Perspective on Adeno-Associated Virus Capsid Modification for Duchenne Muscular Dystrophy Gene Therapy

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Duchenne muscular dystrophy (DMD) is a X-linked, progressive childhood myopathy caused by mutations in the *dystrophin* gene, one of the largest genes in the genome. It is characterized by skeletal and cardiac muscle degeneration and dysfunction leading to cardiac and/or respiratory failure. Adeno-associated virus (AAV) is a highly promising gene therapy vector. AAV gene therapy has resulted in unprecedented clinical success for treating several inherited diseases. However, AAV gene therapy for DMD remains a significant challenge. Hurdles for AAV-mediated DMD gene therapy include the difficulty to package the full-length dystrophin coding sequence in an AAV vector, the necessity for whole-body gene delivery, the immune response to dystrophin and AAV capsid, and the species-specific barriers to translate from animal models to human patients. Capsid engineering aims at improving viral vector properties by rational design and/or forced evolution. In this review, we discuss how to use the state-of-the-art AAV capsid engineering technologies to overcome hurdles in AAV-based DMD gene therapy.

INTRODUCTION

DUCHENNE MUSCULAR DYSTROPHY (DMD) is the most common childhood muscle disease. It is characterized by progressive muscle weakness, loss of ambulation, and premature death caused by respiratory muscle and/or heart failure. DMD results from the loss of dystrophin, an essential cytoskeletal protein that protects muscle from contraction-induced injury (Fig. 1A). Soon after the discovery of the *dystrophin* gene,^{1,2} it was postulated that expression of a functional *dystrophin* gene in muscle may provide a cure for this relentless disease.³ Over the years, a number of nonviral and viral vectors have been explored to deliver the *dystrophin* gene. Currently, adeno-associated virus (AAV) stands out as the leading candidate vector.^{4,5}

AAV is a dependent parvovirus with an ~5 kb single-stranded linear DNA genome.⁶ Wild-type AAV has two major open reading frames (ORFs) flanked by two inverted terminal repeats (ITRs). The 5' and 3' ORFs encode replication and capsid proteins, respectively.⁷ The ITR contains 145 nucleotides and serves as the AAV genome replication

origin and packaging signal (Fig. 1B). In recombinant AAV, viral ORFs are replaced by the exogenous gene expression cassette, while the replication and capsid proteins are provided *in trans* (Figs. 1C and D). AAV has many appealing features as a gene therapy vector. For example, it has broad tissue tropism and high transduction efficiency. AAV can result in long-term persistent episomal expression.^{8,9} In addition, wild-type AAV is not associated with any known human diseases, and recombinant AAV vectors have shown an excellent safety profile in many clinical trials.

Despite many appealing features, recombinant AAV vectors face several challenges for DMD gene therapy. First, AAV has a fairly limited packaging capacity. Two-thirds of the dystrophin coding sequence has to be removed in order to fit into an AAV particle. Second, the dystrophic muscle is an extremely hostile microenvironment for gene transfer (Fig. 1A). Myofiber degeneration and necrosis may result in the loss of the vector genome.^{10,11} Inflammation in the muscle further intensifies the immune response. Third, muscle consists of ~40–50% the body mass and is spread

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Figure 1. Adeno-associated viral vector (AAV) for Duchenne muscular dystrophy (DMD) gene therapy. The ultimate goal of AAV-mediated DMD gene therapy is to deliver a therapeutic gene using the gutless recombinant AAV (rAAV) to achieve bodywide, robust, and persistent gene transfer in highly inflammatory and degenerative dystrophic muscle and heart to improve muscle and heart function, life quality, and lifespan. **(A)** Representative histology images from normal (left panel) and dystrophic (right panel) muscle. Normal muscle has well-organized, uniform myofibers with peripherally placed nuclei. Dystrophic muscle shows myofiber disorganization, centrally localized nuclei, and abundant infiltration of inflammatory cells. **(B)** Schematic outline of the wild-type AAV genome. The wild-type AAV has an ~4.6–4.8 kb genome. It contains a *Rep* gene for viral replication and a *Cap* gene to generate viral capsid. Two inverted terminal repeats (ITRs) are positioned at the ends of the viral genome. ITR serves as the replication origin and packaging signal. **(C)** Schematic outline of the rAAV genome. The AAV vector is essentially gutted. A therapeutic expression cassette replaces the wild-type *Rep* and *Cap* genes. The only viral component in the rAAV genome is ITR. **(D)** The most commonly used AAV vector production method is triple-plasmid transfection in 293 cells. A *cis* rAAV plasmid carries the rAAV genome. A *trans* Rep/Cap plasmid expresses the replication and capsid proteins. Because the reproductive life cycle of AAV requires the help of adenovirus, an adenovirus helper plasmid is included in the transfection cocktail. AAV vector is purified using isopycnic ultracentrifugation and/or chromatography. Color images available online at www.liebertpub.com/hum

throughout the body. An effective gene therapy will require efficient systemic delivery to a variety of muscle groups, including the heart and diaphragm. Bodywide gene transfer brings in the issue of vector genome sequestration in nonmuscle tissues and off-target transduction. Fourth, 30–80% of the human population has preexisting serum antibodies to various AAV serotypes. There is also a high-level cross-reactivity among different AAV serotypes.¹² Capsids with distinctive serological properties will be needed to bypass these obstacles. Last but not least is the species-specific barrier. This not only refers to the scale-up of AAV production and delivery to large species (dogs, pigs, nonhuman primates, and humans), but also includes species-unique AAV transduction biology and possibly the differences in dystrophin structure and function among different species.

The biological properties (such as tropism and immunity) of the viral vectors are mainly determined by the viral capsid. The AAV capsid is composed of three overlapping capsid proteins called viral protein 1 (VP1), VP2, and VP3. The mature virion contains ~5 copies of VP1, ~5 copies of VP2, and ~50 copies of VP3 in a 1:1:10 ratio. Together, 60 copies of capsid proteins form a $T=1$ icosahedral particle with 5-fold, 3-fold, and 2-fold axes of sym-

metry.¹³ There are nine highly conserved regions (defined as regions A–I) in each AAV capsid protein. Region A is an α -helix (α A). Regions B–I are β -sheets. These β -sheets are arranged as a jelly-roll β -barrel. Between the β -sheets are intervening loops. These loops form much of the outer surface of an assembled AAV particle. Importantly, they are composed of variable amino acid sequences and hence are amenable to genetic manipulation.¹⁴

Capsid engineering refers to intentional modification of hypervariable loops (and possibly other regions) of the capsid to achieve a desired biological property. Currently, there are two major approaches: rational design and directed evolution. In rational design, existing knowledge of the capsid structure/function is used to guide genetic engineering to achieve predetermined outcomes. In directed evolution, investigators apply selective pressure(s) to a random capsid library to drive the evolution of the most adapted capsid. The beauty of directed evolution is that it does not require prior knowledge on the structure or function of the capsid. Directed evolution also has the advantage of integrating multiple selective pressures simultaneously to achieve desired features. Most importantly, directed evolution may result in unique capsid variants that are impossible to program using the rational design approach.

In this review, we summarize the state-of-the-art capsid engineering technologies and discuss the potential to apply these technologies to overcome major barriers in AAV-mediated DMD gene therapy. It should be noted that strategies based on

AAV genome engineering (such as the dual AAV vector) and expression cassette optimization (such as the use of a tissue-specific promoter, transgene codon optimization, and microRNA targeting sequence) have also been used to improve AAV vectors for DMD gene therapy. Since these mechanisms are not based on capsid modification, we opt to not include them in this review.

AAV CAPSID ENGINEERING BY RATIONAL DESIGN

Rational design depends on accruing insight into structure–function relationships between the primary amino acid sequence, assembled quaternary structure, and biological phenotype of the AAV capsid. Defined structures for 12 naturally occurring or modified AAV serotype/variants have been resolved in recent years using X-ray crystallography and/or cryo-electron microscopy. These include AAV-1,¹⁵ AAV-2,^{16–22} AAV-3B,^{23,24} AAV-4,^{25,26} AAV-5,^{27–30} AAV-6,^{31,32} AAV-7,³³ AAV-8,^{34–38} AAV-9,^{39–41} AAVrh32.33,⁴² AAV-DJ,^{43,44} and AAV-rh8.^{17,2} The structural information of these AAV capsids as well as their interaction with respective cellular receptor/co-receptors have provided the necessary framework to logically design tailored vectors. Successful application of educated capsid design has been used to alter tissue tropism (targeting or de-targeting), avoid immune recognition, and improve postentry processing.

Engineering to enhance AAV uptake in muscle

Bodywide muscle delivery is a prominent challenge in developing a viable therapy for DMD, especially since muscle occupies such a large volume of the body. Further complicating the picture, significant amount of vectors can be sequestered by non-muscle tissues (such as the liver and spleen), hence reducing the effective dose in muscle. Consequently, an effective gene therapy for DMD will inevitably require a large quantity of AAV vectors (up to 10^{15} – 10^{16} viral genome particles per patient).^{45,46} This not only exposes the body to a large antigen load but also puts an increased demand on vector production. Controlling AAV tropism to favor skeletal and cardiac muscle could minimize some of these concerns.

Two strategies are often used to improve AAV uptake in muscle. In one approach, a muscle homing peptide is inserted on the surface of the capsid to facilitate the entry of AAV into muscle cells. In the second approach, enhanced muscle targeting is achieved by modulating AAV interaction with its natural receptors. In the discussion below, we illustrate both strategies.

Peptide insertion to improve muscle homing. Inserting a tissue-specific peptide ligand to the capsid is a convenient method to alter AAV tropism. When considering peptide insertion, there are two key questions: where should the peptide be inserted and what peptide(s) should be inserted? Ideally, insertion should not alter capsid assembly, vector genome packaging, and the overall yield of vector production. The insertion site should also facilitate proper peptide presentation such that the peptide can effectively interact with its receptor. For muscle gene therapy, apparently, a muscle targeting peptide would be preferred.

Identifying sites amenable to peptide insertion has been the focus of a considerable amount of research. During early years, this was achieved by the “hit-or-miss” approach or scanning mutagenesis. Yang and colleagues inserted a single chain CD34 antibody in front of VP1, VP2, or VP3 of AAV-2.⁴⁷ Fusion to the N-terminus of VP2, but not VP1 and VP3, resulted in particles capable of infecting previously nonpermissible CD34 cells.⁴⁷ It is currently unclear why this strategy did not work for VP1 and VP3. However, in terms of VP1, it may relate to the unique biological property of the VP1 N-terminal region. This region not only contains the nuclear localization signal⁴⁸ but also contains phospholipase A2 activity that is essential for AAV infectivity.^{49,50} Some recent studies suggest that the VP1 N-terminal region undergoes structural changes in the endosome and this change is essential for viral trafficking to the nucleus.^{51,52} To identify regions that are suitable for peptide insertion, Rabinowitz et al. and Wu et al. performed independent scanning mutagenesis across the entire capsid region of AAV-2.^{53,54} They found many sites that can tolerate peptide insertion. For example, Wu et al. successfully enhanced AAV-2 infection in lung epithelial cells by inserting an expanded serpin receptor ligand (10 residues) at residue 138 of the AAV-2 capsid. Warrington et al. inserted larger peptides such as fractalkine chemokine (76 residues) and leptin (146 residues) at exactly the same location (residue 138 of the AAV-2 capsid). Surprisingly, these insertions abolished VP3 production and viral particle assembly.⁵⁵ Collectively, in addition to the location, the size and/or amino acid composition of the peptide may also play an important role.

The resolution of the parvovirus structure has opened the door to rationally select the peptide insertion site.^{56–59} Girod et al. were the first to successfully use this approach.⁶⁰ They inserted an integrin ligand peptide (14 residues) to a putative loop region of AAV-2 and achieved efficient transduction in AAV-2 resistant cells that expressed the

integrin receptor.⁶⁰ Grifman et al. successfully targeted tumor cell lines by inserting an NGR peptide, which binds CD-13, a marker for tumor angiogenesis.⁶¹ In another study, Shi et al. identified potential insertion sites on a computer-simulated AAV structure.⁶² They successfully inserted the 15-residue luteinizing hormone receptor ligand into AAV-2 capsid and enhanced transduction of ovarian carcinoma cells.

With the availability of the AAV-2 atomic structure, it has become possible to precisely select a site for peptide insertion.^{16,17} For example, residues 587 and 588 (AAV-2 numbering) are located within a flexible loop on the external surface of the AAV-2 particle.^{63–73} Insertion at these two locations will likely allow good presentation of the peptide without substantially compromising virion assembly and stability. As the crystal structures of many AAV serotypes are now available, designing tailored peptide insertion in these serotypes may improve their transduction efficiency and specificity.^{74,75}

A major goal of DMD gene therapy is to achieve efficient transduction of skeletal and cardiac muscles. Insertion of muscle-homing peptides may improve targeted delivery of AAV to muscle and at the same time limit uptake in other tissues (Table 1). Ideally, a muscle-targeting peptide should (1) bind with high affinity to a muscle-specific receptor, (2) disrupt binding to the natural AAV receptor/co-receptors (such as the heparan sulfate receptor for AAV-2) (Table 2), and (3) incorporate on the surface of the capsid without altering assembly and infectivity. High-affinity ligands may occur naturally (e.g., transferrin and α -bungarotoxin)⁷⁶ or may be isolated using phage biopanning (Table 2).^{65,67,77–83} The success of *in vivo* biopanning depends on the delivery method (systemic versus local) and selection criteria. For DMD, systemic injection is a preferable selective pressure as a therapeutic vector will most likely be administered intravenously. Phage biopanning has been particularly successful for isolating peptides specific to skeletal and cardiac muscle. For example, Samoylova and Smith isolated a 7-residue muscle-targeting peptide (ASSLNI) (Table 2).⁷⁸ Yu et al. inserted this peptide between residues 587 and 588 in AAV-2.⁶⁸ The modified vector displayed enhanced skeletal and cardiac muscle uptake.⁶⁸ Targeting the heart is particularly important in DMD as ~90% DMD patients develop cardiomyopathy. While intracardiac injection of AAV dystrophin constructs partially restores the dystrophin complex to the heart,⁸⁴ a cardiac muscle targeting peptide could provide a less invasive approach for cardiac delivery. To this end, Ying and colleagues

applied *in vivo* biopanning on organotypic cultures of heart tissue and obtained a unique isolate (VNSTRLP) capable of myocardial targeting.⁶⁷

Altering capsid structural motifs involved in receptor interactions. Ligand–receptor interaction is a major determinant of viral tissue tropism. In the case of AAV, the ligand refers to the special structural motifs on the surface of the capsid that interact with cellular receptor/co-receptors. The ligand is often called the “footprint.”^{20,85} An AAV footprint does not have to be a series of contiguous amino acid residues. More often, a footprint is composed of a cluster of three-dimensionally related amino acids. For example, the footprint of AAV-2 is made of basic residues R484, R487, K527, K532, R585, and R588.^{73,86,87}

Cellular receptors for at least eight different AAV serotypes have been identified (Table 2). These receptors consist of various carbohydrates on the cell surface, in particular, N-linked α -2,3 sialic acid for AAV-1 and AAV-5,^{88,89} heparan sulfate proteoglycan for AAV-2 and AAV-3,^{90,91} O-linked α -2,3 sialic acid for AAV-4,⁹² N-linked α -2,6 sialic acid for AAV-6,⁸⁸ and N-linked galactose for AAV-9^{93,94} (Table 2).

Several studies have explored the possibility of modifying AAV tropism through the alteration of AAV footprint–cellular receptor interaction. The AAV-2 footprint interacts with its receptor heparan sulfate proteoglycan. Heparan sulfate proteoglycan binding correlates with liver transduction.

Table 1. AAV receptor and co-receptor(s)

Serotype	Receptor	Co-receptor(s)	Reference
AAV-1	N-linked α -2,3 sialic acid		Wu et al. ⁸⁸
AAV-2	Heparin sulfate proteoglycan	α 5 β 1-integrin, hFGFR1, α V β 5-integrin, hHGFR, LamR	Qing et al. ¹⁶⁶ , Summerford et al. ⁹¹
AAV-3	Heparin sulfate proteoglycan	hFGFR, hHGFR, LamR	Handa et al. ⁹⁰ , Lerch et al. ¹⁶⁸ , Ling et al. ¹⁶⁷
AAV-4	O-linked α -2,3 sialic acid	Unknown	Kaludov et al. ⁹²
AAV-5	N-linked α -2,3 sialic acid	PDGFR	Di Pasquale et al. ¹⁶⁹ , Walters et al. ⁸⁹
AAV-6	N-linked α -2,6 sialic acid	EGFR	Wu et al. ⁸⁸
AAV-7	Unknown	Unknown	
AAV-8	Unknown	LamR	Akache et al. ¹⁷⁰
AAV-9	Galactose	LamR	Akache et al. ¹⁷⁰ , Bell et al. ⁹⁴ , Shen et al. ⁹³

EGFR, epidermal growth factor receptor; hFGFR1, human fibroblast growth factor receptor 1; hHGFR, human hepatocyte growth factor receptor; LamR, 37/67kDa laminin receptor; PDGFR, platelet-derived growth factor receptor; AAV, adeno-associated virus.

Table 2. Muscle-targeting peptides

Reference	Peptide name	Peptide sequence	Length	Comment
Barry et al. ⁷⁷	Peptide 20.1	TPHSLYEDLKRQMMQLGRH L	20-mer	This peptide targets fibroblasts and myoblasts but not differentiated myotubes <i>in vitro</i> .
Flint et al. ⁷⁹	Peptide T.1	TGGETSGIKKAPYASTTRNR	20-mer	This peptide targets differentiated myotubes.
	Peptide T.2	SHHGVAGVDLGGGADFKSI A	20-mer	This peptide targets differentiated myotubes.
	Peptide (P5)	PYDQLRH	7-mer	This peptide targets C2C12 muscle cell line <i>in vitro</i> .
	Peptide (P6)	KAMHQMQ	7-mer	This peptide targets C2C12 muscle cell line <i>in vitro</i> .
	Peptide (P9)	YASINPM	7-mer	This peptide targets C2C12 muscle cell line <i>in vitro</i> and rat skeletal muscle <i>in vivo</i> .
Samoylova and Smith ⁷⁸ , Yu et al. ⁶⁸		NPSQVKH	7-mer	This peptide targets rat laryngeal muscle <i>in vivo</i> .
		ASSLNIA	7-mer	This peptide targets C2C12 muscle cell <i>in vitro</i> and mouse skeletal and cardiac muscle <i>in vivo</i> .
Ghosh and Barry ⁸⁰	Peptide 12.51	TARGEHKEEELI	12-mer	This peptide targets C2C12 muscle cell line <i>in vitro</i> .
McGuire et al. ⁸¹	Peptide PCM.1	WLSEAGPVVTVRALRGTS W	20-mer	This peptide targets primary cardiomyocytes <i>in vitro</i> and murine heart <i>in vivo</i> .
Seow et al., 2010 ¹⁷¹	Peptide T9	SKTFNTHPQSTP	12-mer	This peptide targets C2C12 muscle cells <i>in vitro</i> and mdx heart and quadriceps muscle <i>in vivo</i> .
Ying et al. ⁶⁷		PSVSPRP and VNSTRLP	7-mers	This peptide targets rat heart slices <i>in vitro</i> and mouse heart <i>in vivo</i> .
		EGRVRPP and GTFSRAP	7-mers	This peptide targets rat cardiomyocytes <i>in vitro</i> and mouse heart <i>in vivo</i> .
Gao et al. ⁸²	Peptide M12	RRQPPRSISSHP	12-mer	This peptide targets C2C12 muscle cell line <i>in vitro</i> and skeletal and heart muscle <i>in vivo</i> .

Theoretically, alteration of the AAV-2 footprint should reduce heparan sulfate proteoglycan binding and hence decrease liver transduction. To test this hypothesis, Asokan and colleagues replaced a section of AAV-2 footprint (from residue 585–590, RGNRQA) with corresponding residues from AAV-8 (QQNTAP).⁹⁵ The chimeric capsids indeed showed reduced liver uptake. AAV-9 enters a cell through binding to N-linked galactose on the surface of the cell. This interaction is thought to underlie systemic transduction of AAV-9. The residues responsible for galactose binding in AAV-9 were identified recently.^{41,96,97} Engraftment of these residues to AAV-2 (Q464V, A467P, D469N, I470M, R471A, D472V, S474G, Y500F, S501A, and D514N) indeed resulted in galactose binding and significantly improved systemic gene transfer efficiency of AAV-2.^{96,97}

Another approach to modulate AAV interaction with its receptor is to conditionally hide the receptor binding residues. Judd et al. invented such a gating system for AAV-2.⁹⁸ To block the interaction of AAV-2 with its receptor, they inserted a short peptide between R585 and R588. They also engineered matrix metallo-protease (MMP) cleavage sites at the end of this short peptide. In the presence of MMP, the inserted peptide was removed and the heparan sulfate proteoglycan binding motif of AAV-2 was reconstituted allowing receptor recognition and viral uptake.⁹⁸ This type of capsid modification will be very useful for DMD gene therapy because inflammation is a prominent

feature of DMD and MMP is highly expressed in inflamed tissues.

Rational modification to circumvent immune responses

As we alluded to earlier, immune responses constitute a critical barrier to DMD gene therapy.^{12,99} Preexisting neutralizing antibodies may nullify AAV in the circulation. Capsid- or transgene product-specific T-cell responses can result in the elimination of AAV-transduced cells. While molecular mechanisms of the immune response remain to be fully elucidated, the immunological hurdle must be addressed in order to achieve successful DMD gene therapy. As the focus of this review is on capsid modifications, we will limit our discussion to capsid-related immune responses.

Alteration of neutralizing antibody binding epitopes to improve transduction in seropositive subjects. Preexisting neutralizing antibodies at levels as low as 1:2 are sufficient to attenuate AAV transduction in animal models.^{100,101} A significant portion of the human population (~30–80%) has preexisting neutralizing antibodies to AAV.¹² This precludes a large percentage of patients from AAV therapy unless new vectors with enhanced antibody evasion become available. Several strategies have been developed to identify capsid epitopes that interact with neutralizing antibodies. Peptide display and scanning have been used to identify linear epitopes that reside in continuous amino acid residues.^{102,103}

However, to fish out conformational epitopes that reside in noncontiguous amino acid residues, one has to use nondenatured intact viral particles.¹⁰³

Capsid mutagenesis has been used to identify both linear and conformational neutralizing antibody binding motifs in AAV. For example, Huttner et al. applied the peptide insertion approach and found that AAV-2 residues 534, 573, and 587 are important for neutralization escaping.¹⁰⁴ Adachi et al. mapped the epitopes for AAV-1 and AAV-9 neutralization antibody binding to 452-QSGSAQ-457 and 453-GSGQN-457, respectively, by screening double-alanine mutants.⁹⁶ Antibody–capsid interactions can also be visualized with cryo-electron microscopy and three-dimensional image reconstruction.^{37,105,106} High-resolution imaging shows that capsid epitopes tend to localize around the threefold protrusions and overlap with key regions involved in receptor recognition and tropism.¹⁰⁵ This information suggests that perhaps capsid engineering to improve tropism may simultaneously alter antibody recognition. In summary, delineating specific antibody recognition sites and targeted mutagenesis may yield new capsid variants that can be used in patients with preexisting immunity to existing serotypes.

Engineering AAV vectors to reduce cellular immune responses. The dystrophic muscle presents a formidable inflammatory environment for AAV gene therapy (Fig. 1A). Expression of major histocompatibility complexes (MHC) is upregulated in dystrophic muscles.¹⁰⁷ Cytokines and chemokines released from degenerative/necrotic myofibers may

recruit more immune cells to muscle and further aggravate inflammation.

Both capsid-specific and dystrophin-specific cellular immune responses have been implicated in transduction loss in dystrophic muscle.^{108–110} Capsid-specific T-cell responses depend on AAV endosomal escape and subsequent proteasome processing (Fig. 2).^{111,112} Therefore, capsid modifications to de-target the proteasome may be a useful method to reduce capsid T-cell responses. Zhong et al. found that AAV capsid was degraded by the ubiquitin–proteasome system upon phosphorylation of surface-exposed tyrosine (Y) residues.^{113,114} Replacement of these residues with phenylalanine (F) aborted phosphorylation.¹¹⁵ Martino and colleagues evaluated immunological benefits of tyrosine mutated AAV-2.¹¹⁶ They found that the mutant vector showed reduced MHC presentation and less killing of hepatocytes in a liver gene transfer study (Fig. 2).¹¹⁶ It is currently unclear whether tyrosine mutants will fare better in dystrophic muscle in terms of the T-cell response. However, some tyrosine-mutated AAV serotypes have shown promise for intramuscular injection in mouse muscle (Y445F or Y731F AAV-6) or body-wide muscle delivery in newborn dogs (Y445F/Y731F AAV-1).^{45,117} We recently found that Y731F AAV-9 resulted in robust local and systemic muscle gene transfer in adult DMD dogs.^{46,118} It is possible that tyrosine-mutated AAV capsids may prove useful to DMD gene therapy. In addition to tyrosine, surface-exposed serine, threonine, and lysine residues are also targets for phosphorylation. Sen and colleagues showed reduced ubiquitination and enhanced hepatic transduction when these

Figure 2. Engineering AAV vector by tyrosine substitution for proteasome evasion. Capsid-induced T-cell response is a major barrier for AAV-mediated DMD gene therapy. Viral capsids are degraded in the proteasome in the host cell cytoplasm. Degraded peptide fragments are transported into the endoplasmic reticulum and loaded into major histocompatibility complexes (MHC) class I molecules on the surface of antigen presenting cells (APC). The peptide–MHC I complex traffics to the cell membrane where peptide is recognized by the T-cell receptor leading to T-cell activation. The tyrosine residue on the surface of the viral capsid plays an important role in this process. Substitution of surface-exposed tyrosine by phenylalanine may reduce MHC presentation and T-cell immunity. Color images available online at www.liebertpub.com/hum

residues were substituted in AAV-1, 5, and 8.^{119–122} Additional studies are needed to determine whether these modifications offer immunological advantages in inflamed dystrophic muscle.

Uptake by antigen-presenting cells (such as macrophages) is a premise for the induction of cellular immune responses.^{123–125} Strategies that can minimize macrophage uptake may improve AAV transduction in dystrophic muscle. CD47 glycoprotein is a surface marker for “self” in all human cells. Interaction of CD47 with signal regulatory protein- α on macrophages prevents uptake of “self” cells by macrophages.¹²⁶ Recently, a 21-residue peptide was found to mediate this self-recognition.¹²⁷ Incorporation of this peptide to nanoparticles reduced phagocytic clearance and enhanced gene delivery.¹²⁷ It is foreseeable that insertion of this peptide to the surface loops of AAV capsids may reduce macrophage uptake and improve AAV transduction in DMD muscle.

AAV CAPSID ENGINEERING THROUGH DIRECTED EVOLUTION

Directed evolution harnesses natural selection to identify capsids with desired phenotypes. In this manner, it is possible to develop AAV vectors capable of coping with multiple selective pressures without any prior knowledge of the capsid structure.¹²⁸ Like natural selection, directed evolution requires a diverse starting population to increase the odds of identifying rare, desirable variants. This translates into the creation of a diverse capsid library. Theoretically, the more diverse the library, the more likely you will find a variant that can meet your need. A key technical challenge of directed evolution is the ability to retrieve the capsid coding sequence based on the phenotype of the capsid protein. For example, one may have identified an attractive neutralization escaping capsid variant. In order to package a therapeutic gene into this capsid variant, the exact coding sequence for this particular capsid variant must be fished out from millions of capsid coding sequences in the library. This could be quite challenging if not impossible. An ingenious solution is to have this capsid variant carrying its own coding sequence in its genome. Genomes can then be extracted from the capsids after selection. We will discuss various strategies used by different groups and a surprising but pleasant new discovery on capsid–genome correlation. The last step in directed evolution is to apply selective pressures to enrich capsid variants with desirable properties, and finally to isolate the fa-

vored capsid variant. We will discuss screening strategies that may help isolation of new AAV capsids for DMD gene therapy.

Methods for library creation

Below we summarized the most commonly used strategies for AAV capsid library generation. More information can be found in a recent review article by Kotterman and Schaffer.¹²⁸

Error-prone polymerase chain reaction. Error-prone polymerase chain reaction (EP-PCR) is a relatively simple method to randomly mutate a DNA sequence. This is achieved by enhancing the inherent polymerase error rate. The Taq polymerase has an inherent cumulative error rate of $\sim 10^{-3}$ per nucleotide favoring A-to-G and T-to-C mutations yielding from 1 to 20 mutations per 1,000 bases.^{129–131} This error rate may be further enhanced by incorporating high levels of Mn^{2+} or Mg^{2+} in the PCR or, alternatively, providing disproportionate amounts of deoxynucleotides. Mn^{2+} and Mg^{2+} help to stabilize noncomplementary strands, while disproportionate deoxynucleotides leads to unequal base pair incorporation. Additionally, nontraditional bases such as 8-oxo-GTP or dITP may be introduced to the PCR products.^{132,133} Using these techniques, it is possible to generate an AAV capsid library with a complexity of approximately 10^7 variants.¹³⁴

Mosaic capsids. The construction of mosaic capsids provides an alternative to EP-PCR for increasing the library diversity. To generate mosaic capsids, cultured cells are transfected with AAV helper plasmids expressing capsid genes from different serotypes, the adenovirus helper plasmid and a rAAV plasmid. This results in the incorporation of viral protein monomers from different serotypes during capsid assembling (Fig. 3A). The resulting viral capsids are mosaic capsids because they contain capsid units from several serotypes. Since not all capsid monomer combinations are viable,¹³⁵ the diversity is limited for this approach.

PCR-based *in vitro* recombination. PCR-based *in vitro* recombination methods create highly diverse chimeric libraries. It takes the advantage of high sequence similarity among different AAV serotypes to develop capsids of various combinations of the original parental sequences. Three most commonly used PCR strategies are staggered extension, overlap extension, and DNA shuffling.

Staggered extension PCR. Staggered extension PCR (StEP) provides a simple method to generate

Figure 3. Generation of mosaic capsid library and DNA-shuffled capsid library. **(A)** Mosaic capsids are produced by co-transfection of the *Cap* gene from several AAV serotypes (in the example here, AAV-3 and AAV-5). During viral production, viral protein monomers from different serotypes are incorporated in the capsids to form mosaic capsids. **(B)** In DNA shuffling, AAV capsid genes from several serotypes are fragmented by DNase I. Homology between different capsid genes allows re-ligation through self-priming PCR. This haphazard re-assembly results in a library of chimeric capsids containing fragments from different serotypes. Color images available online at www.liebertpub.com/hum

Figure 4. Capsid–genome correlation in AAV production. **(A)** Natural AAV production refers to AAV production using a *cis* plasmid that contains a wild-type-like AAV genome. In this genome, the *Rep* and *Cap* genes are flanked by the ITRs. In the presence of multiple different *cis* plasmids, the capsid produced by a particular *Cap* gene will package only the viral genome containing this *Cap* gene. There is a tight capsid–genome correlation. In the example here, two *cis* plasmids are used for AAV production, one carries the *Cap-X* gene (marked in red color) and the other carries the *Cap-Y* gene (marked in purple color). The Rep-2/*Cap-X* genome (green/red) will be packaged only inside capsids X (the red colored capsid). The Rep-2/*Cap-Y* genome (green/purple) will be packaged only inside capsids Y (the purple colored capsid). The Rep-2/*Cap-X* genome (green/red) will not be packaged inside capsids Y (the purple colored capsid), neither will the Rep-2/*Cap-Y* genome (green/purple) be packaged inside capsids X (the red colored capsid). In addition, this system will not generate chimeric viral particles in which the viral genome is packaged in chimeric capsids. **(B)** Recombinant AAV production refers to AAV production using a *cis* plasmid that contains a recombinant AAV genome (a genome in which a foreign expression cassette is flanked by the ITR). In recombinant AAV production, viral replication protein and capsid protein are produced from a *trans* plasmid that does not contain the ITR. In the example here, the recombinant AAV genome can be packaged in the capsid X, the capsid Y, or a mosaic capsid. The capsid–genome correlation is lost during recombinant AAV production. Color images available online at www.liebertpub.com/hum

chimeras by altering the annealing and extension phases in a standard PCR. By reducing the annealing and extension period, staggered extension produces incomplete PCR fragments, which, due to the high sequence homology of AAV capsids, are able to anneal and self-prime on other parent templates.¹³⁶ Through reiterative cycles of staggered extension, capsid sequences from various parent templates are linked together to form chimeras.^{137,138}

Overlap extension PCR. This method is usually used to insert specific mutation at specific points in a sequence or to stitch smaller DNA fragments into a larger fragment. In overlap extension PCR, primers with a 5' overhang complementary to the opposite parent template amplify parent templates yielding fragments with overlapping 5' regions. Primer-less PCR combines the individual amplicons into a larger gene sequence based on the overlapping regions. Terminal flanking primers are then used to amplify the entire sequence.¹³⁹ This method takes advantage of homologous 5' sequences between PCR products allowing parental templates to be sown together. In as few as 5–10 cycles of overlap extension followed by 30 cycles of flanking primer amplification, it is possible to fuse 2–7 parental capsid fragments.¹⁴⁰ Overlap extension PCR is particularly useful for inserting amino acids from one serotype into the capsid of another serotype.

DNA shuffling. DNA shuffling involves the fragmentation of related genetic sequences by DNase I digestion and subsequent recombination based on sequence homology in a reassembly PCR.^{141,142} In recursive cycles of DNA shuffling, selective pressure is applied to enrich the recombinant population that has desirable characteristics (Fig. 3B). To shuffle AAV capsids, parental capsid sequences are PCR amplified with primers containing unique restriction sites on the 5' ends. The unique restriction sites are for cloning each capsid into a common backbone containing the wild-type ITRs and the AAV-2 replication gene. In a subsequent PCR, the capsid sequences are amplified, pooled, and digested briefly with DNase I. Because of sequence homology, fragments are able to undergo template switching and self-priming, allowing the *in vitro* recombination of homologous sequences. Unique restriction sites allow the assembled capsid sequences to be cloned into the common plasmid backbone for virus production.¹⁴³

Capsid–genome correlation

In order to retrieve the AAV genome that encodes the desired capsid, it is important that each capsid encapsulates only its own coding genome during packaging. Several strategies have been developed

to address this issue. Schaffer and colleagues used limiting dilution.¹⁴⁴ Basically, they controlled the amount of plasmids used in transfection to achieve one proviral plasmid per cell. In this case, the AAV vector made in each cell will likely carry only its own genome. Alternatively, Muller and colleagues utilized a shuttle vector system by transfecting the capsid plasmid library with wild-type capsid.⁶⁵ Using a low multiplicity of infection, shuttle vectors theoretically deliver only one genome to each cell. Recently, the Weber laboratory encountered a surprising observation when comparing AAV production using natural or recombinant proviral AAV plasmids (Fig. 4).¹⁴⁵ Natural proviral AAV plasmids contain the 5'-ITR, replication gene, capsid gene, and 3'-ITR. Weber and colleagues found excellent capsid–genome correlation when they transfected a mixture of natural proviral AAV plasmids from different serotypes at the dose of 5,000–50,000 plasmids/cell. Essentially, 75–80% of capsids packaged only their own coding genome (Fig. 4A). This is surprising because there are hundreds of proviral plasmids in each cell and these plasmids encode different capsids.¹⁴⁵ In contrast to natural proviral AAV plasmids, recombinant AAV proviral plasmids contain the 5'-ITR, transgene expression cassette, and 3'-ITR. For the production of recombinant AAV, the AAV replication and capsid genes are provided *in trans* using ITR-deficient plasmids. When Nonnenmacher et al. transfected a recombinant AAV proviral plasmid with ITR-null capsid plasmids from different serotypes, the tight capsid–genome correlation was lost (Fig. 4B). It is currently unclear why a capsid packages only the “correct (its own)” genome in the case of natural AAV reproduction. Nevertheless, this new observation suggests that capsid–genome correlation may not be a big concern as previously thought. If the capsid library is made in natural AAV plasmids, one may not need to perform limiting dilution or to use shuttle vectors.

Evolution of novel capsids for DMD gene therapy

In natural selection, environmental pressure creates a selective advantage for phenotypes with higher fitness. Likewise, selective pressures applied to capsid libraries should force the survival of one or a few dominant capsids that can handle the specific selective pressure. Directed AAV evolution can be performed in cultured cells *in vitro* or in live animals *in vivo*. Selective pressure can also be applied to the capsid library before (e.g., preincubation of the library with antibodies or antisera) or after AAV is delivered to cells/animals.

Directed evolution to improve muscle tropism. Directed evolution is particularly well suited for creating AAV variants with enhanced tissue specificity. Simultaneous application of positive (e.g., muscle uptake) and negative (e.g., restricted liver uptake) pressures can dramatically improve targeting and de-targeting. Using a shuffled capsid library of AAV-1, 2, 3B, 4, 6, 7, 8, and 9, Yang and colleagues isolated a chimeric vector called AAV-M41.¹⁴⁶ AAV-M41 displayed enhanced cardiac tropism comparable to that of AAV-9, the so-called “cardiotropic” AAV. But in contrast to AAV-9, AAV-M41 resulted in minimum liver transduction.¹⁴⁶ The Asokan laboratory utilized EP-PCR to mutate AAV-9 capsids and screened for vectors with enhanced muscle transduction.¹⁴⁷ A resultant vector, AAV-9.45, displayed significantly increased transgene expression in the heart and skeletal muscle. Interestingly, AAV-9.45 was de-targeted from the liver, a feature similar to that of AAV-M41.

Directed evolution has also been used to identify novel capsids that can transduce difficult targets such as nonpermissive melanoma cells.¹⁴⁸ Failure to transduce muscle progenitor cells (such as satellite cells) by conventional AAV serotypes represents a major challenge in DMD gene therapy.¹⁴⁹ Directed evolution may yield capsids with enhanced transduction efficiency in satellite cells.

A major challenge in gene therapy is to reproduce animal study results in human patients.^{150–152} The traditional approach is to confirm rodent results in large animal models before the initiation of human trials. Scaling up from small to large animals gives investigators a good chance to gauge the impact of the body size. However, this cannot address species-specific reactions. The xenograft model may provide a platform to test promising candidate vectors in human tissues or to identify viral capsids with enhanced tropism in human cells. Lisowski and colleagues screened a shuffled capsid library of AAV-1, 2, 3B, 4–6, 8, and 9 in a human liver xenograft model. In this model, human hepatocytes were engrafted in the liver of immune-deficient mice. After several rounds of *in vivo* selection, the authors isolated a chimeric vector called AAV-LK03. This vector showed preferred transduction of human but not mouse hepatocytes.¹⁵³ A human skeletal muscle xenograft model was developed by the Wagner laboratory recently.¹⁵⁴ This model recapitulated dystrophic muscle pathology. Future directed evolution in this model could potentially yield AAV capsids suitable for muscular dystrophy gene therapy.

Directed evolution to modify immune reactivity. Another important application of directed evolution is to develop immune-evasive vectors. By screening against human neutralizing sera, directed evolution has yielded vectors that are resistant to preexisting neutralizing antibodies. Schaffer's group was the first to test this strategy. Maheshri et al. included a neutralizing sera preincubation step before screening EP-PCR- and StEP-derived libraries.¹⁵⁵ This strategy led to the isolation of AAV-r2.4 and r2.15. Both showed improved transgene expression in the presence of the neutralizing antibody in *in vitro* and *in vivo* tests. In another study, Schaffer's lab used a library generated by DNA shuffling. They then selected against pooled intravenous immunoglobulin (IVIG). This led to the isolation of several vectors (AAV-cA2, cA3, cA4) that are highly resistant to IVIG challenge.¹⁴⁴ Similarly, Perabo and colleagues used preincubation with human sera to screen an EP-PCR capsid library for retained infectivity.¹³⁴ Capsids with mutations (R459G/K and N551D) showed increased resistance to human neutralizing sera. Neutralizing antibody screening can also be used in combination with tissue-selective pressures. For example, Grimm and colleagues developed a shuffled library from AAV-2, 4, 5, 8, and 9 and then screened this library on human hepatocytes and antisera.¹⁵⁶ A resultant vector, AAV-DJ, displayed enhanced liver tropism and resistance to neutralizing sera. This study suggests that combined use of multiple selective pressures may yield vectors with multiple desired features. So far, none of these immune evasive capsids has been tested in clinical trials. Future study with sera from DMD patients may reveal the utility of these capsids for DMD gene therapy.

CAPSID MODIFICATION TO INCREASE AAV PACKAGING CAPACITY FOR DMD GENE THERAPY

The *dystrophin* gene is one of the largest genes in the genome. The 2.4 mb gene carries 79 exons and an 11.2 kb protein coding sequence.¹⁵⁷ This greatly exceeds the packaging capacity of AAV. Dual- and tri-AAV vectors are currently under development to deliver a larger *dystrophin* gene via interviral genome recombination.^{158,159} An alternative approach is to increase the payload of each individual viral particle. One study suggests that AAV-5, the phylogenetically most divergent AAV serotype, may package an 8.2 kb vector genome.¹⁶⁰ However, subsequent studies from several laboratories suggest that this is not the case.^{161–163}

AAV belongs to the dependent genera of parvovirus. All parvovirus shares a similar structure. Interestingly, some members appear to have a more flexible capsid.¹⁶⁴ For example, human bocavirus-1 (a member of autonomous genera of parvovirus) carries a viral genome of 5,543 nucleotides. This is 18.5% larger than that of the AAV-2 genome (4,679 nucleotides). Yan et al. recently tested cross-genera packaging of an AAV genome in human bocavirus-1.¹⁶⁵ The chimeric AAV-2/human bocavirus-1 vector indeed packaged a larger genome than conventional AAV. It is possible that further interrogation along this line may lead to the development of oversize AAV vectors suitable for DMD gene therapy.

PERSPECTIVE ON AAV CAPSID ENGINEERING FOR DMD GENE THERAPY

Capsid engineering has contributed significantly to the development of better, more efficient, and specific viral vectors. As we have discussed throughout this review, there is a wide array of methodologies for altering the biological properties of AAV capsids. With the continual understanding of AAV capsid structure–function relationship, improved library generation, and creative screen-

ing methods, we may develop AAV vectors that better fit the needs of DMD gene therapy. In this regard, low-immunogenic, large-capacity, and human muscle-specific capsids will be particularly appealing. It is tempting to speculate that a more integrated approach, combining several rational and directed evolution approaches, may lead to the development of one or several AAV capsids that can meet the needs of DMD gene therapy.

ACKNOWLEDGMENTS

DMD research in the Duan lab is supported by the National Institutes of Health NS-90634, Department of Defense, Muscular Dystrophy Association, Parent Project Muscular Dystrophy, Jesse's Journey—The Foundation for Gene and Cell Therapy, Hope for Javier, Kansas City Area Life Sciences Institute, and the University of Missouri.

AUTHOR DISCLOSURE

D.D. is a member of the scientific advisory board for Solid GT, a subsidiary of Solid Ventures. The Duan lab received funding support from Solid Ventures for research not related to AAV capsid modification.

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Received for publication July 22, 2015;
accepted after revision September 1, 2015.

Published online: September 16, 2015.