anti-capsid antibody binding or airway epithelial structure and polarization, *in vitro* selection strategies using primary cells isolated from tissue samples or immortal cell lines that mimic the behavior of cells in the human body can be effective in creating AAV variants with increased efficiency and/or specificity. *In vitro* selections have thus resulted in the development of improved AAV variants for a number of cell types, including human airway epithelia, human glia, and human embryonic stem cells [17,36,61]. This selection strategy, however, does not accurately model a complete *in vivo* environment, such as immune interactions, organ biodistribution, tissue transport barriers, and the presence of other cell types within a tissue.

In vivo models

To more accurately capture a clinical gene therapy environment, selections can be directly performed in animal models[19,20,38]. AAV libraries can be administered through various dosing routes, including intravenous injection, intravitreal administration, or direct intracranial injection. The tissue or cell type of interest is then harvested to isolate AAV variants that have successfully infected that target. One challenge is that the resulting variants are capable of improved infectivity of the animal used for the selections, which does not necessarily translate to improved infectivity of human cells[62,63]. Human xenograft models can be employed to select for infection of grafted human cells,[20] though this advantage is counter-balanced by absence of an intact immune system in the immunodeficient host and by unknown specificity for a given human target cell in the context of non-human primates or humans.

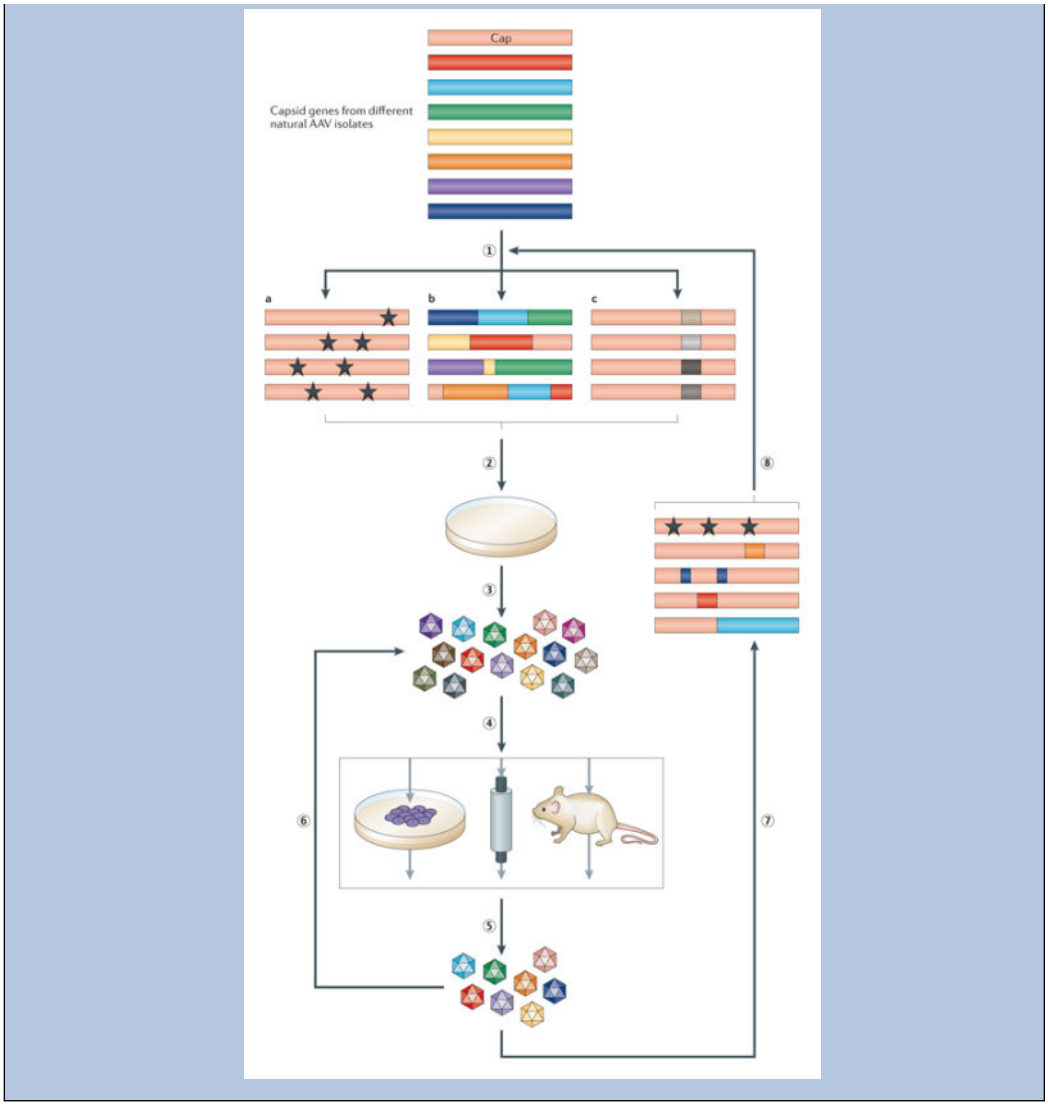
Genome recovery

Adenovirus-mediated replication

Providing successful viral genomes with the opportunity to increase their frequency in the gene pool is an inherent feature of natural selection. Adenovirus has been utilized as a helper virus to amplify AAV vectors that have reached the nucleus of the target cell in a number of *in vitro* selections[17,36,37,55,58,61,64,65]. Rescue by human adenovirus was also utilized *in vivo* to amplify vectors that have infected human hepatocytes, but not mouse hepatocytes[20], an approach that can be effective when the target cells are accessible and permissive to adenovirus superinfection.

PCR amplification

While this approach risks the isolation of genomes that have localized to rather than productively infected a given cell or tissue, PCR has been highly successful in amplification and recovery of enhanced AAV variants in a number of *in vivo* selection studies[19,38,39]. This strategy is well-suited for situations where the cell of interest is not accessible or permissive to adenovirus infection[19] or the use of replication competent libraries raises biosafety considerations.



Directed evolution was first applied to address the neutralizing antibody problem, and several promising studies reported successes, for example the generation of AAV2 variants that could withstand significantly higher levels of neutralizing antibodies *in vitro*[34] and *in vivo*[32] compared with wild-type AAV2. Recent work that involved multiple rounds of directed evolution using several different pools of human anti-AAV antibodies as selective pressures has also yielded new variants capable of enhanced antibody evasion *in vitro* and *in vivo*[35]. Specifically, AAV variants created through either saturation mutagenesis of several amino acids important for antibody binding or DNA shuffling required up to 20-fold higher *in vitro* concentrations of pooled human antibodies for neutralization vs. AAV1 (35-fold vs. AAV2). The antibody neutralization properties also translated to enhanced transduction *in vivo*, where variants were capable of significantly higher liver, heart, and muscle transduction than AAV2 in mice passively immunized with human antibodies.

In parallel, mutant AAV capsids have been evolved for more efficient and specific infection of previously non-permissive cell types. For example, vectors have been engineered for 100-

fold increased transduction of human airway epithelia[36], 50-fold higher transduction of neural stem cells[37], and 3-fold higher transduction of human pluripotent stem cells[17] *in vitro*. Furthermore, directed evolution has increasingly been implemented with *in vivo* models, particularly in situations where *in vitro* culture is an inadequate model, such as for systemic gene delivery or vector transport through complex tissues. Yang *et al*. conducted *in vivo* biopanning for more efficient infection of murine muscle, and a resulting chimeric variant capsid exhibited nearly equal cardiac infectivity, yet statistically significantly decreased liver localization, compared to AAV9[38]. In addition, Gray *et al*. isolated an AAV8 variant for the ability to gain access to regions of the brain in which seizure had compromised the blood-brain barrier[39]. More recently, Lisowski *et al*. used a model involving immunodeficient mice carrying human hepatocyte xenografts to better simulate *in vivo* human hepatocyte infection[20]. Upon administering a chimeric AAV library, human adenovirus (which exhibits tropism for human cells) was added to induce replication of the desired AAV variants and thereby yield an AAV variant capable of efficient and selective human hepatocyte transduction[20]. Future work may extend these studies to large animal models, particularly non-human primates.

Tissue transport barriers to viral infection are also a key limitation for the clinical application of gene therapy (Box 1). For example, the cells most afflicted in retinal disease – photoreceptors and retinal pigment epithelium – lie behind hundreds of microns of dense tissue. Subretinal injections poses surgical risks compared to intravitreal injection and do not transduce the full retina. Hence, Klimczak *et al*. engineered an AAV variant capable of highly specific (94%) and efficient infection of Müller cells, which span across the full retina, upon intravitreal injection[40]. In a rat model of retinitis pigmentosa, transduction of these cells with the engineered AAV variant enabled the broad expression of a neuroprotective factor and slowed retinal degeneration[41]. In a recent study, Dalkara *et al*. used *in vivo* directed evolution to generate an AAV with the ability to transport genetic cargo through the retina and directly infect photoreceptors after intravitreal delivery[19]. The resulting variant was capable of substantially higher gene expression in mouse and non-human primate photoreceptors *in vivo*, and led to the rescue of murine models of X-linked retinoschisis and Leber's congenital amaurosis type 2[19].

Through successful application to a variety of *in vitro* and *in vivo* systems, directed evolution has demonstrated the capacity to overcome a broad range of gene delivery challenges. Future work may increasingly integrate rational knowledge of capsid structure, as well as advances in DNA synthesis and sequencing, to further enhance this technology platform. In addition to the development of improved vectors, advances in their genetic payload promise to further extend the reach of gene therapy.

Engineering genetic payload

Two additional challenges of AAV-mediated gene therapy are the treatment of autosomal dominant genetic diseases – in which an allele must be removed rather than added – and in some cases the limited genomic carrying capacity of AAV (4.7 kb). These problems can potentially be addressed by modifying the genetic cargo rather than the capsid. In particular, the progressive emergence of sequence-specific endonucleases (reviewed in REF. 42) –

including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided engineered nucleases based on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs)/Cas system – may offer innovative answers to such challenges. Endogenous gene repair has been a longstanding goal of genetic therapies, and sequence-specific endonucleases can increase the efficiency of homologous recombination between a defective allele and donor DNA.[43] For example, in a murine hemophilia B model, Li *et al*. used an AAV8 vector encoding a ZFN targeted to the *F9* gene (encoding Factor IX) to induce double-strand breaks in the genome and thereby facilitate homologous recombination with a co-delivered, promoterless Factor IX cDNA fragment[44]. The resulting gene correction was sufficient to improve blood clotting times[44], raising the possibility that fragments of cDNAs that in their entirety are too large for AAV could be used to mediate the repair of focal mutations within large endogenous genes, such as *dystrophin*, *CFTR*, *CEP290*, *ABCA4*, *MYO7A*, *USH2A*, and *F8* (encoding the Factor VIII blood clotting protein). In addition, RNA interference has been implemented for specific knockdown of pathogenic alleles[45], but targeted DNA-binding proteins or nucleases delivered by AAV offer the promise for a more potent transcriptional knockdown or even complete therapeutic knockout of such genes. Although additional investigations must be conducted to elucidate the potential for off-target genotoxicity of this approach, pairing together innovative vehicles and payloads offers the opportunity for further extending the reach of gene therapy.

Conclusions

Clinical trials involving AAV delivery to accessible tissues have enabled successful treatment of several recessive monogenic disorders, which has provided strong momentum to the field. That said, considerable challenges with both delivery and payload remain. Fortunately, viruses, like many biomolecules, are highly plastic, and the engineering and evolution of ‘designer’ vectors with properties that are tailored to specific clinical needs may bring progressively more therapeutic targets within AAV’s reach. Moreover, the development of new cargoes – especially site-specific DNA endonucleases – raises the possibility of gene correction or even the treatment of dominant genetic disorders. Recent advances in human disease biology, AAV virology and engineering, and therapeutic payloads thus promise to extend clinical successes to additional monogenic and complex disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Glossary

Parvovirus	linear, non-segmented single-stranded DNA viruses whose genome size is typically in the range of 5000 nucleotides
Leber’s congenital amaurosis type 2	rare, monogenic inherited eye disorder caused by mutations in the *RPE65* gene that encodes a protein needed for the

	isomerohydrolase activity of the retinal pigment epithelium that results in loss of photoreceptor function
Choroideremia	X-linked recessive disease caused by a mutation to the *CHM* gene and subsequent absence of the Rab escort protein-1 (REP1) that leads to progressive loss of vision due to degeneration of the retina and choroid
Rational design	a capsid engineering approach that utilizes knowledge of AAV biology and structural analysis to guide capsid changes
Directed evolution	a capsid engineering approach that emulates natural evolution through iterative rounds of genetic diversification and selection processes, thereby enabling the accumulation of beneficial mutations that progressively improve the function of a biomolecule
Tropism	the cell or tissue type that can be infected by a virus or gene delivery vector
Biopanning	*in vivo* method for selection of AAV variants from a library for more efficient infectivity of a cell or tissue type of interest
Müller cells	glial cells that support neurons in the vertebrate retina

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Biographies

Melissa A. Kotterman received her B.S. in Chemical Engineering and Biomedical Engineering from Carnegie Mellon University and her Ph.D. in Chemical and Biomolecular Engineering from University of California, Berkeley. Dr. Kotterman's research goals focus on the development of gene delivery vectors as tools for use in both basic research and clinically therapeutic applications. Specifically, she has developed AAV vectors that improve gene delivery to human embryonic stem cells and adult neural stem cells and prevent neutralization by antibodies.

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Online summary

- Gene delivery vectors based on adeno-associated virus (AAV) have emerged as safe and effective for numerous clinical gene therapy applications.
- AAV-based gene therapy must overcome challenges that arise because the properties that constitute success for natural viral infections can be distinct from those needed for most medical applications. As a result, vectors based on natural AAV variants have both positive qualities, and many shortcomings.
- Vector engineering can release viruses from the constraints of natural evolution and thereby enable them to acquire novel and biomedically valuable phenotypes, which may otherwise not serve their interests in a natural setting.
- Advances in AAV biology and structural analysis have led to the rational design of AAV variants capable of reduced proteasomal degradation, decreased neutralization by anti-AAV antibodies, and increased tropism for certain cell types.
- Directed evolution – genetic diversification and selection for greatly improved function – has led to the development of novel AAV variants capable of enhanced receptor binding, decreased neutralization by anti-AAV antibodies, and increased (and in some cases targeted) tropism for many specific cell types *in vitro* and *in vivo*.
- Recent advances in AAV vector development via rational design and directed evolution, as well as in novel genetic payloads, promise to extend clinical successes of AAV gene therapy.

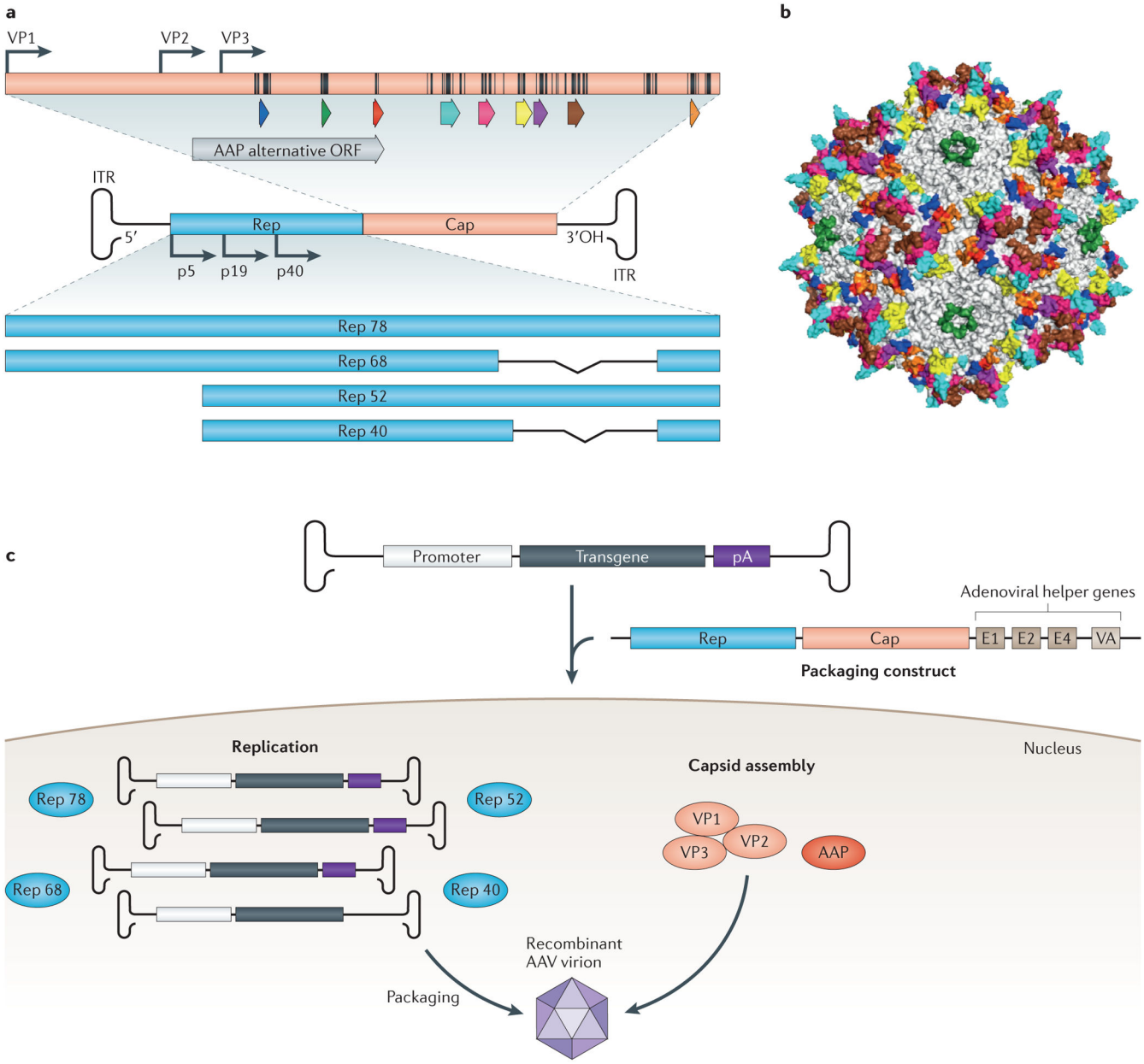

Figure 1. Adeno-associated virus (AAV) biology and variant generation

(a) Schematic of the 4.7 kb single-stranded DNA genome. The AAV genome, which is packaged within a nonenveloped, icosahedral capsid, contains three open reading frames (ORFs) flanked by inverted terminal repeats (ITRs), which form T-shaped hairpin ends. The *rep* ORF encodes four nonstructural proteins (Rep 40, Rep 52, Rep 68, and Rep 78) that are essential for viral replication, transcriptional regulation, genome integration, and virion assembly[66]. The *cap* ORF encodes three structural proteins (VP1-3), that form the 60-mer viral capsid[66] with the aid of the assembly-activating protein (AAP; gray arrow)[67,68], which is encoded in an alternate ORF located within *cap*. Hypervariable regions are denoted by colored arrows. Surface-exposed amino acids are indicated on the capsid protein (black lines). (b) Crystal structure of the AAV capsid, with VP3 hypervariable regions colored to

match the corresponding genetic regions.[69] (c) To generate recombinant versions of AAV, a gene of interest is inserted between the ITRs, replacing *rep* and *cap*, which are provided *in trans*, on so-called 'packaging constructs', along with adenoviral helper genes needed for replication[70]. The viral capsid governs the resulting vector's ability to transduce cells, from initial cell surface receptor binding to nuclear entry and genome release, which can lead to stable transgene expression in post-mitotic tissue[71]. Eleven naturally occurring serotypes and over 100 variants of AAV exist, which differ in their amino acid sequence and thus in their gene delivery properties[72,73].