Engineering adeno-associated viruses for clinical gene therapy

Melissa A. Kotterman and David V. Schaffer

Abstract | Clinical gene therapy has been increasingly successful owing both to an enhanced molecular understanding of human disease and to progressively improving gene delivery technologies. Among these technologies, delivery vectors based on adeno-associated viruses (AAVs) have emerged as safe and effective and, in one recent case, have led 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.

The vast majority of the ~7,000 monogenic disorders — which collectively afflict millions of people worldwide with often debilitating personal and societal consequences — have no treatment options. Sequencing efforts so far have identified the genes that are responsible for ~50% of these disorders; with the rapidly progressing advances in next-generation sequencing technologies, the remainder are likely to be identified within the next 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, including chronic conditions such as heart disease, neurodegenerative disorders, stroke and diabetes mellitus. The prospect of single-administration treatments for monogenic and complex human diseases, which could be developed by integrating knowledge of disease genetics and pathology with effective gene therapy, has the potential to be paradigm shifting for health care.

Therapeutic success so far 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) (FIG. 1). In particular, an increasing number of phase I–III clinical trials using AAV vectors have yielded promising results (see Supplementary information S1 (table) for an overview of published clinical trials using AAV vectors, their achievements and associated limitations). For example, in clinical trials for familial lipoprotein lipase (LPL) deficiency, an AAV1-based vector encoding the gain-of-function LPL^{S447X} variant resulted in persistent gene expression and protein activity, which led to sustained decreases in the incidence of pancreatitis[2–4]. On the bases of these outcomes and its safety profile, this product — alipogene tiparvovec (Glybera; uniQure) — received marketing approval in the European Union in October 2012, albeit under "exceptional circumstances" (see the European Medicine Agency's Summary of product characteristics), and this represents 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 (REFS 5–10), choroideremia[11] and haemophilia B[12] (see Supplementary information S1 (table)). In parallel to successes with monogenic disorders, AAV vectors have been applied to idiopathic diseases. For example, administration of an AAV1 vector containing *ATP2A2* (also known as *SERCA2*, which encodes sarcoplasmic/endoplasmic reticulum calcium ATPase 2) resulted in improvements of various key outcomes in patients with advanced heart failure[13,14]. Gene therapy using AAV vectors is thus showing increasing promise for both Mendelian inherited diseases and complex diseases.

However, the effective delivery of genetic material has been and will continue to be a major challenge in the research field (BOX 1) as, in numerous cases, the naturally evolved infectious properties of viral 'vehicles' are mismatched with the delivery needs of many therapeutic indications. Various 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 both AAV capsid library development[17,18] and screening methodology[19,20] is enabling directed evolution of AAV capsids. Furthermore, although gene therapy so far has primarily been successful in gene replacements for recessive disorders, advances with therapeutic 'payloads' may soon enable treatment of dominant genetic diseases.

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. We discuss successful applications of several vector engineering strategies that have created novel AAV variants to overcome some of the current challenges of AAV-mediated gene delivery, particularly recent developments in directed evolution approaches for capsid engineering.

Engineering delivery systems

Challenges of gene delivery using AAV vectors (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 advances in vector engineering can be grouped into rational design and directed evolution efforts.

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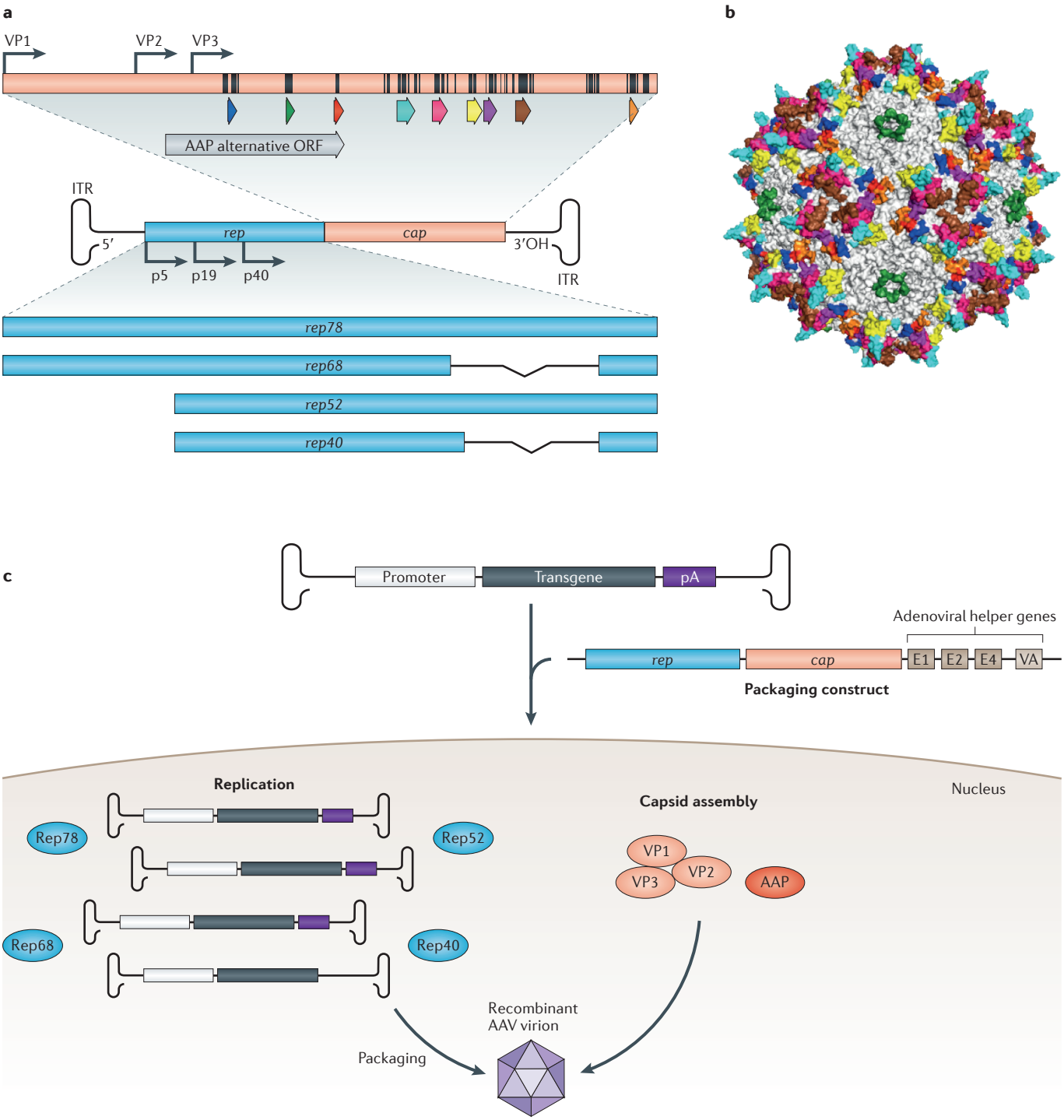

Figure 1 | **Adeno-associated virus biology and variant generation.** **a** | The 4.7-kb single-stranded DNA genome of adeno-associated virus (AAV) is shown. The AAV genome is packaged within a non-enveloped icosahedral capsid and contains three open reading frames (ORFs) flanked by inverted terminal repeats (ITRs), which form T-shaped hairpin ends. The *rep* ORF encodes four non-structural proteins (Rep40, Rep52, Rep68 and Rep78) that are essential for viral replication, transcriptional regulation, genome integration and virion assembly[66]. The *cap* ORF encodes 3 structural proteins (VP1, VP2 and VP3) that form the 60-mer viral capsid[67] with the aid of the assembly-activating protein (AAP)[67,68], which is encoded by an alternative ORF (grey arrow) located within *cap*. Hypervariable regions are denoted by coloured arrows. Regions encoding surface-exposed amino acids are indicated on *cap* (black lines). **b** | Crystal structure of the AAV capsid[69] is shown, and hypervariable regions of VP3 are coloured to match the corresponding genetic regions. **c** | To generate recombinant versions of AAV, a gene of interest is inserted between the ITRs and replaces both *rep* and *cap*, which are provided in *trans* on 'packaging constructs' along with adenoviral helper genes that are needed for replication[70]. The viral capsid determines the ability of the resulting AAV vector to transduce cells, from initial cell surface receptor binding to nuclear entry and genome release, which can lead to stable transgene expression in postmitotic tissue[71]. There are 11 naturally occurring serotypes and more than 100 variants of AAV, which differ in their amino acid sequence of the capsid and thus in their gene delivery properties[72,73]. pA, poly(A) tail.

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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 tyrosine residues in capsids results in ubiquitylation and promotes proteasomal degradation of AAV virions[21] led to the development of vectors in which tyrosines were mutated to phenylalanines by 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]. This approach was recently used to engineer a novel AAV2 tyrosine-to-phenylalanine mutant capsid with a potentially reduced risk of cytotoxic T lymphocyte immune responses, which is a key limitation of clinical AAV-mediated gene therapy[23] (BOX 1). Specifically, major histocompatibility complex (MHC) class I presentation of AAV capsid epitopes is thought to underlie cytotoxic T lymphocyte reactions against AAV-transduced hepatocytes in clinical trials for haemophilia B[12,24]. As the tyrosine mutations affect proteasomal processing of capsids, they also have the potential to reduce MHC class I presentation of capsid antigens — a process that generally begins with proteasomal degradation of cytosolic proteins.

Rational design approaches have also been pursued to address challenges of pre-existing neutralizing antibodies (BOX 1). 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 such therapies. Several strategies have been used to discover and mutate the epitopes that result in capsid-specific antibody binding. Linear and conformational epitopes that are responsible for neutralizing antibody binding to the AAV capsid have been mapped for several antibodies[25,26]. One study[27] 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*. A more recent alternative approach[28] involved the generation of empty AAV2-based capsid particles with mutations that ablate primary cell receptor binding. When mixed with recombinant vectors that carry therapeutic transgenes, these empty capsids functioned as decoys to bind to neutralizing antibodies at low to moderate levels and thereby enhance transduction of the co-administered vector in mice and non-human primates[28].

In another example of rational design, the incorporation of high-affinity ligands into the AAV capsid can confer binding to alternative cell surface receptors and thereby restrict or redirect viral tropism. A recent preclinical study inserted designed ankyrin repeat proteins that are specific to human epidermal growth factor receptor 2 (HER2; also known as ERBB2) at the amino terminus of the VP2 region of the AAV2 capsid (FIG. 1), thereby increasing the specificity of the vector to tumour cells that overexpress the HER2 receptor by ~30-fold *in vitro* and ~20-fold *in vivo*[29]. In addition, structural alignment and knowledge of regions involved in receptor binding can enable shifts in tropism. For example, site-directed mutagenesis was used to incorporate the amino acids that are responsible for AAV9 binding to galactose residues at the corresponding sites in the AAV2 capsid, which generated dual glycan-binding AAV vectors that could use both heparan sulphate and galactose to enter cells[30]. As a result of this dual receptor binding, the vector showed significantly higher infectivity of the liver than AAV2 and greater specificity to the liver than AAV9.

Box 1 | **Challenges of adeno-associated virus 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. The majority of the human population has been naturally exposed to adeno-associated viruses (AAVs), and natural AAV variants and serotypes show considerable sequence identity[20]. Widespread natural exposure to AAVs has resulted in a large portion of the population with neutralizing antibodies specific to capsids in the blood and other body fluids, which markedly limit gene delivery by many natural vectors[48] (see the figure). The percentages of population with antibodies against the individual AAV serotypes are indicated in parentheses. Furthermore, following cellular transduction, AAV capsid epitopes can become cross-presented on major histocompatibility complex (MHC) class I molecules, which leads to the elimination of transduced cells by capsid-specific cytotoxic T lymphocytes and the corresponding loss of gene expression, as evident in the decline in coagulation factor IX expression observed in an early clinical trial for haemophilia B[24]. Many human $CD4^+$ and $CD8^+$ T cell epitopes have been identified for AAV2 (REFS 24,49,50) and AAV8 (REF. 51), and MHC loci are among the most polymorphic in the human genome, which makes 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 TAP (transporter associated with antigen processing) proteins. Of note, another approach to reduce the immunogenicity of AAV vectors through elimination of CpG motifs from the vector genome has recently been reported[52].

Transport to and tropism for target cells

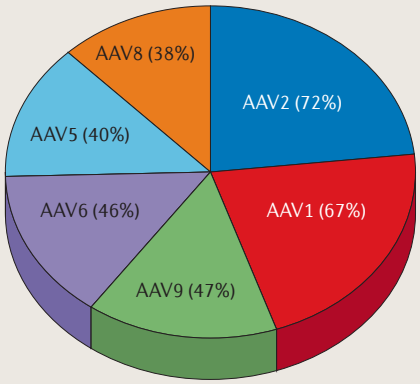
For systemically administered viruses, the liver is often the default destination, which can represent a barrier when other organs are the intended targets. In addition, endothelial cell layers, especially those within the blood–brain barrier, pose a physical barrier for entry into a tissue. A vector that gains access to an organ, or that 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[53] (for example, heparan sulphate[54]).

Cellular barriers

The surface of a target cell may lack the primary and/or secondary receptors that are necessary for vector binding and internalization. Furthermore, endosomal escape, proteasomal escape, nuclear entry and vector unpackaging all represent barriers to transduction.

Packaging capacity

Natural AAVs have a single-stranded DNA genome of 4.7 kb. Gene delivery vectors based on AAVs have been shown to be capable of packaging genomes of up to ~5 kb at near wild-type titres and infectivity, beyond which packaging efficiency markedly decreases, and genomes with 5′ truncations become encapsidated[55,56].



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However, in many situations, knowledge of the viral structure–function relationships that underlie a given gene delivery problem is insufficient to enable rational design of the complex virion of an AAV. 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). 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[31,32] (BOX 2). Importantly, the mechanistic basis underlying a gene delivery problem does not need to be known for directed evolution of function, which can thus accelerate the development of enhanced vectors.

Directed evolution was first applied to address the problem of neutralizing antibodies (BOX 1), and several promising studies reported successes, for example, the generation of AAV2 variants that could withstand significantly higher levels of neutralizing antibodies *in vitro*[33] and *in vivo*[31] than wild-type AAV2. Recent work that involved multiple rounds of directed evolution using several different pools of human AAV-specific antibodies as selective pressures has also yielded new variants that can enhance antibody evasion *in vitro* and *in vivo*[34]. Specifically, AAV variants that were created either through saturation mutagenesis of several amino acids that are important for antibody binding or through DNA shuffling required up to 20-fold higher *in vitro* concentrations of pooled human antibodies for neutralization than AAV1 (and 35-fold higher than AAV2). The antibody neutralization properties also led to enhanced transduction *in vivo*: AAV variants could achieve significantly higher rates of transduction in the liver, heart and muscle than AAV2 in mice that have been 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 transduction rates that are higher by 100-fold in human airway epithelial cells[35], 50-fold in neural stem cells[36] and 3-fold in human pluripotent stem cells[17] *in vitro*. Furthermore, directed evolution has increasingly been implemented using *in vivo* models, particularly in situations in which *in vitro* culture is an inadequate model, such as for systemic gene delivery or vector transport through complex tissues. One study[37] carried out *in vivo* biopanning for more efficient infection of murine muscle, and a resulting chimeric variant capsid showed nearly equal cardiac infectivity yet significantly decreased liver localization compared to AAV9. Another study[38] isolated an AAV8 variant for the ability to gain access to regions of the brain in which seizure had compromised the blood–brain barrier. More recently, a model involving immunodeficient mice that carried human hepatocyte xenografts was used to better simulate *in vivo* human hepatocyte infection[20]. Upon administering a chimeric AAV library, human adenovirus (which shows tropism for human cells) was added to induce replication of the desired AAV variants and thereby yield an AAV variant that could efficiently and selectively transduce human hepatocytes[20]. Future work may extend these studies to large animal models, particularly to 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 most afflicted cells in retinal disease — photoreceptor cells and retinal pigment epithelial cells — lie behind hundreds of microns of dense tissue. Subretinal injections pose surgical risks compared with intravitreal injection and do not transduce the full retina. Hence, one study[39] engineered an AAV variant that was capable of highly specific (94%) and efficient infection of Müller cells, which span across the full retina, upon intravitreal injection. 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[40]. A recent study[19] used *in vivo* directed evolution to generate an AAV that could transport genetic cargo through the retina and directly infect photoreceptor cells after intravitreal delivery. The resulting variant had substantially higher gene expression levels than both wild-type AAV2 and AAV2 with tyrosine mutations in mouse and non-human primate photoreceptor cells *in vivo*, and led to the rescue of murine models of X-linked retinoschisis and Leber's congenital amaurosis type 2 (REF. 19).

Through successful application to various *in vitro* and *in vivo* systems, directed evolution has shown 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.

Engineering genetic payload

An additional challenge of AAV-mediated gene therapy is the treatment of autosomal dominant genetic diseases, in which an allele must be removed rather than added. In some cases, the limited packaging capacity of the AAV vector (4.7 kb) can also be challenging. These problems can potentially be addressed by modifying the genetic cargo rather than the capsid.

Genome engineering. The progressive emergence of sequence-specific endonucleases (reviewed in REF. 41) — including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases that are based on the CRISPR (clustered regularly interspaced short palindromic repeat)–Cas (CRISPR-associated protein) system — offers innovative answers to such challenges. Endogenous gene repair has been a long-standing goal of gene therapy, and sequence-specific endonucleases can increase the efficiency of homologous recombination between a defective allele and donor DNA[42]. For example, in a murine haemophilia B model, an AAV8 vector encoding a ZFN targeted to the coagulation factor IX (*F9*) gene was used to induce double-strand breaks in the genome and thereby facilitate homologous recombination with a co-delivered, promoterless *F9* cDNA fragment[43]. The resulting gene correction was sufficient to improve blood clotting times[43], which raises the possibility that fragments of cDNAs that in their entirety are too large for AAV vectors could be used to mediate the repair of focal mutations in large endogenous genes, such as dystrophin (*DMD*), the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, *CEP290* (which encodes centrosomal protein 290 kDa), *ABCA4* (which encodes a retinal-specific ATP-binding cassette transporter), myosin VIIA (*MYO7A*), the Usher syndrome 2A (*USH2A*) gene and *F8*. In addition, RNA interference has been implemented for specific knockdown of pathogenic alleles[44], but targeted DNA-binding proteins or nucleases delivered by AAV vectors offer the promise for a more potent transcriptional knockdown or even complete therapeutic knockout of such genes. Although additional investigations

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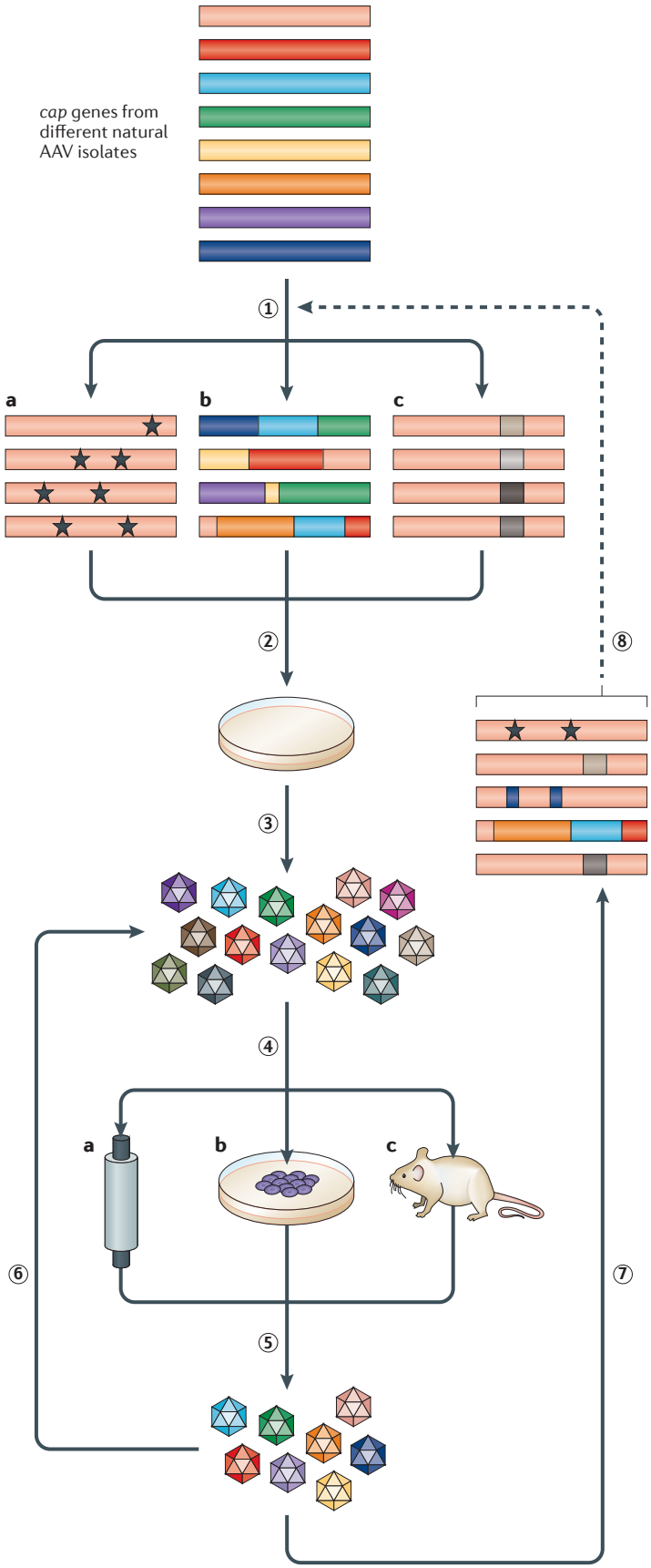
Box 2 | **Directed evolution for capsid engineering**

A viral library is created by mutating the *cap* gene (which encodes structural proteins of the capsid) using a range of techniques (see the figure, step 1). Error-prone PCR introduces random point mutations (black stars) into the adeno-associated virus (AAV) *cap* open reading frame (ORF) at a predetermined, modifiable rate[34] (step 1a). Using an *in vivo* viral recombination method[57] or, more commonly, DNA shuffling[38,58–60], random chimaeras of AAV *cap* genes can be generated, which yields a gene library with multiple serotypes (step 1b). 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 sequences can be inserted into defined sites of the viral capsid by ligation of degenerate oligonucleotides into the *cap* ORF (step 1c). Conversely, defined peptide-encoding sequences can be inserted into random locations of the AAV *cap* ORF using transposon mutagenesis[61,62]. Diversity can also be concentrated onto multiple hypervariable regions of the AAV capsid, which lie on surface-exposed loops. Such 'loop swap' libraries are generated, for example, by replacing four surface loops of AAV2 with libraries of peptide sequences that are bioinformatically designed based on the level of conservation of each amino acid position among natural AAV serotypes and variants[63]. 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. Viruses are then packaged (step 2) — such that each particle is composed of a mutant capsid surrounding the *cap* gene encoding that capsid — and purified (step 3). The capsid library is placed under selective pressure *in vitro* or *in vivo* (step 4). AAV variants can be selected using affinity columns[31] (step 4a), in which elution of different fractions yields variants with altered binding properties. Although this approach allows rapid selection of AAV variants with novel receptor binding affinity or specificity, it does not take into consideration other important aspects of the infection pathway, such as extracellular or intracellular trafficking. When cell culture models (step 4b) can emulate key aspects of an *in vivo* context, such as capsid-specific 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 behaviour of cells in the human body can be effective in creating AAV variants with increased efficiency and/or specificity. To more accurately capture a clinical gene therapy environment, selections can be directly carried out in animal models[19,20,37] (step 4c). The tissue or cell type of interest is harvested to isolate AAV variants that have successfully infected that target. One challenge is that the resulting variants have improved infectivity of the animal used for the selections, which does not necessarily translate to improved infectivity of human cells[64,65]. Human xenograft models can be used to select for infection of grafted human cells[20], although this advantage is counter-balanced by the absence of an intact immune system in the immunodeficient host and by the unknown specificity for a given human target cell in the context of non-human primates or humans. Successful viruses are amplified and recovered by adenovirus-mediated replication (step5) or PCR amplification (not shown). Although the PCR amplification risks the isolation of AAV genomes that have localized to, rather than productively infected, a given cell or tissue, this strategy is well suited when the target cell of interest is not accessible or permissive to adenovirus infection[19], or when the use of replication competent libraries raises biosafety considerations. Successful clones are enriched through repeated selection (step 6), and viral DNA is isolated to recover selected *cap* genes (step 7). Selected *cap* genes can be mutated to serve as a new starting point for further selection steps to iteratively increase viral fitness (step 8). In some cases, successful capsids have been generated without additional mutation.



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are needed 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.

Dual vectors. Another approach to expand the packaging capacity of recombinant AAVs is based on the generation of dual vectors. Transgene expression cassettes that are >4.7 kb in size are split into two, and each half is packaged within a normal-sized AAV vector. Upon successful transduction of the same cell, expression of the full-length transgene is achieved by viral inverted terminal repeat (ITR)-mediated recombination (dual AAV *trans*-splicing vectors), homologous recombination (dual AAV overlapping vectors) or both (dual AAV hybrid vectors). Although this strategy has shown some success in various recent preclinical studies[45–47], its efficiency varies markedly in diverse species, which raises questions about its applicability for the clinic.

Glossary

Biopanning
An *in vivo* method for selection of adeno-associated virus variants from a library for more efficient infectivity of a cell or tissue type of interest.

Choroideremia
An X-linked recessive disease caused by a mutation in the choroideremia (*CHM*) gene and the subsequent absence of Rab escort protein 1 (REP1) that leads to progressive loss of vision due to degeneration of the retina and choroid.

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.

Leber's congenital amaurosis type 2
A rare monogenic inherited eye disorder caused by mutations in the *RPE65* gene (which encodes a protein needed for the isomerohydrolase activity of the retinal pigment epithelium) that result in loss of photoreceptor function.

Müller cells
Glial cells that support neurons in the vertebrate retina.

Parvovirus
A linear, non-segmented single-stranded DNA virus with a genome size that is typically ~5 kb.

Rational design
A capsid engineering approach that uses knowledge of adeno-associated virus biology and structural analyses to guide capsid changes.

Tropism
The cell or tissue type that can be infected by a virus or a gene delivery vector.

Conclusions

Clinical trials involving AAV-mediated gene delivery to accessible tissues have enabled successful treatment of several recessive monogenic disorders, which has provided strong momentum to the research field. However, considerable challenges in both delivery and payload remain. Fortunately, similar to many biomolecules, viruses 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 the reach of AAVs. 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.

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Competing interests statement
The authors declare competing interests: see Web version for details.

FURTHER INFORMATION
Summary of product characteristics: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002145/WC500135472.pdf

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