

Chapter 11

Development of Next-Generation Muscle Gene Therapy AAV Vectors



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Abstract Recombinant adeno-associated virus (AAV)-based gene delivery is a promising approach to treat muscle diseases. However, body-wide muscle delivery and pre-existing immune responses pose significant challenges to AAV muscle gene therapy. While the determinants of tissue tropism and immunogenicity of AAV are amenable to traditional molecular engineering, the development of a muscle-specific, immunosilent AAV vector has remained elusive. Recent advances in understanding the relationship between capsid structural motifs and functional domains have created exciting developments in the search for a muscle-specific AAV. Novel approaches to generate unique AAV properties through forced evolution have resulted in capsids with improved immune properties and/or muscle-targeting efficiency. Optimization of the gene cassette to restrict expression to mature muscle fibers provides another level of control. These reengineered AAV vectors have the potential to greatly increase efficacy and reduce off-target effects for body-wide muscle gene therapy. In this chapter, we discuss recent advances in the development of a next-generation, muscle-specific AAV vector.

Keywords AAV · Capsid · Muscle · Directed evolution · Rational design

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11.1 Introduction

Muscular dystrophies are a diverse group of inherited muscle diseases characterized by muscle weakness and dystrophic muscle pathology. Histologically, dystrophic muscle shows degeneration/regeneration, inflammation, and fibrotic/fatty infiltration. These microscopic changes result in variable clinical manifestations involving skeletal, smooth, and cardiac muscle and sometimes non-muscle organs such as the brain or skin. While our understanding of disease pathogenesis is still evolving, many genetic determinants underlying muscular dystrophies have been identified [1]. Thus far, defects associated with muscular dystrophies are primarily related to genes that encode extracellular matrix proteins, sarcolemma-associated proteins, nuclear membrane proteins, sarcomeric proteins, and proteins with enzymatic functions [1]. Before the 1990s, treatment options for muscular dystrophies were limited to mainly supportive therapies without hope for correcting the underlying gene mutation. This therapeutic gap has its origins in our incomplete understanding of the genetics of each disease and, also, our limited ability to modify the genetic landscape. In more recent years, exciting progress in the areas of gene replacement, gene supplementation, and gene editing have increased potential for a viable genetic treatment in the near future.

The ability to efficiently and stably deliver the therapeutic gene via a vehicle to the diseased muscle all over the body is a premise for muscular dystrophy gene therapy. This vehicle can be either a viral or non-viral vector. From a pharmacokinetic standpoint, the vector must traffic through the muscle vasculature, traverse the endothelium and extracellular space, and then, finally, enter the target cell. For an effective therapy for many muscular dystrophies, the target cells would include not only mature muscle fibers but also muscle progenitor cells. Gene delivery to muscle is further complicated by off-target uptake in the reticuloendothelial organs such as the spleen and liver and activation of local and systemic immune responses [2].

After decades of research, adeno-associated virus (AAV) is now considered the premier gene delivery vector for muscle. AAV is a dependent virus and requires a helper virus such as adenovirus or herpesvirus for productive infection. In the absence of a helper virus, AAV establishes a latent infection capable of stable long-term gene expression in dividing and nondividing cells. AAV was discovered more than 50 years ago and has been studied as a gene transfer vector in mammalian cells for over 30 years [3–6]. To date, there are 12 known AAV serotypes with hundreds of AAV variants having been engineered in the laboratory or isolated from nature [7, 8]. Despite this diversity, none of the existing AAV can fulfill all the needs of muscle gene therapy such as selective and body-wide muscle targeting and the capability to efficiently transduce muscle progenitor cells. In this chapter, we introduce AAV biology in the context of muscle gene therapy and then discuss current progress toward developing next-generation, muscle-targeting AAV vectors. Additionally, we highlight key issues and potential solutions in the field of AAV capsid engineering.

11.2 AAV Biology

11.2.1 *The Basic Biology of AAV*

AAV is a 25 nm non-enveloped, icosahedral particle with a 4.7 kb single-stranded DNA genome. The AAV genome is flanked by 145 base pair inverted terminal repeats which are necessary for AAV replication and packaging [9]. Within the genome, there are two major open reading frames (ORFs) that encode nonstructural Rep proteins and structural Cap proteins. Rep proteins are involved in viral replication and packaging. Cap proteins are responsible for forming the viral capsid. Recently, a small ORF was discovered in the AAV genome. This ORF encodes a protein called AAV assembly-activating protein [10]. An intact AAV particle consists of 60 monomers formed by Cap proteins. Each monomer contains a conserved core structure with an alpha helix and eight antiparallel beta sheets ($\beta\text{B}-\beta\text{I}$) with intervening hypervariable loops [11, 12]. The hypervariable loops represent the primary source of diversity and convey the tropic/immunological properties. For this reason, these loops have been the focus of AAV capsid modification.

11.2.2 *AAV Infection Biology in the Context of Muscle and Muscle Stem Cells*

While the mechanisms of AAV gene transfer have been extensively studied over the past couple of decades, detailed knowledge of AAV infection in muscle remains incomplete [13, 14]. At the cellular level, AAV infection is initiated with the attachment of the viral particle to the cell surface followed by entry and trafficking to the nucleus for gene expression. Various types of cell surface glycans have been identified as the primary binding receptor for different AAV serotypes. Of particular interest is the discovery of galactose as the AAV-9 receptor because this may contribute to the superior cardiotropic property of AAV-9 [15–19]. AAV entry is mediated by a secondary binding event to a co-receptor (often a transmembrane protein such as integrin) that recycles between the endosomal compartment and plasma membrane. More recently, a generic AAV receptor named AAVR was discovered [20]. AAVR interacts with multiple AAV serotypes through its extracellular immunoglobulin domains. Characterization of the expression profile of AAVR in muscle will help to better understand AAV transduction in muscle and, if needed, to increase AAVR expression for enhanced muscle transduction. AAV internalization is primarily through clathrin-independent carriers/GPI-enriched endocytic compartment (CLIC/GEEC) endocytic pathway [21]. Following endocytosis, AAV appears to utilize the microtubule network to transport to the perinuclear region prior to nuclear entry. This may be important in muscular dystrophies where the microtubule network may be disrupted [22, 23]. In the absence of a helper virus, AAV establishes a latent infection in muscle as a double-stranded circular episomal molecule [24, 25].

Following intramuscular injection, AAV gradually spreads through the endomysial compartment between muscle fibers [26]. By 4 h, AAV particles are readily detected three myofibers away from the injection site. AAV capsids become detectable as early as 2 h after injection at the injection site. Nucleus-associated AAV particles increase by tenfold at 4 h after injection. However, by 6 days after injection, AAV capsids become largely undetectable in myonuclei although they are still readily visible in the endomysial compartment [26]. A study in human patients suggests that AAV capsid can persist in myonuclei for at least 12 months after local injection [27]. In contrast to AAV capsid proteins, the AAV vector genome can persist for many years after gene transfer in muscle. This leads to continuous transgene expression [28].

Skeletal muscle has the unique property of high regeneration upon chemical/physical injuries or in diseased status (such as muscular dystrophy). Satellite cells are responsible for muscle regeneration [29]. Effective targeting of satellite cells has clear advantage for gene editing therapy because genetic defects in all regenerated muscle cells should theoretically be corrected. AAV gene transfer to satellite cells has been the topic of great interest in past several years, yet the ability of various AAV serotypes to efficiently infect satellite cells remains uncertain. Satellite cells are a challenging target due to their (1) sub-laminar location, (2) cellular quiescence, (3) differential gene expression including cellular receptors and downstream trafficking molecules, and (4) low frequency in adult muscle. To study AAV-mediated satellite cell transduction, several labs used reporter AAV vectors [30, 31]. A study in adult mice from the Chamberlain Lab suggests that AAV-6 and AAV-9 do not transduce satellite cells and AAV-8 transduces ~5% satellite cells following local injection in mice [30]. However, AAV-8 did not appear to transduce satellite cells to a detectable level following systemic delivery [30]. Stitelman et al. tested in utero transduction of satellite cells with AAV-9 in mice and observed an efficiency reaching ~28% when AAV was delivered on embryonic day 16 [31]. More recently, Tabebordbar and colleagues used CRISPR editing to track AAV-9 transduction in satellite cells. Using fluorescence-activated cell sorting, they found slightly over 3% of satellite cells were transduced irrespective of AAV delivery route (intramuscular injection or intraperitoneal injection) [32]. Collectively, except for in utero delivery, postnatal AAV delivery using existing AAV serotypes appears very inefficient in transducing satellite cells.

11.3 Rational Design of Muscle-Targeting AAV Capsids

11.3.1 Naturally Existing Muscle-Tropic AAV Serotypes

Since the initial isolation of AAV as a contaminant of adenoviral stocks nearly 50 years ago [6], the explosion of knowledge and isolation of novel AAV serotypes have propelled muscle gene therapy forward from bench to clinic in a relatively short period of time. While many initial studies focused on AAV-2 [5], the discovery

of AAV serotypes with inherent tropism for muscle tissue has been instrumental in the development of vectors for treating muscular dystrophies [33].

While a number of AAV serotypes (such as AAV-1, AAV-5, AAV-6, AAV-7, AAV-8, and AAV-9) have been shown to efficiently transduce muscle following local injection [33], recent studies suggest that tyrosine-mutated AAV-6 may likely represent the most potent serotype for local muscle delivery [34]. Efficient muscle transduction has been detected following systemic administration of AAV-1, AAV-6, AAV-7, AAV-8, AAV-9, rh10, and rh74 in rodents and large mammals [33, 35]. Among these, AAV-8, AAV-9, and rh74 have received particular attention for human use to treat neuromuscular diseases [36]. Several systemic AAV gene therapy clinical trials have been initiated using these serotypes to treat type 1 spinal muscular atrophy, X-linked myotubular myopathy, and Duchenne muscular dystrophy [36]. The recent report on the spectacular clinical efficacy and high tolerability of systemic AAV-9 treatment in type 1 spinal muscular atrophy patients is a historical milestone for the entire field of gene therapy [37].

11.3.2 Improving Muscle Targeting with Rational Design

Despite robust muscle transduction with naturally existing AAV serotypes, these vectors usually display broad tropism to a number of tissues. This makes it a challenge to achieve muscle-specific transduction. Rational design of muscle-tropic AAV uses knowledge of high-resolution AAV capsid structure, receptor and co-receptor binding footprints, and known muscle-homing peptides. Most commonly, a muscle-targeting peptide is inserted to a specific location on the surface of a naturally existing AAV serotype. In this regard, the hypervariable loops located on the capsid threefold protrusions have been found extremely effective. Yu et al. inserted muscle-targeting peptide ASSLNIA following either 587 or 588 amino acid residues in AAV-2 [38]. Interestingly, the authors found that insertion after 587 ablated the intrinsic heparin binding of unmodified AAV-2, while insertion after 588 did not. Systemic delivery of a modified capsid inserted after 587 resulted in significantly improved heart and skeletal muscle transduction as well as reduction in non-muscle tissues. Work et al. inserted the peptide EYHHYNK following 587 and achieved great targeting of AAV-2 to human venous and arterial smooth muscle cells [39]. Ying et al. tested several cardiac-targeting peptides they discovered through in vitro screening of a random peptide library [40]. Insertion of these peptides after residue 588 in AAV-2 indeed significantly increased myocardial transduction in mice [40].

Besides muscle-homing peptides, swapping the tissue tropic footprint from one serotype to another has also resulted in dramatic change in tissue tropism. AAV-2 cannot efficiently transduce muscle. The AAV-2 heparan sulfate receptor footprint is a hexapeptide located between residues 585 and 590. The Asokan lab replaced the AAV-2 footprint with the corresponding residues from AAV-8, a serotype that can efficiently transduce muscle following systemic delivery. The resulting chimeric capsid AAV2i8 displayed significantly enhanced muscle and heart transduction [41].

Adachi et al. took a more radical approach in designing a muscle-tropic AAV [42]. They performed a double alanine scan in AAV-9 and identified residues critical for the tissue tropic phenotype. Based on this knowledge, they successfully reprogrammed AAV-2 for muscle targeting using a minimum number of noncontiguous mutations.

11.4 Evolving Novel Muscle-Tropic AAV Capsids Through Directed Evolution

Despite the success of rational design for improving muscle targeting of AAV capsids, it is suggested that the determinants of the AAV tropism are likely spread widely over the entire capsid surface. Modification in one location, or even one residue, may lead to unexpected changes in AAV properties. This often makes structure-function correlation difficult. For this reason, investigators have turned to directed evolution [43, 44]. The fundamental concept in directed evolution is the isolation of desired individuals from a diverse population to meet desired selection criteria. Similar to natural selection, selective pressures drive the emergence of beneficial traits, which at the protein level amounts to desirable amino acid polymorphisms. In-depth discussions on the applications of directed evolution to AAV and DMD therapy are reviewed in [45]. Here, we limit our discussion to a brief introduction of basic concepts, and we then focus on the application of directed evolution for AAV muscle gene therapy.

11.4.1 Basic Concepts

The methods of AAV plasmid library construction and selection platform are of utmost importance in the initial planning phase for a directed evolution experiment. Traditional library construction has typically focused on one approach for diversification. Moving forward however, combining multiple approaches is becoming more popular to further increase diversity (e.g., point mutations, small insertions, and DNA shuffling [45]). Point mutations are introduced with conventional molecular biology techniques such as error-prone PCR. Small insertions can be achieved by inserting a small stretch of random nucleotides at a particular location. DNA shuffling leverages sequence homology between parental AAV serotypes to derive novel, diverse capsid genes through in vitro recombination [46]. Theoretically, higher diversity correlates with a greater probability in isolating a beneficial mutation(s). However, it also increases the risk of defective variants, for example, variants that contain nonsense mutations and variants that affect assembly and packaging. According to an estimation by PacBio, sequencing a plasmid library with $\sim 10^7$ diversity may lose around 99% of its diversity resulting in $\sim 10^5$ in the packaged virus library [47].

Following diversification and virus production, the virus library is applied in recursive cycles to a selection platform for the desired phenotype. For muscle targeting, *in vivo* selection will likely provide the most realistic selection parameters. Screening methods should take into account the requirements of (1) intravenous delivery, (2) endothelial barrier translocation, (3) body-wide muscle targeting, (4) reduce non-muscle, especially liver, uptake, and (5) immune evasion. Another criteria may include species, which is difficult to address since viral libraries cannot be directly screened in human patients.

11.4.2 Retaining Muscle Tropism While Avoiding the Liver

From a reductionist perspective, primary muscle cells are the most straightforward platform for selecting muscle-targeting AAV capsids. However, *in vitro* screening methods often fail to address key desired traits for *in vivo* application. A vector that performs well in cell culture may be dependent upon a surface expression profile that does not occur naturally in the intact muscle and vice versa. For this reason, *in vivo* selection is preferred.

Following systemic delivery, the vast majority of AAV accumulates in the liver. Hence, de-targeting from the liver will allow more AAV to transduce muscle. Although, theoretically, minimizing liver retention should enhance muscle transduction, it may not always be the case. For example, Yang and colleagues identified a variant called AAVM41 from a DNA-shuffled virus library [48]. AAVM41 displayed reduced liver tropism but also had reduced muscle transduction when compared to AAV-9. Focusing on mutations to the threefold protrusion, Pulicherla and colleagues isolated an AAV-9 variant, AAV-9.45. This variant displayed reduced expression in the liver [49]. The muscle tropism of AAV-9 was retained but not enhanced. These studies suggest that there may be a trade-off in terms of cost-benefit when the entire capsid gene is mutated. It should be pointed out that although these variants did not show increased muscle transduction, they are still highly very useful for systemic muscle gene therapy due to reduced toxicity from liver de-targeting.

In a recent study from the Sena-Esteves laboratory, Choudhury et al. described a novel variant called AAV-B1 [50]. This variant was isolated from a shuffled library consisting of 11 parental serotypes. Similar to AAVM41 and AAV-9.45, AAV-B1 showed reduced liver transduction. However, it also showed robust transduction in the central nervous system and motor neurons. Of relevance to muscle gene therapy, AAV-B1 displayed skeletal muscle and heart transduction at least tenfold higher than that of AAV-9 in mice. These results were extended and consistent following systemic delivery in cats. If these results can be extended to human patients, AAV-B1 may become a preferred vector for systemic muscle gene therapy in clinical trials in the future.

11.4.3 Evading Immunity to Enhance Muscle Delivery

Muscle-specific and systemic humoral and cellular immune responses are impediments to muscle gene delivery with AAV vectors. It is estimated that 30–80% of the human population has pre-existing neutralizing antibodies from childhood exposure with a high cross-reactivity between serotypes [51]. IgG appears to be the most important neutralizing antibody. In one of the earliest studies investigating the evolution of vectors capable of escaping antibody neutralization, Maheshri et al. developed a screening method by preincubating shuffled virus with increasing concentrations of rabbit anti-AAV-2 sera with each round of selection in HEK-293 cells. Interestingly, this approach identified mutant capsids with improved gene expression in the mouse hind-limb muscle compared to wild-type following preincubation with rabbit anti-AAV-2 sera [52]. While these results were promising, an important question is how relevant are antigenic variants isolated when screening with pooled animal sera versus human sera and, more stringently, to patient-specific sera? Further is preincubation with sera contextually relevant to a viral-antibody encounter within the vasculature? In a recently published study by Tse et al., the authors used a combined rational and directed evolution approach focused on mouse monoclonal antibody epitopes to derive antibody-resistant AAV vectors [53]. The resulting vector (AAV-CAM130) displayed improved antibody resistance to not only mouse anti-sera but pooled nonhuman primate and human anti-sera while maintaining the tissue tropism of parental AAV-1. Therefore, there is likely antigenic conservation between species to an extent.

The capsid composition, in terms of the individual structural motifs, derived from each parental serotype may have a large impact on the ability of chimeric capsids to avoid antibody neutralization. Conspicuous epitopes, such as the threefold protrusions, are formed by the apposition of GH loops from adjacent monomers. In theory, GH loop segments could derive from different serotypes in an evolved chimeric AAV capsid. Such a capsid may still react to the neutralizing antibody against the parental serotype. For example, a capsid containing segments of GH loop from AAV-1 may not be able to evade neutralizing serum against AAV-1. Li et al. observed that shuffled vectors screened with human sera performed variably depending on a patient-to-patient basis [54]. This phenomenon appeared to be related to the parental origin of certain segments of the VP3 viral protein monomer. Therefore, the origin of the micro-architectural domains within the capsid could have a significant influence on important vector properties. With this notion, another consideration would be the ability of shuffled vectors to retain the tissue transduction efficiency of native vectors. Several approaches may help overcome this limitation. One solution is to co-inject the shuffled capsid library and neutralizing serum into skeletal muscle. While this approach incorporates two selective pressures, it fails to mimic the natural route of infection and neutralization, i.e., intravascular delivery. Furthermore, it would be important to consider the effect of injecting high doses of serum into the muscle and how this alters the environment and muscle transduction. Indeed, muscle-specific vectors isolated from this approach failed to exceed the heart and

muscle transduction of parental serotype AAV-9 [54]. Another solution is to pre-infect the host with a parental AAV capsid, then deliver the virus library and anti-sera intravenously. This approach incorporates more realistic selection. Tse et al. identified capsid variants with improved antibody evasion and robust muscle expression using this approach [53].

While one of the driving principles behind directed evolution is the ability to achieve improved vectors without prior knowledge of the structure of the evolved vector, analysis of the primary amino acid sequence from advantageous capsids can help to rationally identify capsid segments responsible for functional improvements. Maheshri identified a T716A mutation in the C-terminus of VP-1 to confer improved antibody resistance [52]. Likewise, muscle tropism in chimeric vectors may be enhanced by inserting the AAV-6 VP-1 sequence from amino acid 347–446 [54].

To summarize, directed evolution is a powerful platform to develop AAV variants with enhanced muscle targeting and immune evasion. However, despite the immense effort to evolve novel vectors, the preclinical results have, thus far, not translated to the clinic. Likely combined approaches or, at the least, focused evolution of specific capsid regions will be an important area of research development for muscle gene therapy.

11.5 Future Directions and Conclusions

AAV capsid engineering is an alembic process whereby years of research are beginning to produce promising novel vectors for muscle gene therapy. In a sense, the immense power of directed evolution and multitude of potential rational modifications provides gene therapists with an open canvas for endless creative possibilities. However, there are several additional questions (among many others) that remain for capsid engineers.

11.5.1 *Can We Use Capsid Engineering to Expand the AAV Capsid Packaging Capacity?*

While capsid engineering approaches have been applied to retargeting vectors and reducing immune responses, it is relatively unknown if these techniques may be utilized to enhance AAV packaging capacity. Increasing the packaging capacity opens the door to more complex gene expression cassettes which may accommodate larger genes and more sophisticated elements for selective muscle expression. Several studies since the early 2000s [55–57] determined that the packaging capacity of AAV is limited to ~5.2 kb, although larger packaging size was reported. Interestingly, AAV genomes may also be cross packaged in other human parvovirus capsids including parvovirus B19 (up to 5.6 kb) [58] and human bocavirus-1 (up to 5.5 kb) [59]. The molecular interactions, specifically protein and nucleic

moieties, involved in cross packaging AAV genomes to the capsids of other parvoviruses have yet to be determined. Identification of these signal/interaction domains may allow rational cross packaging to other, larger icosahedral capsids. A high degree of homology exists between AAV serotypes, underlying the fundamental recombinogenic mechanism in directed evolution. The ability of AAV genomes to cross package to other human parvovirus capsids suggests the path to an effective muscle-targeting vector with increased packaging may lie in the choice of human parvovirus B19 or human bocavirus-1 as starting platforms for directed evolution or rational design. In this scenario, endogenous tropism may be ablated and vectors retargeted through in vivo bio-panning or retargeting with muscle-specific ligands.

11.5.2 Is Directed Evolution in Large Animal Models Possible?

When considering library selection in animal models, the amount of library injected should be large enough that each individual clone is represented multiple times [60]. Similarly, injecting too much virus results in saturation and isolation of nonspecific variants. The use of approximately 1000-fold more particles than the estimated library diversity has been reported as ideal for a mouse [60]. This factor provides a limitation on the use of larger animal models such as dogs, pigs, or nonhuman primates. Inevitably, directed evolution in larger animals may be justified, despite the costs. In an attempt to bypass this limitation, translational models such as human xenografts in immune-deficient mice may allow selection in a more relevant in vivo model while limiting the amount of virus needed for screening [61].

In conclusion, next-generation muscle-targeting vectors may dramatically improve the translation of gene therapy efforts for treating muscle diseases. Not only will more selective vectors improve uptake in the muscle tissue, but they may reduce unwanted toxicity in the other tissues and curtail untoward immune responses that have significantly hindered moving preclinical success into the clinic. With the increase in our understanding of AAV biology and the need to treat different muscle diseases using a tailored vector, capsid engineering certainly warrants dedicated research in the coming years.

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