Molecular Therapy
Review

Systemic AAV Micro-dystrophin Gene Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal muscle disease caused by dystrophin gene mutation. Conceptually, replacing the mutated gene with a normal one would cure the disease. However, this task has encountered significant challenges due to the enormous size of the gene and the distribution of muscle throughout the body. The former creates a hurdle for viral vector packaging and the latter begs for whole-body therapy. To address these obstacles, investigators have invented the highly abbreviated *micro-dystrophin* gene and developed body-wide systemic gene transfer with adeno-associated virus (AAV). Numerous microgene configurations and various AAV serotypes have been explored in animal models in many laboratories. Preclinical data suggests that intravascular AAV micro-dystrophin delivery can significantly ameliorate muscle pathology, enhance muscle force, and attenuate dystrophic cardiomyopathy in animals. Against this backdrop, several clinical trials have been initiated to test the safety and tolerability of this promising therapy in DMD patients. While these trials are not powered to reach a conclusion on clinical efficacy, findings will inform the field on the prospects of body-wide DMD therapy with a synthetic micro-dystrophin AAV vector. This review discusses the history, current status, and future directions of systemic AAV *micro-dystrophin* therapy.

Introduction

Duchenne muscular dystrophy (DMD) is an inherited X-linked recessive muscle-wasting disease caused by mutations in the *dystrophin* gene.[1,2] DMD affects approximately 1 in every 5,000 newborn boys.[3] Patients start to show symptoms of muscle weakness at 2 to 3 years of age.[4] Muscle function deteriorates rapidly at ~7 years of age.[5–7] Most patients lose ambulation at ~10–12 years of age and die in the second to third decade of their life due to diaphragm failure and/or cardiac complications.[4,8]

For a disease caused by mutation(s) in a single gene, a highly appealing therapy is to replace the diseased gene with a normal gene using gene therapy. Luxturna, an adeno-associated virus (AAV) gene therapy drug recently approved by the Food and Drug Administration (FDA) for treating Leber congenital amaurosis (LCA) provides an outstanding proof-of-concept for gene-replacement therapy.[9] LCA is a hereditary recessive blindness disease caused by mutations in the *retinal pigment epithelium-specific 65-kDa protein* (*RPE65*) gene. AAV delivery of a normal *RPE65* gene to the retina partially restores vision in patients.[10]

Tremendous efforts have been made over the last three decades to develop gene-replacement therapy for DMD. Although conceptually similar to that of AAV *RPE65* gene therapy for LCA, DMD gene-replacement therapy has turned out to be a much more challenging task. The first problem is the size of the gene. Unlike the *RPE65* gene, the *dystrophin* gene greatly exceeds the packaging capacity of the AAV vector. The second problem is the distribution of the diseased tissue. Unlike the retina, muscle spreads all over the body. Direct muscle injection offers limited benefits. Furthermore, the heart and the diaphragm, two muscles that are most important for the prognosis, are located deep inside the body and not readily accessible from the surface. *Micro-dystrophin* genes and systemic AAV delivery are developed to solve these problems.[11,12] Below, I review the progress and current status of preclinical and clinical development of AAV *micro-dystrophin* gene therapy for DMD. I also discuss unanswered questions and future research directions.

Preclinical Development of Systemic AAV Micro-dystrophin Gene Therapy

The history of AAV *micro-dystrophin* gene therapy can be traced back to the growth of two fascinating research disciplines, DMD genetics and AAV biology (Figure 1). Studies on the genetics of DMD not only resulted in the discovery of the *dystrophin* gene but also provided the rationale for treating DMD with an abbreviated gene. Studies on AAV biology open the door to the development the AAV vector, an enormously powerful viral vector for human gene therapy (Figure 2).[13]

DMD-like symptoms have been described in the literature since 1850.[2] The disease got its name following the publication of a clinical monograph in 1968 by French physician Duchenne de Boulogne.[14] The gene name was coined in 1987 by Louise Kunkel following the discovery of the gene and its protein product by the Kunkel

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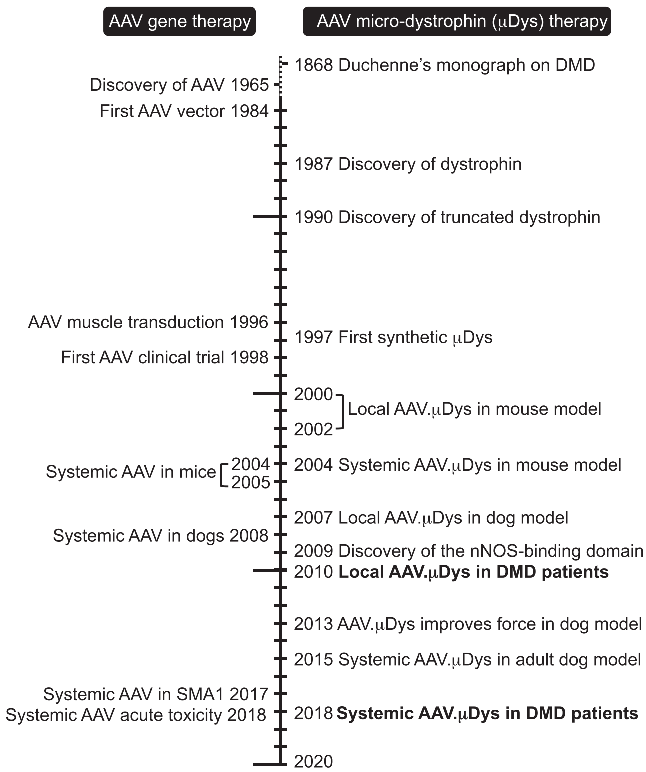

Figure 1. Historical Milestones in the Development of Systemic AAV Micro-dystrophin Gene Therapy

laboratory.[1,15,16] The full-length *dystrophin* gene is 2.6 mb. It encodes 79 exons. The 11.5-kb coding sequence translates into a 427-kD protein. Dystrophin can be divided into four major domains, including the N-terminal domain, rod domain, cysteine-rich domain, and C-terminal domain. The rod domain can be further divided into 24 spectrin-like repeats and four hinges (Figure 3).

The availability of the *dystrophin* coding sequence immediately raised hopes for curing DMD at its genetic root.[17] Indeed, *dystrophin* gene-replacement therapy has been on the very top of the research agenda ever since the discovery of the gene. Because the full-length *dystrophin* cDNA exceeds the packaging capacity of the first generation adenoviral and retroviral vectors, early proof-of-principle studies used simple intramuscular injection of plasmid DNA, which is an inefficient method for *in vivo* muscle gene therapy.[18] The discovery of the highly functional *Δ17–48 mini-dystrophin* protein by Kay Davies's laboratory in 1990[19] changed the situation. Despite being a half-size protein, it provided excellent muscle protection. One patient was ambulant at age 61, and another was a body builder at age 25.[19] The therapeutic potential of mini-dystrophin has since been confirmed in numerous genotype-phenotype correlation studies in human patients (Table 1).[20–29] The 6.2 kb *Δ17–48 minigene* was evaluated in a series of studies using adenoviral and retroviral vectors and in transgenic mice.[30] While encouraging results were achieved in animal models, these viral vectors have not been translated to DMD patients. Adenoviral vectors have strong immunotoxicity, while retroviral vectors are difficult to use for *in vivo* gene therapy. Notably, these vectors are ineffective for systemic delivery. DMD gene therapy was thus in need of more suitable vector systems.

AAV was discovered as a contaminating particle in an adenovirus stock in 1965 (Figures 1 and 2).[31] In 1984, the Nicholas Muzyczka laboratory built the first recombinant AAV virus for gene transfer[32]. In 1998, the first AAV gene therapy clinical trial was conducted in cystic fibrosis patients.[33] AAV has also been tested for muscle gene transfer. In contrast to adenovirus and retrovirus, direct muscle injection of the AAV vector produced high-level and persistent gene transfer in muscle.[34,35] Most importantly, a single intravenous delivery of AAV-6, -8, and -9 resulted in whole-body muscle transduction in rodents and large mammals.[36–38] These desirable features encouraged the development of AAV as a vector for muscle gene therapy.[39,40] However, there is a major hurdle to applying AAV for DMD gene therapy. The maximal packaging capacity of an AAV virus is 5 kb, smaller than the 6.2 kb *mini-dystrophin* gene.

The problem was addressed with the development of synthetic microgenes that are less than 4 kb. In 1997, Yuasa et al.[41] published the 3.7 kb *ΔDysM3* gene (Figure 3; Table 2). This microgene encodes the N-terminal domain, hinges 1 and 4, a single spectrin-like repeat, the cysteine-rich domain, and C-terminal domain. Unfortunately, the *ΔDysM3* gene did not reduce dystrophic phenotype in the mouse model.[42] The first series of protective AAV micro-dystrophin vectors were reported in 2000 and 2002 by the Xiao lab[43] and Chamberlain lab,[44] respectively (Table 2). The therapeutic potency of these microgenes has been significantly improved with subsequent modifications, in particular, codon optimization by the Dickson lab in 2008[45] and inclusion of the dystrophin spectrin-like repeats 16 and 17 (R16/17) neuronal nitric oxide synthase (nNOS)-binding domain by the Duan lab in 2009.[46] Hitherto, more than 30 different configurations of synthetic microgenes have been published (Table 2).[41,43,44,46–57] Among these, the *Δ3990-*, *ΔR4–23/ΔC-* (deletion of repeats 4 to 23 and deletion of the C-terminal domain) and *R16/17-containing micro-dystrophin* are particularly noteworthy given the extensive safety and efficacy data on these constructs in the murine and canine models. As exemplified in the representative data from two publications (Figure 4),[58,59] a large body of preclinical studies from many laboratories has now provided compelling evidence that AAV delivery of a rationally designed synthetic micro-dystrophin gene can significantly reduce muscle pathology, increase muscle force, enhance cardiac function, and prolong lifespan in animal models.[12,60]

The next critical achievement in the development of DMD gene therapy is vascular AAV delivery for body-wide therapy.[40] This is of utmost importance for DMD in order to significantly change the clinical course of the disease. Treating a single muscle or a group of muscles can only improve the function of the treated muscle. This may improve life quality (for example treating forearm flexors can help wheelchair-bound patients to grasp), but it will not slow down the progression of the disease and will not reduce mortality. Except

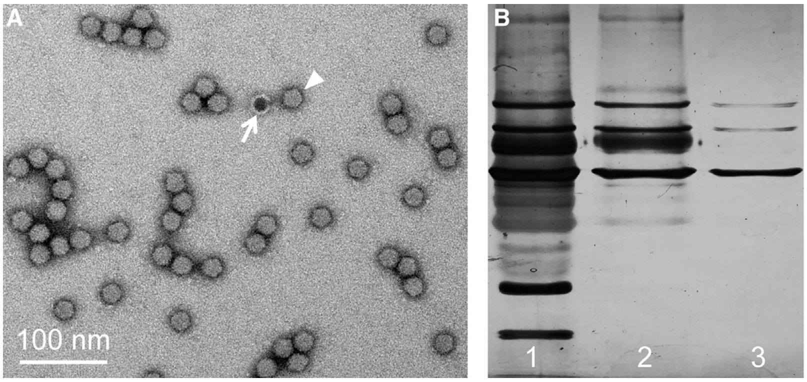

Figure 2. Adeno-associated Viral Vector

(A) A representative electron microscopic image of the AAV vector. Arrowhead, a fully packaged AAV particle. Arrow, an empty AAV particle. (B) Examination of AAV purity with SDS-PAGE silver staining. Lanes 1, 2, and 3 show a gradual increase of the purity after one, two, and three rounds of purification. The highly pure AAV stock has three viral proteins (VPs) at the ratio of VP1:VP2:VP2 ≈ 1:1:10.

for the extra-ocular muscle, essentially all body muscles undergo continuous deterioration in DMD. Diaphragm necrosis results in respiratory failure. Myocardial dystrophy leads to heart failure and sudden cardiac death. The breakthrough in systemic gene transfer occurred in 2004 and 2005 when the Chamberlain lab[36] and Xiao lab[37] showed effective whole-body muscle transduction in rodents with AAV serotype-6 and -8, respectively. In 2008, the Duan lab[38] demonstrated that systemic AAV transduction also worked in canines using AAV-9. Currently, systemic delivery has been achieved using a large collection of AAV capsids that are either isolated from nature or engineered in the laboratory.[40,61]

In parallel with the development of systemic AAV delivery technology, AAV *micro-dystrophin* vectors were tested in the mouse and dog DMD models.[12,60,62] In 2004, the Chamberlain lab[36] showed widespread systemic AAV *micro-dystrophin* transduction in muscles and an improvement of limb muscle function in young mdx mice, a commonly used mouse DMD model. However, young mdx mice are poor models because they do not show dystrophic symptoms.[62] To more stringently test systemic AAV therapy in clinically relevant models, experiments were conducted in a number of more severely affected mouse models, such as aged mdx mice, utrophin and dystrophin double knockout mice, and DBA/2J-mdx mice.[57,59,63–66] These studies have provided unequivocal evidence for body-wide improvement with systemic AAV *micro-dystrophin* therapy in the mouse model. Systemic AAV *micro-dystrophin* therapy in the canine DMD model was first performed in newborn puppies (Table 3).[67] Despite widespread *micro-dystrophin* expression in muscles throughout the body, treated puppies displayed weight loss and clinical signs of inflammatory myopathy.[67] In light of the concern on high-dose systemic AAV delivery to a dystrophic large mammal, regional perfusion was proposed as an intermediate step to derisk whole-body administration.[40] The technique was tested in dogs, non-human primates, and human patients. Results from these studies revealed several limitations, including (1) uncertainty on consistently delivering AAV to the intended limb, (2) failure to reach the heart and diaphragm, two most critical targets, and (3) safety concerns on applying a tourniquet to a DMD patient to achieve vascular escape of the vector. The first successful systemic AAV *micro-dystrophin* therapy in young adult dystrophic dogs was published in 2015 (Table 3).[68] The authors treated affected dogs at 2 months of age and observed body-wide muscle transduction and amelioration of muscle pathology.[68] A recent study further confirmed the safety and durability of systemic AAV *micro-dystrophin* therapy in juvenile affected dogs (Table 3).[69] Importantly, this study demonstrated a dose-dependent improvement in clinical score and dog gait. The safety, durability, histological, and functional amelioration, and the dose response of systemic AAV *micro-dystrophin* therapy were further confirmed in two additional studies (Table 3).[70–73]

Prelude to Systemic AAV *Micro-dystrophin* Gene Therapy Trials in DMD Patients

The first AAV *micro-dystrophin* gene therapy in human patients was initiated on March 28, 2006 by Jerry Mendell and colleagues at the Nationwide Children's Hospital in Columbus, Ohio USA[74] (Figure 5). In this study, a cytomegalovirus (CMV) promoter-driven *Δ3990* minigene cassette was packaged in AAV-2.5 and injected to the biceps of six 5- to 11-year-old patients (2×10^{10} vg/kg or 1×10^{11} vg/kg) (Figure 3; Tables 2 and 4).[74,75] Four hours before injection, all patients received methypredinisolone (2 mg/kg). A biopsy was performed at 42 or 90 days after injection. 3∼4 positive myofibers were detected in one low-dose patient at day 42, and one positive myofiber was detected in one high-dose patient at day 42 (Table 4).[74] No positive myofiber was detected in the remaining patients. Among these "non-responders," one showed T cell response to micro-dystrophin, another showed T cell response to revertant fibers (rare dystrophin positive fibers in patient muscle), and a third patient showed T cell response to AAV capsid (Table 4).[74,75] Collectively, the level of *micro-dystrophin* expression seen in this trial is far from sufficient for therapy. Several explanations have been suggested for the negative outcome in patients, such as immunity to new antigenic epitopes in micro-dystrophin, immunity to preexisting antigenic epitopes in revertant dystrophin, and immunity to viral capsid. The use of the ubiquitous (rather than the muscle-specific) promoter and failure to screen patients for preexisting anti-AAV antibodies may have also contributed to the immune response. Addressing these issues may likely lead to a successful trial in the future. In summary, this first in-patient study has provided critical insights for the design of future human trials, especially, in regards to AAV serotype, expression cassette, immune response, and patient selection.

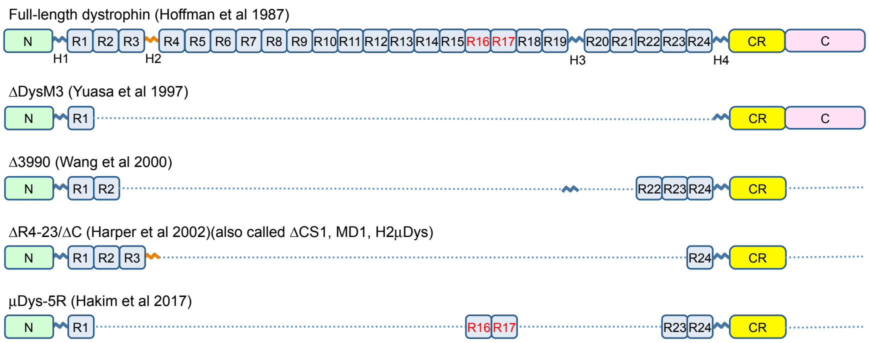

Figure 3. Full-Length Dystrophin and Representative Micro-dystrophins

Full-length dystrophin contains an N-terminal domain (N), 24 spectrin-like repeats (R1 to R24), four hinges (H1 to H4), a cysteine-rich domain (CR), and a C-terminal domain (CT). ΔDysM3 is the first synthetic micro-dystrophin. Δ3990, ΔR4–23/ΔC and μDys5R are three micro-dystrophins currently in use in clinical trials. H2 is marked in orange to indicate that it compromises micro-dystrophin function in the mouse DMD model (see Banks et al.[53] for details). R16 and R17 are marked in red to indicate that they are the nNOS-binding domain (see Lai et al.[46,98] for details).

Favorable results from a series of recently published high-dose systemic AAV therapy clinical trials have instilled enthusiasm for systemic *micro-dystrophin* therapy.[76–79] The first trial is for treating spinal muscular atrophy type 1 (SMA1). SMA1 is a motor neuron disease caused by mutations in the *survival motor neuron 1* (*SMN1*) gene. Patients die before 2 years of age. Jerry Mendell and colleagues[76] delivered a CAG promoter (CMV enhancer-chicken β-actin promoter)-driven *SMN1* gene cassette with AAV-9 intravenously to 15 patients (0.9- to 7.9-month-old) at the dose of 6.3×10^{13} vg/kg (three patients) and 2.0×10^{14} vg/kg (12 patients) (trial number NCT02122952). Gene therapy significantly changed the course of the disease and improved motor function. All patients were alive and event-free at 20 months of age. The longest survivor has reached 33 months of age and remained healthy. Two patients (one at low dose and one at high dose) had treatment-related severe adverse events (serum aminotransferase >30 times normal upper limit) and two patients showed non-serious adverse events (serum aminotransferase > normal upper limit but < 10 times). Nonetheless, none of these events was accompanied with abnormal liver function. Further, all events were controlled with prednisolone treatment. The unprecedented clinical success of the SMA1 trial suggests that systemic AAV therapy can be tolerated, is safe, and effective for treating certain neuromuscular diseases.

A second systemic AAV trial for neuromuscular diseases was initiated in August 2017 by Audentes Therapeutics to treat X-linked myotubular myopathy (XLMTM) (trial number NCT03199469). XLMTM is a fatal congenital muscle disorder caused by mutations in the *myotubularin* (*MTM1*) gene. Mortality reached 64% for patients ≤18 months and 31% for patients >18 months.[80] In this phase 1/2 (phases 1 and 2) open-label randomized trial, patients receive intravenous injection of an AAV-8 vector expressing the human *MTM1* gene from the muscle specific desmin promoter. Three doses (four patients for each dose) have been planned, including 1.0×10^{14} vg/kg, 3.0×10^{14} vg/kg, and 5.0×10^{14} vg/kg. Audentes Therapeutics recently released the data from the low-dose cohort.[77,78] All four patients (9 months to 4.1 years) tolerated AAV injection well. Two patients showed asymptomatic elevation in liver enzymes, and one patient had elevated troponin levels. All adverse events responded well to steroid treatment. Motor and respiratory function assay were clearly improved in patients that have received the therapy for 8 weeks or longer.[77,78]

Besides neuromuscular diseases, intravenous high-dose systemic AAV delivery has also been used to treat hemophilia A.[79] Seven adult hemophilia A patients were treated with 6.0×10^{13} vg/kg of an AAV-5 vector. Treatment resulted in significant and persistent clinical benefits for at least one year. Moderate asymptomatic elevation of transaminase was observed, but there was no clinical sequelae. The only serious adverse event was progression of preexisting arthropathy in one patient.[79]

In summary, these new results have raised the hope of treating inherited diseases with systemic AAV delivery. However, whether they can be translated to DMD patients remain to be tested due to the differences in disease nature, target tissue, transgene, and patient population.

Clinical Development of Systemic AAV Micro-dystrophin Gene Therapy

Preclinical data in the murine and canine DMD models, as well as promising findings from SMA1, XLMTM, and hemophilia A trials suggest that systemic AAV *micro-dystrophin* gene therapy may represent a viable approach to treat DMD. It is against this background that three clinical trials were initiated in DMD patients in the USA in December 2017 and one more trial has been planned in Europe (Table 5).[81] While all these trials aim to establish the safety and gene transfer efficiency of an AAV *micro-dystrophin* vector, there are important differences in the details with regards to AAV serotype and dose and methods of AAV production and purification, promoter, micro-dystrophin configuration, patient age, and gene mutation (Table 5). AAV-9 is used in trials by Solid Biosciences and Pfizer, and AAV-rh74 (a serotype very similar to AAV-8) is used in the Mendell trial (Nationwide Children's Hospital). AAV is produced with a scalable herpesvirus-based system, a scalable transient transfection system using suspension cell culture and the traditional transient transfection system using adherent cell culture in the Solid trial, Pfizer trial, and Mendell trial, respectively.[82,83] A muscle-specific promoter is used in all three trials. Specifically, the Solid trial uses the CK8 promoter,[57] the Mendell trial uses the MHCK7 promoter,[84]

Table 1. Dystrophin Large In-Frame Deletion and Clinical Phenotype

Genotype	% Lost	Level of Expression	Clinical Phenotype	Reference
Full-length	0%	+++	normal	16
Δ17–48	46%	+++	BMD	19
Δ13–47	47%	++ ∼+++	BMD	23
Δ10–44	48%	++	DMD	143
Δ10–44	48%	N/A	BMD	28
Δ10–44	48%	N/A	BMD	28
Δ13–48	49%	N/A	BMD	27
Δ13–48	49%	++ ∼+++	BMD	24
Δ13–48	49%	++	BMD	26
Δ4–41	50%	+	DMD	24
Δ4–41	50%	++	DMD	145
Δ4–41	50%	–	DMD	26
Δ3–41	51%	++	DMD	26
Δ3–41	51%	++	IMD	144
Δ3–41	51%	+	DMD	143
Δ3–42	52%	+	IMD	24
Δ11–48	52%	N/A	DMD	142
Δ5–44	54%	N/A	DMD	28
Δ10–53	60%	N/A	DMD	25
Δ10–53	60%	+++	DMD	141
Δ14–60	61%	N/A	DMD	140
Δ2–50 (Δ2–44)[a]	63%	++	DMD	139

Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; IMD, intermediate muscular dystrophy (clinical phenotype between BMD and DMD); N/A, information not available.
[a]The patient has a Δ2–44 deletion in DNA but a Δ2–50 deletion in mRNA due to alternative splicing.

and the Pfizer trial uses a minimized *murine muscle creatine kinase* (*MCK*) promoter.[85] The Solid trial and Pfizer trial have a dose-escalation design. The Mendell trial has only one dose. Ambulatory patients will be recruited in all trials. In addition, the Solid trial will recruit non-ambulatory patients and the Mendell trial will recruit infant patients (Table 5). The Solid trial and Pfizer trial are open to all patients irrespective of mutation. The Mendell trial only takes patients who have frameshift or nonsense mutation within exons 18–58 (Table 5). The rational for restricting patients to a particular mutation region is not explicitly stated in the trial protocol published in Clinicaltrials.gov (NCT03375164). But it may likely relate to the configuration of the microgene construct proposed in this trial.

A major difference among these trials is the configuration of the particular microgene used in the trial (Figure 3). At least three different microgenes have been proposed. These include a five-repeat microgene (*Δ3990*), a four-repeat microgene (*ΔR4–23/ΔC*) and a different five-repeat microgene (μDys5R) (Figure 3).[43,44,57] The common features of these micro-dystrophins are the presence of the N-terminal domain, the cysteine-rich domain, spectrin-like repeats 1 and 24, hinges 1 and 4, as well as the absence of the C-terminal domain (Figure 3). The differences are in the central hinges and R16/17 nNOS-binding domain. Δ3990 micro-dystrophin contains hinge 3 and ΔR4–23/ΔC micro-dystrophin contains hinge 2. μDys5R micro-dystrophin has no central hinge. Only μDys5R micro-dystrophin carries the R16/17 nNOS-binding domain (Figure 3).

It is worth pointing out that the differences in the rod domain of the microgene may have important clinical implications. Of particular interests are hinge 2 and R16/17. Banks et al.[53] found that the polyproline site in hinge 2 profoundly influenced the functional capacity of micro-dystrophin in the mouse model. Specifically, it altered the normal structure of the muscle tendentious junction and neuromuscular junction, reduced myofiber size, and resulted in the formation of abnormal ring-shaped myofibers in some muscles.[53]

Dystrophin accomplishes its biological function through assembly of the dystrophin-associated glycoprotein complex (DGC). A pivotal DGC component in skeletal muscle is nNOS.[86,87] nNOS is involved in a number of muscle activities, including metabolism, regeneration, mitochondrial biogenesis, muscle perfusion, fatigue, and atrophy.[88] During contraction, elevated cytosolic calcium activates sarcolemmal nNOS to produce nitric oxide (NO). Diffusion of NO to the surrounding vasculatures counteracts sympathetic vasoconstriction and hence allows sufficient blood perfusion in working muscle (Figure 6). In DMD, nNOS is delocalized from the sarcolemma.[86,87] This has two consequences. First it compromises the ability of muscle to counteract functional ischemia and hence leads to focal ischemic damage (Figure 6).[46,89–91] In fact, focal ischemic muscle injury is not only the first observable lesions on histological examination in affected dogs and DMD patients (Figure 6),[92] but also a characteristic feature that distinguishes DMD from other types of muscular dystrophy.[92] Second, mislocalized nNOS elicits nitrosative stress, which directly compromises force production in dystrophic muscle.[93,94] Becker muscular dystrophy (BMD) is a mild form of DMD caused by in-frame deletion of the dystrophin gene. To study the clinical consequence of sarcolemmal nNOS delocalization, Gentil et al.[94] performed a genotype-phenotype correlation study in BMD patients. The authors found that patients with sarcolemmal nNOS consistently showed much milder clinical manifestations.[94] Collectively, sarcolemmal nNOS plays an essential role in the initiation and progression of muscle disease in DMD. Restoration of nNOS homeostasis should be considered in dystrophin replacement therapy.[95]

The molecular mechanism underlying dystrophin-mediated nNOS membrane localization has been perplexing. Early studies suggest that nNOS is recruited to the sarcolemma by the dystrophin C-terminal domain via interaction with syntrophin.[96] Surprisingly, we found that the presence of the dystrophin C-terminal domain and/or membrane-associated syntrophin is not sufficient to anchor nNOS to the sarcolemma.[97] Our recent studies suggest that dystrophin spectrin-like repeats 16 and 17 are the nNOS-binding domain.[46] Specifically, alpha-helices 2 and 3 from R16 and R17 frame a 10-residue peptide in the α-helix 1 of R17 for direct interaction with the groove region

Table 2. Structural and Functional Features of Micro-dystrophins Developed between 1997 and 2017

Year	Name	Other Name	MW	% Lost	NT	Rod (Hinges)	Rod (Repeats)	CR	CT	Pathology	Force	Comments	Reference
1997	ΔDysM3	M3	125	71%	Yes	2 (H1, H4)	2 (R1,[a] R24)	Yes	Yes	reduced	no improvement	this is different from M3 reported in 2009	41
1998	ΔDysH1		103	76%	Yes	1 (H1)	0	Yes	Yes	N/A	N/A		47
1998	ΔDysH4		103	76%	Yes	1 (H4)	0	Yes	Yes	N/A	N/A		47
1998	ΔDysAH3		138	68%	Yes	2 (H1, H4)	3 (R1, R2,[a] R24)	Yes	Yes	N/A	N/A		47
1998	ΔDysAX2		150	65%	Yes	2 (H1, H4)	4 (R1, R2, R3,[a] R24)	Yes	Yes	N/A	N/A		47
1998	ΔDysAX11	AX11	150	65%	Yes	2 (H1, H4)	4 (R1, R2,[a] R23, R24)	Yes	Yes	reduced	no improvement		47
2000	Δ3849		∼130	70%	Yes	2 (H1, H4)	5 (R1, R2, R22, R23, R24)	Yes	No	reduced	improved		43
2000	Δ3990		∼140	67%	Yes	3 (H1, H3, H4)	5 (R1, R2, R22, R23, R24)	Yes	No	reduced	improved	clinical trial candidate	43
2000	Δ4173		∼140	67%	Yes	2 (H1, H4)	6 (R1, R2, R3, R22, R23, R24)	Yes	No	reduced	N/A		43
2002	ΔR1–R24		108	75%	Yes	2 (H1, H4)	0	Yes	Yes	no imprоment	N/A		44
2002	ΔR4–R23/ΔCT	ΔCS1, MD1, H2μDys	138	68%	Yes	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	improved	clinical trial candidate	44
2002	ΔR4–R23	CS1	167	61%	Yes	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	Yes	reduced	improved		44
2002	ΔR2–R21		165	61%	Yes	2 (H1, H4)	4 (R1, R22, R23, R24)	Yes	Yes	reduced	improved		44
2002	ΔR2–R21+H3		169	60%	Yes	3 (H1, H3, H4)	4 (R1, R22, R23, R24)	Yes	Yes	reduced	improved		44
2002	Δ3788	ΔAB/ΔR3–18/ΔCT	144	66%	Yes[a]	3 (H1, H3, H4)	8 (R1, R2, R19, R20, R21 R22, R23, R24)	Yes	No	reduced	improved		48
2002	CS1		165	61%	Yes	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	Yes	reduced	improved		49
2004	ΔCS1	ΔR4–R23/ΔCT, MD1, H2μDys	138	68%	Yes	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	improved	clinical trial candidate	50
2007	ABS1,2μDys		N/A	>60%	Yes[a]	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	no imprоment		51
2007	ABS1μDys		N/A	>60%	Yes[a]	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	no imprоment		51
2009	ΔR2–15/ΔR18–23/ΔC	R16–17/ΔC, YL90	132	69%	Yes	2 (H1, H4)	4 (R1, R16, R17, R24)	Yes	No	reduced	improved		46
2009	ΔR3–15/ΔR18–23/ΔC	YL93	144	66%	Yes	2 (H1, H4)	5 (R1, R2, R16, R17, R24)	Yes	No	reduced	improved		46
2009	ΔR3–15/ΔR17–23/ΔC	YL113	132	69%	Yes	2 (H1, H4)	4 (R1, R2, R16, R24)	Yes	No	N/A	N/A		46
2009	M1		127	73%	Yes	2 (H1, H4)	1.5 (R23,[a] R24)	Yes	Yes	reduced	N/A		52
2009	M2		125	71%	Yes	2 (H1, H4)	1.5 (R1, R24[a])	Yes	Yes	reduced	improved	no force increase in similar constructs (ΔDysM3, AX11)	52
2009	M3		125	71%	Yes	2 (H1, H4)	1 (R24)	Yes	Yes	reduced	N/A	this is different from M3 reported in 1997	52
2009	M4		115	70%	Yes	2 (H1, H4)	0.5 (R24[a])	Yes	Yes	reduced	N/A		52
2010	ΔH2-R24/ΔCT		N/A	>60%	Yes	2 (H1, H4)	3 (R1, R2, R3)	Yes	No	reduced	N/A		53

(Continued on next page)

Table 2. Continued

Year	Name	Other Name	MW	% Lost	NT	Rod (Hinges)	Rod (Repeats)	CR	CT	Pathology	Force	Comments	Reference
2010	ΔH2-R23+H3/ΔCT	H3μDys	N/A	>60%	Yes	3 (H1, H3, H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	improved	H2 is bad	53
2010	ΔR2–R23+R18-H3/ΔCT		N/A	>60%	Yes	3 (H1, H3, H4)	4 (R1, R18, R19, R24)	Yes	No	reduced	N/A		53
2010	ΔR4–R23/ΔCT/ΔpolyP		N/A	>60%	Yes	3 (H1, H2,[a] H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	N/A	polyproline site in H2 is bad	53
2011	MD1	ΔR4–R23/ΔCT, ΔCS1, H2μDys	138	68%	Yes	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	improved	clinical trial candidate	54
2011	MD2		154	64%	Yes	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	Yes[a]	reduced	improved		54
2011	ΔCS2		140	67%	Yes	3 (H1, H2, H4)	4 (R1, R2, R3, R24)	Yes	No	reduced	improved	ΔCS1+Dys2 epitope	55
2012	R16–17/H3/ΔC	ΔR2–15/ΔR18–19/Δ20–23/ΔC, YL196	133	69%	Yes	3 (H1, H3, H4)	4 (R1, R16, R17, R24)	Yes	No	reduced	improved		56
2017	mDys5R	ΔR2–15/ΔR18–19/Δ20–22/ΔC, XP42, μDys5	147	66%	Yes	2 (H1, H4)	5 (R1, R16, R17, R23, R24)	Yes	No	reduced	improved	clinical trial candidate	57

MW, molecular weight (in kD); N/A, information not available; Yes, The domain is present in the construct; No, The domain is absent in the construct.
[a]The domain is truncated.

of the nNOS post synaptic density protein, Drosophila disc large tumor suppressor and zonula occludens-1 protein (PDZ) domain.[98] R16/17-containing synthetic dystrophin genes successfully restored sarcolemmal nNOS expression in the murine and canine models, effectively enhanced muscle perfusion, prevented functional ischemia, and significantly improved muscle force and exercise capacity.[46,57,58,68,99,100] In a nutshell, R16/17-containing micro-dystrophin offers much better protection than those without this domain in animal models.

Safety and Immune Response to High-Dose Systemic AAV Administration

As the pace of DMD gene therapy continues to accelerate, it is important to reflect on the lessons learned from patients and animal models. There is no doubt that the most important question is whether systemic AAV micro-dystrophin therapy can be tolerated and whether it is safe in human patients. Systemic AAV delivery has been performed in numerous studies at various dose ranges in mouse models of neuromuscular diseases.[40] No safety concern has arisen in these studies. However, toxic responses have been noted when high-dose ($\geq 7.5 \times 10^{13}$ vg/kg) AAV was delivered intravenously in large mammals. Kornegay et al.[67] delivered an AAV-9 vector to three 4-day-old dystrophin null dog puppies at the dose of 1.5×10^{14} vg/kg. This vector expressed a human *micro-dystrophin* gene from the ubiquitous CMV promoter. One puppy showed persistent lethargy and was euthanized 9 days later (Table 6). Congenital liver steatosis found at necropsy was thought to be the culprit. The remaining two puppies developed muscle atrophy and contracture resembling clinical manifestations of inflammatory myopathy. These two puppies were euthanized at 16 weeks of age (Table 6). Blood chemistries were unremarkable. $CD4^+$ and $CD8^+$ T cells were not observed in micro-dystrophin-positive muscle, suggesting the observed toxicity was likely due to an innate immune response rather than the T cell response.[67] Two recent studies from the Wilson laboratory[101,102] further cautioned potential toxicity of high-dose systemic AAV delivery in large-animal models. In one study, Hordeaux et al.[102] delivered 7.5×10^{13} vg/kg vectors to two 4-year-old rhesus macaques, one with AAV-9 and the other with an AAV-9 variant called AAV-PHP.B (Table 6). The vector expressed the GFP gene from the ubiquitous CB7 promoter. The subject injected with AAV-PHP.B developed acute liver toxicity and thrombocytopenia on day 3 and was euthanized on day 5 due to diffuse hemorrhage. The AAV-9 injected subject also showed liver enzyme elevation on day 3 (Table 6).[102] In another study, Hinderer et al.[101] delivered a different AAV-9 variant called AAV-hu68 to three 14-month-old rhesus macaques and three 3- to 30-day-old piglets at the dose of 2×10^{14} vg/kg. The vector expressed the human *SMN1* gene from the ubiquitous CB7 promoter. Of three macaques, one developed acute liver failure and was euthanized on day 5 due to disseminated intravascular coagulation (DIC). This animal also had marked elevation of inflammatory cytokines on day 4. The remaining two animals showed liver enzyme elevation and thrombocytopenia on day 5. Histological evidence of sensory neuron toxicity was observed at the scheduled necropsy on day 28. Three piglets did not show liver enzyme

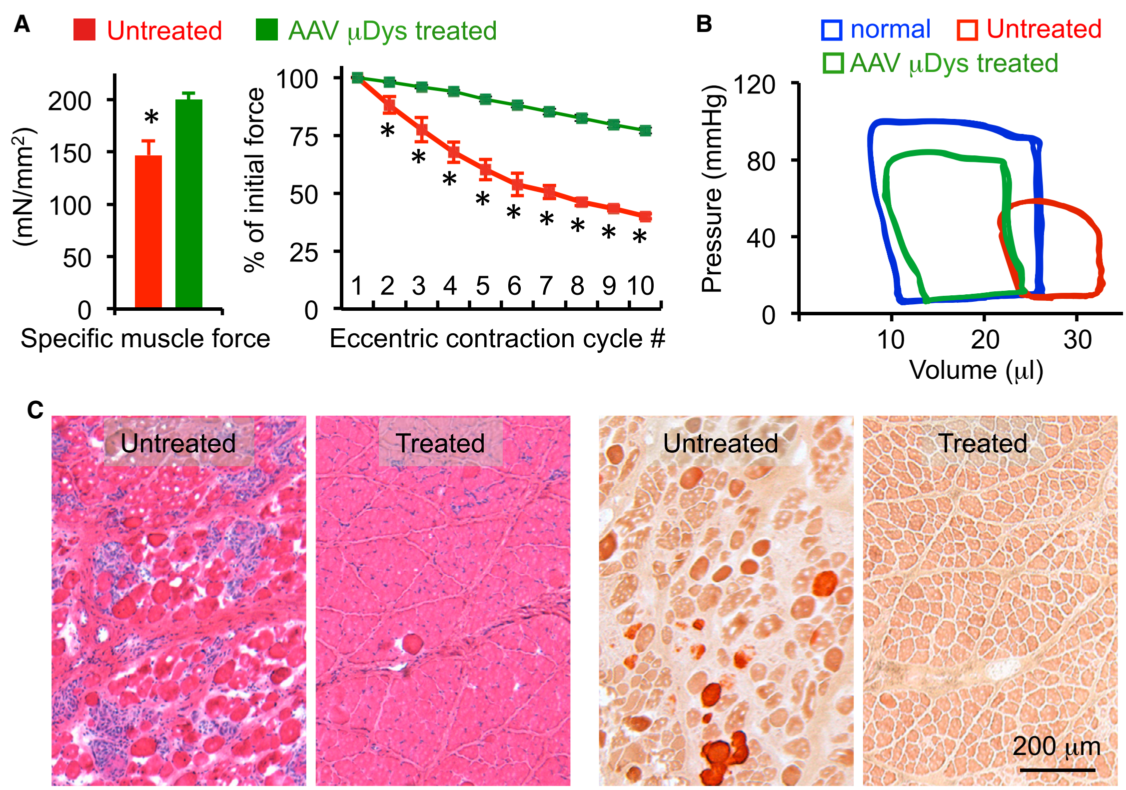

Figure 4. AAV Micro-dystrophin Gene Therapy Ameliorated Muscle Disease in the Murine and Canine DMD Models

(A) Systemic AAV micro-dystrophin injection improved skeletal muscle function in mdx mice. Treatment significantly improved specific tetanic force and resistance to eccentric contraction-induced force drop in the extensor digitorum longus muscle (see Shin et al.[58] for details). Error bar, mean ± SEM. (B) Systemic AAV micro-dystrophin injection improved cardiac hemodynamics in mdx mice (see Bostick et al.[59] for details). (C) AAV micro-dystrophin therapy improved histology (left) and reduced pathological muscle calcification (right) in the extensor carp ulnaris muscle in affected dogs (see Shin et al.[58] for details).

elevation, but all showed signs of sensory neuron toxicity within 2 weeks after injection and were euthanized on days 13–14.[101] In light of the acute onset of the toxicity and lack of strong evidence supporting a cellular immune response, the authors reasoned that the activation of innate immune response might be responsible for the observed liver toxicity and coagulopathy in nonhuman primates. The cause of sensory neuropathy remained unclear. Collectively, all three papers point to the activation of the innate immune response as an important concern for high-dose systemic AAV gene therapy.[103–105]

The innate immune response is a well-recognized barrier for adenovirus, but not AAV, gene therapy.[106,107] Indeed, AAV vectors are much weaker than adenoviral vectors in activating genes involved in the innate immune response.[108,109] However, recent studies suggest that the innate immune response may likely play an important role in shaping the outcome of AAV gene therapy.[103,105,110,111] AAV capsids and the vector genome can be sensed by toll-like-receptor-2 and -9, respectively.[112,113] Zaiss et al.[108] found a dose-dependent transient induction of chemokine expression at 1 hr following intravenous injection of AAV-2. Importantly, the level of induction was comparable to that of adenovirus at this time point. Martino et al.[114] reported a transient but profound induction of pro-inflammatory cytokines at 2 hr following intravascular injection of self-complementary AAV-2 and -8. The authors also showed that higher doses resulted in significantly stronger induction.[114] These results suggest that high-dose systemic AAV delivery faces a unique innate immunity challenge that is absent or marginal in low-dose intravascular AAV administration.

Recently, Solid Biosciences announced the hold of its clinical trial due to suspected unexpected serious adverse reaction.[115,116] Specifically, 5×10^{13} vg/kg of an AAV-9 *micro-dystrophin* vector was injected intravenously to a non-ambulatory adolescent DMD patient. Several days later, the patient showed platelet count reduction, followed by red blood cell count reduction and transient renal impairment. There was also evidence of complement activation. Nevertheless, there were no signs of bleeding or clotting abnormalities. The liver function was not altered from the baseline either. The patient recovered from the event smoothly, and the trial was resumed.[116] The exact reason(s) and/or trigger(s) of this unexpected response are yet to be investigated. However, the rapid onset of the reaction (within days) is reminiscent of the time course observed in nonhuman primates by the Wilson laboratory,[101,102] suggesting innate immune response may have played a role. This notion is further supported by the observation of complement activation in the patient.[115] The complement system

Table 3. Systemic AAV Micro-dystrophin Study in the Canine DMD Model

References	Injection Age (Months)	Sample Size	AAV Serotype	AAV Dose ($\times 10^{14}$ vg/kg)	Expression Cassette		Follow-up (Months)	μ-Dystrophin Expression		Disease Amelioration
					Promoter	Transgene		Bodywide	Persistent	
[67]	0.13	3	AAV-9	1.5	CMV	human m-Dys	4	confirmed	confirmed	N/A
[68]	1.8	2	Y731F AAV-9	5.0, 6.2	CMV	canine m-Dys	4	confirmed	confirmed	improvement in muscle histology
[69]	2∼2.5	8	AAV-8	0.2, 1.0	Spc5-12	canine m-Dys	6.5∼24	N/A	confirmed	improvement with clinical score and gait in 1.0 $\times 10^{14}$ vg/kg group
[71,72]	2.5∼3.5	5	AAV-9	0.5, 1.0, 3.0	CK8	canine m-Dys	8, 30	confirmed	confirmed	improvement in muscle histology and force
[70]	3	9	AAV-9	0.1, 1.0, 2.0	CK8	canine m-Dys	3	confirmed	confirmed	dose-dependent improvement in muscle histology and function

is a primary component of innate immunity. An *in vitro* study suggests that AAV capsid not only interacted with various components of the complement system, but also directly activated the complement system in a dose-dependent manner.[117] Alternatively, the complement system can also be activated through the classic pathway by the immune complex formed from anti-AAV capsid antibodies.[102,118] Activated complement not only promotes inflammation but also damages cells that are in constant contact with plasma such as red blood cells, platelets, and endothelial cells.[118,119] Injured endothelium, activated platelets, and hemoglobin released from red blood cells can in turn further activate the complement system.[120–123] If unchecked, this malicious feedback cycle may lead to severe thrombocytopenia and anemia, organ injury, bleeding, and death. Together, data from nonhuman primates and a DMD patient indicate that careful monitoring and management of the innate immune response should be included in the protocol in ongoing systemic micro-dystrophin gene therapy trials.

Despite the concern on acute and/or sub-acute toxicity of the innate immune response, it should be pointed out that high-dose systemic AAV delivery remains a viable and highly promising therapy to improve all affected muscles in DMD patients. This positive attitude is backed up by (1) safety and efficacy data from high-dose systemic AAV therapy in SMA1, XLMTM, and hemophilia A patients,[76–79] (2) lack of toxicity in young adult DMD dogs at dose as high as 5×10^{14} vg/kg,[68–73] (3) encouraging preliminary results in DMD boys who have already received systemic AAV *micro-dystrophin* gene therapy.[124–126] Clearly, a dystrophic body and a high AAV dose are not sufficient to induce systemic toxicity within days after injection in large mammals. A careful comparison of the protocols from different studies should give a hint on the cause(s) and solution(s) to this important safety concern.

In contrast to innate immunity, the adaptive immune response is more frequently discussed in AAV gene therapy.[127–132] T cell response to AAV capsid has been reported in hemophilia B trials that have used a relatively low dose of the AAV vector ($<1 \times 10^{13}$ vg/kg).[133–135] In these cases, the loss of transgene expression is often accompanied by an elevation of transaminase.[127,131] Transient application of high-dose glucocorticosteroids has been shown to effectively control liver enzyme and maintain expression of the therapeutic protein (factor IX).[134] Transaminase elevation has also been observed in high-dose systemic AAV gene therapy for SMA1, XLMTM, and hemophilia A.[76–79] However, it is currently unclear whether liver enzyme elevation is caused by T cell immunity to the AAV vector in these studies. Besides viral capsid, the cellular immune response may also target transgene product expressed from the AAV vector. The anti-dystrophin T cell response was highlighted in the local injection trial (Table 4).[74] Interestingly, using the enzyme-linked immunospot assay, Flanigan et al.[136] revealed a high prevalence of anti-dystrophin T cell immunity in DMD patients (up to 53% in corticosteroid naive patients). The implication of this finding to systemic AAV *micro-dystrophin* therapy is unclear but should be carefully investigated in the future.

The humoral response is a hurdle for patients with preexisting AAV neutralizing and/or binding antibodies.[132,137] Patients with high titers are usually excluded from the trial (Table 4). For patients that have received AAV therapy, the antibody response from initial exposure creates a barrier for re-administration. Considering the degenerative nature of the disease, it is very likely DMD patients may have to receive repeated therapy. Several strategies are currently under development to overcome the humoral response, such as plasmapheresis, AAV capsid engineering, sheltering capsid with encapsulation, the use of decoy empty capsid, and pharmacological modulation of the B cell and/or T cell activation.[129,132,137,138] Each of these methods has its advantages and shortcomings. Ultimately, a combinatorial approach may be required to overcome this important challenge.

Micro-dystrophin Structure Optimization

An equally important question is whether micro-dystrophin can alleviate disease in human patients. The concept of treating DMD with an abbreviated gene originates from observations that patients with large in-frame deletions display a mild clinical course[19] (Table 1). However, it should be noted that in these cases, only $\leq$49% the dystrophin protein is lost.[19,23,24,26–28,139–145] In other words, the patients still carry a half-size protein. Patients who have lost $\geq$50% of dystrophin due to larger in-frame deletions often show the severe DMD clinical manifestation rather than the milder BMD phenotype (Table 1).[24–26,139–145] In 1996, Fanin et al.[141] proposed the dystrophin

Figure 5. First AAV Micro-dystrophin Clinical Trial
Direct injection of the AAV vector to the biceps of a patient by Dr. Jerry Mendell (asterisk). The injection was assisted by an interventional radiologist (triangle) and a neurologist (square). The radiologist and the neurologist guided and monitored the injection process with ultrasound and electromyography, respectively, to make sure AAV was delivered into viable muscle (see Mendell et al.[74] for details).

length threshold theory. The authors presented a case in which a high level of an ∼160-kD truncated dystrophin protein was detected on immunostaining and western blot using an antibody that recognizes the C-terminal domain (Table 1). Despite the abundant presence of this micro-size dystrophin, the patient displayed a clinical phenotype of DMD instead of BMD. After reviewing a large collection of in-frame deletion patients, they found that smaller dystrophins (deletion larger than 36 exons) were always associated with a severe phenotype. They hypothesized that a dystrophin protein of at least 200 kD might be needed for muscle protection.[141] Mouse studies suggest that there may indeed exist a length threshold (∼150 kD instead of 200 kD in mouse muscle). For example, the highly truncated ΔDysM3 and ΔR1–R24 micro-dystrophins cannot reduce dystrophic phenotypes in mice (Table 2).[42,44] It is currently unclear where the line should be drawn for the length threshold in human patients. Nevertheless, the existing AAV *micro-dystrophin* literature in the murine and canine models suggests that a rationally designed micro-dystrophin can certainly protect mice and dogs. Ongoing trials should offer critical clinical efficacy data regarding the effectiveness of micro-dystrophin in human patients (Table 5).

Besides the length, the configuration of the microgene is also of paramount importance. Among three candidate micro-dystrophins (Figure 3), two contain five repeats and one has four repeats. A side-by-side comparison of the five-repeat microgene versus the four-repeat microgene has yet to be published. Harper et al.[44] previously demonstrated that the rod domain functions better when it has an even (rather than odd) number of repeats. This is in agreement with the evolution of spectrin family proteins, which are stemmed from a common four-repeat ancestor.[146,147] However, a counter-argument in support of the five-repeat microgene is that a larger micro-dystrophin would carry more genetic information.

Another complexity is the central hinge. Two micro-dystrophins used in clinical trials have a hinge in the middle, and one has no hinge (Figure 3). The question is whether a central hinge should be included in micro-dystrophin and, if yes, which hinge should be included. So far, a side-by-side comparison has not been performed using AAV micro-dystrophin vectors. However, we may gain some hints from a transgenic study.[44] Harper et al.[44] compared two transgenic mdx lines: one expressed the *ΔR2–R21* microgene (without a central hinge), and the other expressed the *ΔR2-R21+H3* microgene (with a central hinge). Although the ΔR2-R21 line expressed less dystrophin, this line outperformed the ΔR2–R21+H3 line on histological and function assays. Specifically, the ΔR2–R21 line showed less degeneration and produced a higher force. This result suggests that in the context of micro-dystrophin, a construct without a central hinge may function better. However, the result may also suggest that hinge 3 negatively influences micro-dystrophin function. A study in patients seems to support this second view. Carsana et al. examined 108 unrelated DMD and BMD patients and found that in-frame deletion of hinge 3 yielded significantly much milder disease.[142] They concluded that "when hinge 3 is lost, the protein, though shorter, is able to function better."[142] But it should be pointed out that evidence also exists suggesting inclusion of hinge 3 may lead to better rescue in the context of a larger minigene. Specifically, Harper et al.[44] showed that the hinge 3-containg *ΔH2-R19* minigene was much more effective in ameliorating muscle pathology and boosting muscle force than a similar minigene (*ΔH2–H3* minigene) that is identical to the *ΔH2-R19* minigene except without hinge 3. While the jury is still out on whether hinge 3 should be included, results from Banks

Table 4. First AAV Micro-dystrophin Clinical Trial (Local Injection)

Patient	Dose (vg/kg)	Cortico Steroid	Pre-Nab to AAV	Positive Myofiber	AAV Genome	CTL to m-Dystrophin	CTL to Revertant Fiber	CTL to AAV Capsid
1	2×10^{10}	yes			+			+
2	2×10^{10}	yes	1:800				+	±
3	2×10^{10}	no		3∼4 (day 42)	+			±
4	1×10^{11}	no						±
5	1×10^{11}	yes	1:100		++	+		
6	1×10^{11}	yes		1 (day 42)	++			

AAV genome: +, 0.5∼1 copy/nucleus; ++, 1.5∼3 copies/nucleus. Abbreviations: CTL, cytotoxic T cell response; Pre-Nab, preexisting neutralizing antibody.

Table 5. Overview of Systemic AAV Micro-dystrophin Clinical Trials

	Solid Biosciences	Nationwide Children's Hospital	Pfizer
ClinicalTrials.gov Identifier	NCT03368742	NCT03375164	NCT03362502
Trial name	microdystrophin gene transfer study in adolescents and children with DMD (IGNITE DMD)	systemic gene delivery clinical trials for Duchenne muscular dystrophy	a study to evaluate the safety and tolerability of PF-06939926 gene therapy in Duchenne muscular dystrophy
Start date (actual)	December 6, 2017	December 11, 2017	January 23, 2018
Completion date (estimated)	March, 2021	January, 2021	June 7, 2024
Location	University of Florida	Nationwide Children's Hospital and Washington University	Duke University, UCLA and University of Utah
Responsible party	Solid Biosciences	Jerry R. Mendell	Pfizer
Study nature	phase 1 and 2, open-label, randomized, controlled	phase 1 and 2, open-label, non-randomized	phase 1b, open-label, non-randomized
Drug name	SGT-001	rAAVrh74.MHCK7.Micro-dystrophin	PF-06939926
AAV-serotype	AAV-9	AAV-rh74	AAV-9
Dose	3 doses (start at 5×10^{13} vg/kg)	1 dose (2×10^{14} vg/kg)	2 doses
Patient number	16	12	up to 12
Patient age	4 to 17 years	3 months to 7 years	5 to 12 years
Disease stage	both ambulatory and non-ambulatory	ambulatory only	ambulatory only
Corticosteroid use	daily for $\geq$2 years	no prior use for $\leq$3 years old; >12 weeks use for $\geq$4 years old	$\geq$6 month use and $\geq$3 month daily use
Dystrophin gene mutation	any mutation	Frameshift or nonsense mutation within exons 18-58	any mutation
Pre-Nab to AAV	negative	$\leq$1:400	negative
Primary outcome	safety and micro-dystrophin expression in biopsy	safety	safety and tolerability
Secondary outcome		micro-dystrophin expression in biopsy and motor function	micro-dystrophin expression in biopsy

et al.[53] have made it clear that hinge 2 should be excluded because it impairs normal muscle structure.

In above discussions, I touched on the issues related to the length of *micro-dystrophin*, the number of repeats, and the central hinge. However, it should be kept in mind that dystrophin is not a simple protein; it interacts with many different cellular proteins such as γ-actin (in both skeletal muscle and heart) and α-actin (in heart only), various types of intermediate filaments (e.g., keratin 8 and 19, synemin, and synemin 2), tubulin (microtubule), ankyrin, myospryn, plectin, dystrobrevin, syntrophin, nNOS, dystroglycan, polarity-regulating kinase partitioning-defective 1b, cavin-1, ahnak1, cipher, and crystalline αB. Dystrophin also interacts with the membrane lipid bilayer. It is unlikely that a highly truncated micro-dystrophin will establish all these interactions, especially in light of the fact that the region(s) that bind to some of the above proteins remain to be identified. For the development of next-generation *micro-dystrophin*, the question is to determine which interactions are absolutely required for dystrophin function and which ones are less essential.

Alone the same line, recent studies have revealed many previously unappreciated or not fully appreciated aspects of dystrophin biology. Of particular interest is the recent discovery of new dystrophin membrane-binding domains.[148] It is well established that dystrophin binds to the sarcolemma via its cysteine-rich domain. However, there also exists evidence suggesting that truncated dystrophins (either naturally occurring or synthetic) may directly bind to the sarcolemma in the absence of the cysteine-rich domain. To clarify this issue, we conducted a comprehensive *in vivo* screening in both mouse and dog models.[148] Our data suggest that in addition to the cysteine-rich domain, dystrophin indeed contains additional membrane-binding domains that can independently anchor to the sarcolemma in skeletal muscle and the heart.[148] Since sarcolemmal interaction is essential for dystrophin to protect muscle, it is likely that inclusion of more membrane-binding domains may result in better muscle protection. In support, a recent transgenic study found better resistance to eccentric contraction injury for micro-dystrophin with two, rather than one, membrane-binding domains.[149]

The C-terminal domain (exons 71–79) has a length of 975 bp and translates into 325 amino acid residues (~36 kD). It contains the α1-and β1-syntrophin binding site and α-dystrobrevin binding site. These binding sites span exons 73 to 75 (450 bp). The C-terminal domain is absent in all candidate microgenes proposed for clinical trials (Figure 3). The decision to remove the C-terminal domain originates from a study by Crawford et al.[150] The authors created a

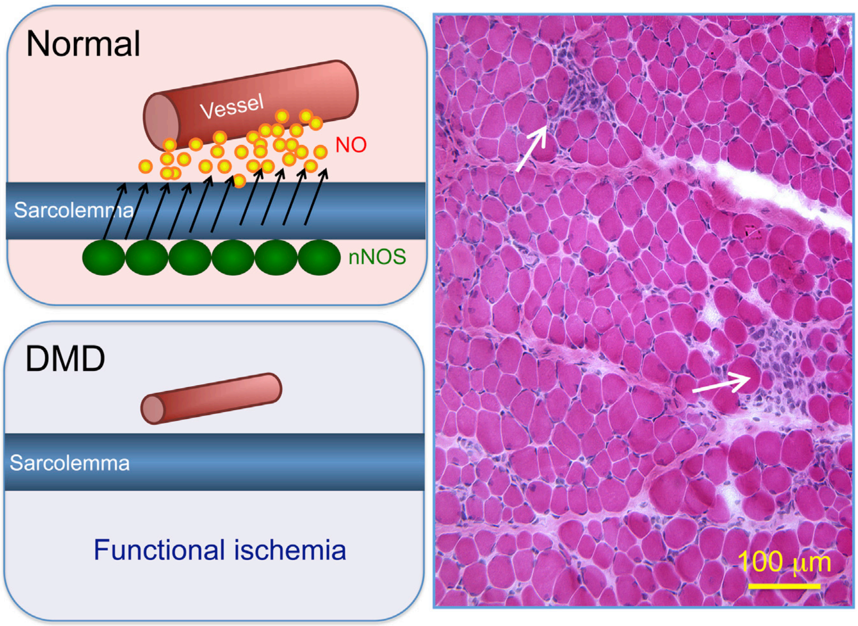

Figure 6. Sarcolemmal nNOS Delocalization Contributes to DMD Pathogenesis
In normal muscle, nNOS is localized at the sarcolemma. This allows immediate diffusion of nitric oxide (NO) to the vasculature and vasodilation in contracting muscle. In DMD, the loss of sarcolemmal nNOS compromises this process and leads to functional ischemia. The H&E-stained image illustrates focal ischemic lesions (arrow) as the first observable histological change in a 3-week-old affected dog. Despite the absence of dystrophin, histologically, the majority of myofibers appeared normal at this age.

transgenic mdx mouse that expressed a C-terminal truncated *dystrophin* gene (ΔC mice). Muscle histology and force of young adult (≤6-month-old) ΔC mice were identical to that of normal mice, suggesting that the C-terminal domain might be disposable. This notion is further supported by morphological improvement and functional rescue by AAV micro-dystrophin in animal models. While there is no doubt that C-terminal truncated dystrophins could be highly protective in skeletal muscle, a recent clinical study raised the possibility that the C-terminal domain might be critical for the heart. Tandon et al.[151] performed a genotype-phenotype correlation study in 274 patients using cardiac magnetic imaging. Interestingly, they found that the presence of the C-terminal domain is linked with a milder cardiac phenotype, suggesting a cardiac protection role of this domain.[151] Besides the heart, patient studies suggest that the C-terminal domain may also be important for cognitive function.[152,153] It is worth pointing out that even in skeletal muscle, ΔC dystrophin may become less competent as animals age. For example, in the Crawford et al.[150] paper, the percentage of centrally nucleated fibers in ΔC mice was 1% at 4 months of age, and it increased to 10% at 12 months of age. Due to the size restriction of the AAV vector, it is unlikely that a highly functional AAV microgene vector can be generated to carry the entire C-terminal domain. Future studies are needed to identify smaller functional motifs that can be included in the AAV micro-dystrophin vector.[54]

The basic function of dystrophin is to protect muscle from contraction-induced damage. The heart is the only muscle in the body that contracts constantly. Theoretically, the heart should be the earliest and most severely damaged muscle. Surprisingly, cardiomyopathy becomes apparent only at late stages of the disease.[154] This suggests that dystrophin in the heart may cope with mechanical stress using a different mechanism. In support of this, it was recently discovered that cardiac dystrophin interacts with a distinctive set of cellular proteins.[155] We also found that modification of the rod domain configuration substantially improved myocardial protection.[156,157] Heart rescue has only been studied in a few microgenes in mdx mice, and most of these studies have used the *ΔR4–23/ΔC* microgene.[55,59,63,65,158–160] As more patients survive to the third and fourth decade of their life due to modern medicine, cardiomyopathy is becoming a more prominent issue. Considering the scarce data available on DMD cardiomyopathy gene therapy, there is an urgent need to study AAV micro-dystrophin in the heart, especially to compare the potency of different microgenes and to evaluate efficacy in the heart of affected dogs.[161–163]

Improving Micro-dystrophin Therapy with the Optimized Expression Cassette and Engineered AAV Capsids

Another area that has room for improvement is the regulatory elements that are used to drive micro-dystrophin expression. To minimize toxicity and immunogenicity from untoward expression in non-muscle tissues, a muscle-specific promoter is strongly recommended. A number of muscle-specific promoters have been tested for muscle gene therapy such as the CK6, CK8, desmin, MHCK7, miniMCK, myoglobin, and SPc5-12 promoter.[164] Among these, the CK8, MHCK7, miniMCK, and SPc5-12 promoter have been proposed for systemic micro-dystrophin therapy in DMD patients.[57,84,85,165] Except for the SPc5-12 promoter,[165] all other promoters are derived from naturally existing muscle promoters.[166] Animal studies suggest that all these promoters can drive muscle-specific expression. However, these studies have also raised issues on fiber type specificity and leaky expression in antigen-presenting cells (APCs).[84,85,167,168] Skeletal muscle is composed of at least five different types of myofibers that express type I, IIa, IIb, IIx, and embryonic myosin heavy chains. Cardiac muscle is composed of myofibers that express α and β myosin heavy chains. Fiber type preference has been noted in some promoters in mouse studies.[84,85] Upcoming clinical trials will help to elucidate whether some promoters can drive robust expression in all types of myofibers in DMD patients. Leaky expression in APCs is a major concern for any tissue specific promoter. Unfortunately, some muscle promoters

Table 6. Side Reactions to High-Dose Systemic AAV Injection in Large Animal Models and Human Patients

	Subject				AAV				Side Reaction			
	Species	Disease	Age	n	Type	$\times 10^{14}$vg/kg	Promoter	Transgene	Onset	Presentation	Suspected Cause	Outcome
[67]	dog	DMD	neonatal	1	9	1.5	ubiquitous	hum-μDys	days	lethargy and anorexia	congenital liver disease	euthanized
[67]	dog	DMD	neonatal	2	9	1.5	ubiquitous	hum-μDys	weeks	weight loss, muscle atrophy, and contracture	innate immune response and inflammatory response	euthanized
[102]	NHP	normal	adult	1	PHP.B	0.75	ubiquitous	GFP	days	liver enezyme elevation, platelet reduction, hemorrahge	innate immune response and systemic inflammation	euthanized
[102]	NHP	normal	adult	1	9	0.75	ubiquitous	GFP	days	liver enezyme elevation	innate immune response	scheduled euthanization
[101]	NHP	normal	adolescent	1	Hu68	2	ubiquitous	hum-SMN	days	liver failure, DIC	innate immune response and systemic inflammation	euthanized
[101]	NHP	normal	adolescent	2	Hu68	2	ubiquitous	hum-SMN	days and weeks	liver enezyme elevation, platelet reduction, neural toxicity	innate immune response and systemic inflammation; unknown for neural toxicity	scheduled euthanization
[101]	pig	normal	neonatal	3	Hu68	2	ubiquitous	hum-SMN	weeks	neural toxicity	unknown	euthanized
[76]	human	SMA1	neonatal	3	9	0.67	ubiquitous	hum-SMN	weeks	liver enzyme elevation (1 patient)	cellular immune response	resolved
[76]	human	SMA1	neonatal	12	9	2	ubiquitous	hum-SMN	weeks	liver enzyme elevation (3 patients)	cellular immune response	resolved
[77,78]	human	XLMTM	neonatal and preschool	4	8	1	muscle-specific	hum-MTM1	weeks	liver enzyme elevation (1 patient), troponin elevation (1 patient)	N/A	resolved
[115,116]	human	DMD	adolescent	1	9	0.5	muscle-specific	hum-μDys	days	platelet and red blood cell reduction, complement activation and transient renal impairment	innate immune response?	resolved

Abbreviations: DIC, disseminated intravascular coagulation; DMD, Duchenne muscular dystrophy; Dys, dystrophin gene; Hu68, an AAV-9 variant; MTM1, myotubular myopathy gene; NHP, nonhuman primate; PHP.B, an AAV-9 variant; SMA1, type 1 spinal muscular atrophy; SMN, survival of motor neuron gene; XLMTM, X-linked myotubular myopathy.

(such as the desmin and SPc5-12 promoter) have been shown to drive expression in APCs.[167,168] A promising solution to this problem is through post-transcriptional silencing using the hematopoietic miRNA-142-3p binding site.[167,169,170] Alternatively, one may consider the use of small viral genes that interfere with antigen presentation.[171] Inclusion of these strategies in future AAV micro-dystrophin vectors may help reduce micro-dystrophin-specific immune reactions. It is worth mentioning that there is still room to increase the specificity and activity of current muscle promoters by molecular engineering.

Based on preclinical studies, it has been suggested that an effective systemic AAV therapy for DMD may require dosing $\geq 10^{15}$ vg particles per patient.[11] This creates a significant burden for the production and purification of good manufacturing practice (GMP) grade vectors for trials and future commercialization.[172,173] The high cost associated with AAV production will undoubtedly increase the price tag of the gene therapy drug, and this may further heat the already hotly debated price issue.[174] Most importantly, it causes important safety concerns, such as the immune response to the administration of large quantities of viral capsid proteins.[175] Since AAV transduction properties are largely dependent on the viral capsids, targeted engineering and/or forced *in vivo* evolution in patient muscle should provide clues to a cost-effective way to generate novel AAV capsids that are more potent than the ones used in the current trials.[40,61,176] In this regards, several newly engineered AAV variants (such as AAV-B1 and AAV-6 tyrosine mutant) have shown significantly improved muscle transduction efficiency in rodent studies.[177,178]

Level and Duration of Micro-dystrophin Expression

Another important question is the level of expression needed for treatment. The threshold has to be defined in regards to the percentage of dystrophin-positive cells, level of expression in each positive cell, and the total amount of dystrophin in whole muscle. The threshold is likely going to be different for heart and skeletal muscle, for different skeletal muscles, for different aspects of the disease (histology versus force), for different stages of the disease (early versus late), and for different therapeutic goals (halting progression versus reversing the disease). The threshold may also need to be adjusted to meet growth needs. Although marginal level (~3 to 5%) expression has been shown to improve the outcome in mice and patients,[179–186] a substantial correction may likely require the total muscle dystrophin level to reach 20%–30% of normal and mosaic expression in ~50% myofibers.[187–196] Based on comprehensive necropsy in canine studies, this may likely be reachable in human patients.[67,68,71,72]

Last but not least is the durability of the therapy. DMD is a chronic disease and requires continuous dystrophin expression.[197] One study in the canine model suggests that intravenous AAV-8 micro-dystrophin injection in the absence of immune suppression can lead to persistent expression for 2 years without a detectable T cell response to either micro-dystrophin or AAV capsid.[69] Based on the literature, the chance is likely low to translate such an ideal scenario to human patients. For example, intravascular AAV factor IX therapy in hemophilia B dogs was not associated with cytotoxic T cell responses.[198–200] However, a similar approach in human patients resulted in a significant T cell response to AAV capsid.[133] Several studies suggest that the dystrophic microenvironment (leaky membrane and oxidative stress) may promote the cellular immune response and even cause the loss of AAV in dystrophic muscle.[201–205] Re-administration is very likely needed to provide life-long therapy in DMD patients. Several strategies proposed for treating patients with preexisting neutralizing antibodies (such as the use of alternative AAV capsid, pharmacological modulation of the immune system, and plasmapheresis) may also help with re-administration.[137,206–209] Future studies in the canine DMD model will reveal the suitability of these approaches in dystrophic large mammals.

Conclusion

In summary, research over the last three decades has laid the foundation for systemic AAV micro-dystrophin gene therapy for DMD. Several clinical trials have been initiated to test the safety and tolerability in patients. Results from these trials will shed critical light on this promising therapeutic modality. However, there is still a long way to go. It took ~10 years from the report of the successful phase I trial to the approval of the AAV drug Luxturna by the FDA.[9,10,210] Considering the disease complexity, broad distribution of muscle and the need for systemic high-dose AAV administration, it is very likely that the path forward will be much more challenging for eventual regulatory approval and commercialization of AAV *micro-dystrophin* gene therapy for DMD. Nevertheless, given the rapid development of the entire gene therapy field, clinical success of AAV in multiple diseases and results from the murine and canine DMD models, there are reasons to be cautiously optimistic. It should also be pointed out there is clearly room for improving the current generation of AAV *micro-dystrophin* therapy. Future studies are needed to improve AAV capsid, maximize micro-dystrophin potency, and minimize immunological risk. Efforts in these directions may also benefit other AAV-based DMD gene therapy approaches such as reframing the mutated *dystrophin* gene at the RNA levels by U7 small nuclear RNA therapy and at the DNA level by short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-mediated genome editing.

AUTHOR CONTRIBUTIONS

D.D. is the sole author of the paper. D.D. is responsible for all aspects of the paper.

CONFLICTS OF INTEREST

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