Duchenne Muscular Dystrophy Gene Therapy in the Canine Model

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked lethal muscle disease caused by dystrophin deficiency. Gene therapy has significantly improved the outcome of dystrophin-deficient mice. Yet, clinical translation has not resulted in the expected benefits in human patients. This translational gap is largely because of the insufficient modeling of DMD in mice. Specifically, mice lacking dystrophin show minimum dystrophic symptoms, and they do not respond to the gene therapy vector in the same way as human patients do. Further, the size of a mouse is hundredfolds smaller than a boy, making it impossible to scale-up gene therapy in a mouse model. None of these limitations exist in the canine DMD (cDMD) model. For this reason, cDMD dogs have been considered a highly valuable platform to test experimental DMD gene therapy. Over the last three decades, a variety of gene therapy approaches have been evaluated in cDMD dogs using a number of nonviral and viral vectors. These studies have provided critical insight for the development of an effective gene therapy protocol in human patients. This review discusses the history, current status, and future directions of the DMD gene therapy in the canine model.

Duchenne Muscular Dystrophy and the Canine Duchenne Muscular Dystrophy Model

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a fatal muscle disease caused by null mutations in the *dystrophin* gene, a 2.4 mb gene in the X-chromosome.^{1,2} DMD occurs in ~1 in 5,000 male births.³ Affected boys show delayed motor skill development between ages 2 and 5. They lose ambulation in their early teens and die around age 20 because of cardiorespiratory failure (Table 1).⁴ The current standard of care includes steroids, palliative support, and symptom management.^{5,6} Unfortunately, these therapies cannot solve the fundamental problem of dystrophin deficiency in DMD. Gene therapy has the potential to bring back the missing protein and radically change the disease course.⁷

Dystrophin is a 427 kDa subsarcolemmal protein. It has four major functional domains: the N-terminal, rod, cysteine-rich, and C-terminal domains (Fig. 1). The N-terminal domain binds to γ -actin. The rod domain constitutes of 24 spectrin-like repeats and four intervening hinges. Repeats 1–3 have been suggested to interact with the membrane lipid bilayer. Repeats 11–15 form the second actin-binding domain. Repeats 16 and 17 contain the neuronal nitric oxide synthase (nNOS)-binding motif. Repeats 20–23 interact with microtubule. Hinges are thought to provide flexibility

to the dystrophin protein. The cysteine-rich domain and a part of hinge 4 bind to the transmembrane glycoprotein dystroglycan that interacts with laminin in the extracellular matrix (ECM). The C-terminal domain interacts with dystrobrevin and syntrophin. Through interaction with dystroglycan and actin/microtubule, dystrophin links the ECM with the cytoskeleton and provides mechanic stability to muscle cells during contraction. Dystrophin also mediates muscle signaling through its interaction with nNOS, syntrophin, and dystrobrevin. Transmembrane protein sarcoglycans and sarcospan further strengthen the structure connection between the cytoskeleton and the ECM. The dystrophin-associated glycoprotein complex (DGC) formed by dystrophin and its partners provides essential support for normal muscle structure and function (Fig. 1).

More than 60 dystrophin-deficient animal models have been reported in the literature.⁸ These models have played a pivotal role in elucidating the biological function of dystrophin and pathogenic mechanisms of DMD. They are also essential for the establishment of the scientific premise for DMD gene therapy.^{9–11} The majority of the proof-of-principle gene therapy studies are conducted in the mdx mouse, a spontaneous dystrophin-deficient mouse strain with a nonsense mutation in the exon 23 of the dystrophin gene.^{12,13} Several gene therapy strategies have effectively

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TABLE 1. A COMPARISON OF CANINE AND HUMAN DUCHENNE MUSCULAR DYSTROPHY

	<i>Canine DMD</i>	<i>Human DMD</i>
General		
Mutation type	Point mutations, deletions, insertions	Mainly deletions (~60%) and duplications (~10%)
Lifespan reduction	By 75%	By 75%
Disease course	Progressive and severe	Progressive and severe
Birth body weight	Same as a normal puppy	Same as a normal baby
Neonatal death	15–30%	Rare
Onset of disease	Birth (weak milk sucking or death) to 3 months (activity reduction)	2–5 years; patients cannot reach motor development milestones
Ambulation	Rarely lost	Wheelchair-bound by early teenage
Growth retardation	Common	Rare unless the patient is on steroids
Kyphosis	Yes	Yes
Muscle wasting	Yes	Yes
Limb muscle hypertrophy	Cranial sartorius	Calf muscle
Histopathology		
Pathology at birth	Minimal	Minimal
Limb muscle fibrosis	Yes	Yes
Centronucleation	Limited	Limited
Limb muscle MRI		
Abductor	N/A	Affected
Biceps femoris	Affected	Affected, prominent fat replacement
Cranial tibialis	N/A	Relatively spared
Gluteus	N/A	Affected
Gracilis	Affected	Often preserved
Sartorius	Hypertrophic	Relatively spared
Semitendinosus	Severely affected	Less affected
Heart		
Abnormal ECG	Frequent	Frequent
Function reduction	Detectable by 6 months of age	Evident by 16 years of age
Death from heart failure	Seldom	More common than used to
Cognitive defect		
Prevalence	N/A	One-third of patients
Correlation with gene mutation	N/A	Often involves dystrophin C-terminus
Correlation with muscle disease	N/A	No correlation
Gene therapy tested		
Gene replacement and RNA repair	Full-length dystrophin plasmid, adenovirus minidystrophin, gutted adenovirus full-length dystrophin, AAV microdystrophin, AAV exon skipping, AON exon skipping	Full-length dystrophin plasmid, AAV microdystrophin, AON exon skipping
DNA repair	Only been tested in one GRMD dog	N/A
Dystrophin independent	N/A	AAV follistatin tested in BMD patients

AAV, adeno-associated virus; AON, antisense oligonucleotide; BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy; N/A, no information available.

ameliorated muscle pathology and enhanced muscle force in mdx mice. However, translation to patients has encountered great difficulties. A major reason for the delay in translation is the inherent limitations of the mdx model. For example, mdx mice show very mild clinical symptoms and they cannot accurately model the immune response to the gene therapy vector. The huge body size difference between a mouse and a boy also presents a significant scale-up challenge.

Concurrent with the discovery of the mdx mouse,¹⁴ a canine DMD (cDMD) model was established.^{15,16} This dystrophic dog is a golden retriever. Hence, it is called the golden retriever muscular dystrophy (GRMD) dog. The GRMD dog carries a point mutation (adenine to guanine transition) in the intron 6 of the *dystrophin* gene. This mutation disrupts nor-

mal splicing. As a consequence, exon 7 is excluded from the final messenger RNA. Connection of exons 6 and 8 introduces a frameshift mutation. Dystrophin translation is aborted in exon 8 because of the premature stop codon in the mutated transcript.¹⁵ Since then, dystrophin-deficient dogs have been described in many other breeds.^{15,17–33} The majority of these reports are descriptive case studies. *Dystrophin* mutations have been determined in some breeds. However, research colonies have only been established in a few breeds (GRMD, beagle with GRMD mutation, corgi with intron 13 insertion, Labrador retriever with intron 19 insertion, and Cavalier King Charles spaniel with exon 50 point mutation).^{8,34} DMD is a worldwide disease occurring in every race and every country. The genetic background of

FIG. 1. Dystrophin, minidystrophin, microdystrophin, and the DGC. Dystrophin and its associated proteins constitute the DGC. The DGC provides mechanical support and signaling function for muscle. Nitric oxide generated by nNOS dilates the vasculature during muscle contraction to meet metabolic needs of the muscle. Minidystrophins are about half the size of full-length dystrophin. The representative $\Delta H2-R15$ minidystrophin protein contains all the known functional domain of the full-length protein (see reference 73). Microdystrophins are about one-third the size of full-length dystrophin. The representative $\Delta R2-15/\Delta R18-19/\Delta R20-23/\Delta C$ microdystrophin protein restored sarcolemmal nNOS expression in mdx mice and improved muscle function in adult dystrophic dogs (see reference 40). C, dystrophin C-terminal domain; CR, dystrophin cysteine-rich domain; DGC, dystroglycan complex; ECM, extracellular matrix; H, hinge in the middle rod domain of dystrophin; N, dystrophin N-terminal domain; SG, sarcoglycan complex; SS, sarcospan. Numerical numbers refer to the number of spectrin-like repeats in the dystrophin rod domain. Note, the lipid-binding property of spectrin-like repeats 1–3 is not depicted.

the patients is highly variable and complex. A pure breed cannot model this heterogeneity. To overcome this shortcoming, we have generated hybrid DMD dogs.^{35–40} These dogs carry the genetic information from several breeds and thus can better reflect the human condition.

In contrast to mdx mice, dystrophin-deficient dogs share many clinical features of human patients (Table 1). At birth, affected puppies are often weak and cannot compete with littermates for milk. As they reach 2–3 months of age (~3 years of age in humans), they begin to show signs of limb muscle weakness such as frequent rests, difficulty in walking, and reduced activity. The condition continues to deteriorate. Conspicuous muscular dystrophy is seen around 6 months of age. Typical symptoms at this age include excessive salivation, stunted growth, muscle wasting, abnormal gait, joint contracture, dysphagia, and aspiration pneumonia. By 3 years of age (~20 years of age in humans), affected dogs either die from cardiorespiratory complications or are euthanized because of poor health condition (Table 1). The cDMD model not only shares symptomatic similarity to human patients, but also has histological lesions resembling those of human patients. For example, limb muscle fibrosis is a common feature in DMD patients. This is observed in cDMD dogs but not in mdx mice. Centronucleation is not a prominent feature in patients because of poor muscle regeneration. This is reflected in cDMD dog muscle but not in mdx muscle.

Besides clinical manifestations and histology, the dog also has the advantage to simulate the immune response observed

in DMD patients in gene therapy. Adeno-associated virus (AAV) is the most advanced viral vector for DMD gene therapy. However, AAV-mediated DMD gene therapy has been deterred by the cellular immune response.^{41,42} For example, muscle injection results in persistent AAV transduction in mdx mice. But nominal transduction is detected in DMD patients following direct injection.⁴³ Similar to human patients, intramuscular injection also induces robust immune rejection in affected dogs (Table 2).^{44–46} For this reason, the canine model will be very useful to dissect the underlying mechanisms of the immune response and to develop creative strategies to evade immune surveillance.

Scale-up is a significant challenge in human gene therapy. There are issues related to vector purity, procedure safety, vector dose and dose regimen, host response, metabolic rate and body weight of the host, and so on. Large-scale vector production may amplify contaminations that are negligible in small-scale preparations.⁴⁷ Infusion of trillions of viral particles to a dystrophic boy may lead to unexpected inflammatory and/or immune response and possibly fatal complications.⁴⁸ A phenotypic large animal model (such as cDMD dogs) will be ideal to address these issues.

Collectively, given the biological and immunological similarities between dystrophic dogs and DMD patients, also given the advantage for scale-up, the cDMD model represents a highly valuable tool for the development and fine-tuning of gene therapy protocols before the human trial.

TABLE 2. CELLULAR IMMUNE RESPONSE TO ADENO-ASSOCIATED VIRUS IN THE CANINE DUCHENNE MUSCULAR DYSTROPHY MODEL

<i>Serotype</i>	<i>Dog age</i>	<i>Route of delivery</i>	<i>CTL</i>	<i>Comments</i>	<i>References</i>
AAV-1	Young adult	Local limb muscle injection and limb perfusion	No	The vector does not express a protein.	Vulin et al. (2012) ⁹
AAV-2	Not tested in cDMD dogs	Local limb muscle injection ^a	Yes ^a	CTL to either capsid (Wang et al. 2007) ⁴⁴ or transgene (Yuasa et al. 2007) ^{a,45}	Yuasa et al. (2007) ^{a,45} Wang et al. (2007) ^{a,44}
AAV-6	Adult	Local limb muscle injection	Yes	CTL to capsid. CTL is reduced by immune suppression and elimination of the contaminating capsid gene.	Wang et al. (2007) ^{44,124} , 2014 ¹²⁹), Shin et al. (2012) ³⁷
AAV-6	Young adult	Local injection to heart	No	The vector does not express a protein.	Bish et al. (2012) ¹⁰² , Barbash et al. (2013) ¹⁰¹
AAV-8	Young adult	Local limb muscle injection and limb perfusion	Yes	Local injection resulted in at least 1 m expression. Intravascular delivery led to at least 2 m expression but there was a clear trend of expression reduction over time.	Ohshima et al. (2008) ¹³¹
AAV-8	Young adult	Local limb muscle injection	No	Single-dog study. Expression lasted for at least 2 m.	Koo et al. (2011) ¹³³
AAV-8	Young adult	Limb perfusion	No	The vector does not express a protein.	Le Guiner et al. (2014) ¹⁰⁰
AAV-9	Neonatal	Intravenous injection	No?	Severe innate immune response. No CD4+ and CD8+ T cell infiltration.	Kornegay et al. (2010) ⁸⁴
Y731F AAV-9	Adult	Local limb muscle injection	Yes	CD4+ and CD8+ T cell infiltration despite transient immune suppression. But saturated expression was observed for at least 2 m.	Shin et al. (2013) ⁴⁰

^aStudy performed in normal dog muscle.
cDMD, canine DMD; CTL, cytotoxic T lymphocyte.

Current Status of DMD Gene Therapy in the Canine Model

Discovery of the *dystrophin* gene opens the door to correct DMD by gene therapy.⁴⁹ The 2.4 mb full-length *dystrophin* gene contains 79 exons, and it produces a 11.5 kb cDNA. Since dystrophin deficiency underlies DMD pathogenesis, the majority of gene therapy approaches have been centered on the restoration of dystrophin expression. Currently, there are three distinctive classes of approaches, including gene replacement, gene repair, and dystrophin-independent gene therapy. All these approaches have been evaluated in the cDMD model.

Dystrophin replacement therapy in the cDMD model

Direct injection of a plasmid to muscle is perhaps the simplest method. However, it is very inefficient. Only a few dystrophin-positive cells (less than 1%) were observed after intramuscular injection of dystrophin plasmids to either newborn or adult GRMD dogs.^{50–52} Limited expression and immune cell infiltration were observed following electro-transfer of canine dystrophin plasmids to GRMD muscle.^{53,54}

Adenovirus is the first viral vector used for delivering dystrophin to the canine muscle. Since the first-generation adenoviral vector has a packaging capacity of 8.2 kb,⁵⁵ investigators used a 6.2kb, minimized *dystrophin* gene called the $\Delta 17$ –48 minigene.⁵⁶ This minigene is isolated from a very mild patient who was ambulant at age 61.⁵⁷ A large portion of the rod do-

main (from exon 17 to 48) is absent in this minidystrophin because of an in-frame deletion. Compared with plasmid injection, adenoviral delivery to neonatal GRMD puppies resulted in significantly much more efficient transduction.^{58,59} However, minidystrophin expression did not last long because of strong cellular immune responses to the adenoviral vector and human minidystrophin. Application of immune suppressive drug cyclosporine only moderately prolonged gene transfer.⁵⁸ Gutted adenoviral vector has a carrying capacity up to 35 kb.⁶⁰ It offers a great opportunity to deliver the full-length cDNA. Gilbert et al. generated a full-length human dystrophin gutted adenoviral vector and tested it in GRMD puppies. Unfortunately, only limit transduction was observed.⁶¹

AAV is a 4.7kb single-stranded DNA virus. AAV-based gene replacement therapy has shown unprecedented clinical success in treating inherited diseases.⁶² However, there is a major limiting factor to use AAV in DMD gene therapy. The maximum packaging capacity of the AAV vector is 5 kb. This excludes the possibility of delivering the full-length dystrophin cDNA or even a truncated *minidystrophin* gene with the AAV vector.⁶³ To overcome this hurdle, investigators engineered super small microdystrophin. The microgene carries only one-third of the dystrophin coding sequence (~ 4 kb).^{64,65} Unlike the $\Delta 17$ –48 minigene, there is no human precedent of a functional microgene. Although patients with super-large in-frame deletions have been identified and expression of micro-size dystrophin has been confirmed in some cases, unfortunately, these patients invariably displayed severe clinical

disease.^{66–69} Since a spontaneous functional microgene does not exist, researchers have built a series of artificial microgenes based on our understanding on dystrophin. To determine the therapeutic potential of the microgene, various configurations of AAV microdystrophin vectors were injected in mdx mice. The majority of these rationally designed microgene vectors significantly protected mouse skeletal muscle and the heart.^{70–82} Surprisingly, when microdystrophin was initially tested in the cDMD model, it did not deliver therapeutic benefits.⁸³ In one case, the phenotype of the treated dogs even became much worse.⁸⁴

Over the last few years, several critical but previously unrecognized aspects of dystrophin biology are elucidated. Specifically, it is found that (1) minidystrophins with paired spectrin-like repeats are functionally superior to the ones with odd number repeats⁸²; (2) hinge 2 negatively influences microdystrophin function⁸⁵; (3) spectrin-like repeats 16 and 17 (R16/17) are required to anchor nNOS to the sarcolemma

to prevent functional ischemia^{73,86}; and (4) codon optimization can significantly improve microdystrophin function.⁸⁷ To determine whether incorporation of these new developments can enhance the performance of the microgene in muscles of large mammals, we generated the $\Delta R2-15/\Delta R18-19/\Delta R20-23/\Delta C$ microgene.^{73,88} This microgene has four spectrin-like repeats. Among which, two of the repeats are R16 and R17. We have also replaced hinge 2 with hinge 3 (Fig. 2). We delivered the codon-optimized canine version of this microgene to mdx mice and dystrophic dogs using tyrosine-engineered AAV-9.⁴⁰ Systemic gene transfer restored sarcolemmal nNOS expression and enhanced muscle function in mdx mice. (Fig. 2B and C)⁴⁰

In six random-bred dystrophic dogs (10–28 months old), direct muscle injection resulted in saturated microdystrophin expression and dramatic histological improvement. Macrophage infiltration, fibrosis, and calcification were all greatly reduced (Fig. 2D).⁴⁰ Importantly, treatment significantly protected the

FIG. 2. Y731F AAV-9-mediated $\Delta R2-15/\Delta R18-19/\Delta R20-23/\Delta C$ microdystrophin expression ameliorated skeletal muscle disease in mdx mice and adult DMD dogs. (A) Schematic outline of the $\Delta R2-15/\Delta R18-19/\Delta R20-23/\Delta C$ microgene ($\Delta R2 \mu Dys$) AAV vector. The microgene is driven by the ubiquitous CMV promoter. A flag tag is fused to the N-terminal end of the microgene for unequivocal determination of microgene expression. (B) Evaluation of the $\Delta R2 \mu Dys$ expression in mdx mice. Representative serial muscle sections were stained for the Flag tag, hinge 1, repeats 6–8, and nNOS activity. *The same myofiber in serial sections. R6–8 is absent in $\Delta R2 \mu Dys$. (C) $\Delta R2 \mu Dys$ therapy significantly reduced pathological central nucleation and enhanced specific muscle force in mdx mice. * $p < 0.05$. (D) $\Delta R2 \mu Dys$ therapy greatly improved overall histology, reduced muscle inflammation, and fibrosis in adult dystrophic dogs. The untreated and AAV-treated sides are the left and right sides of the extensor carpi ulnaris muscles of the same dog, respectively. (E) $\Delta R2 \mu Dys$ therapy significantly preserved muscle force during eccentric contraction in affected dogs. * $p < 0.05$. AAV, adeno-associated virus; DMD, Duchenne muscular dystrophy.

dystrophic muscle from eccentric contraction-induced force loss, a physiological hallmark of DMD (Fig. 2E).⁴⁰ Our data suggest that microdystrophin may ameliorate muscular dystrophy in a large mammal, potentially in human patients. Our data also suggest that the $\Delta R2-15/\Delta R18-19/\Delta R20-23/\Delta C$ microgene is an excellent candidate gene for treating DMD. Interestingly, soon after the publication of our study, Baroncelli et al. discovered a 3-year-old dog with a mild Becker muscular dystrophy (BMD) phenotype.²⁹ On Western blot, the authors detected a micro-size dystrophin migrating at 130–140 kDa. Further investigations may reveal the location of the deletion in this BMD dog and provide critical insight to the design of next-generation microgene.

Dystrophin repair therapy in the cDMD model

The reading-frame rule explains the correlation between the mutation in the *dystrophin* gene and the clinical presentation in patients.^{9,90} Frame-shift mutation results in a complete loss of dystrophin and severe phenotype. However, patients with in-frame mutation often express a smaller but partially functional protein. These patients manifest a much milder clinical disease and are classified as BMD. The reading-frame rule suggests that lethal DMD can be converted to less severe BMD if an out-of-frame transcript can be converted to an in-frame transcript. Based on this theory, investigators have developed exon skipping. In this strategy, antisense oligonucleotides (AONs) are used to modulate the splicing machinery so that certain exons are excluded from the mRNA. The modified mRNA produces an internally deleted dystrophin protein similar to that observed in BMD patients.

Initial exon skipping studies were conducted using 2'-O-methylated phosphorothioate (2OMe-PS) and phosphorodiamidate morpholino oligomers (PMO). These AONs worked very well in cultured muscle cells obtained from different breeds of dystrophic dogs.^{19,91,92} Local injection also resulted in exon skipping in GRMD and beagle-background GRMD dogs.^{92,93} While a single AON seems sufficient for exon skipping in myoblasts,^{19,92} interestingly, a cocktail of AONs is required for efficient exon skipping in canine muscle *in vivo*.⁹² To test whole-body exon skipping, Yokota et al. delivered the PMO cocktail to beagle-background GRMD dogs by intravenous injection.⁹² Systemic therapy resulted in widespread dystrophin expression and clinical improvement. Short half-life and limited tissue penetration are the major limitations of 2OMe-PS and PMO. To overcome these obstacles, various modified AONs are developed.⁹⁴ These modified AONs are conjugated with cell-penetrating peptides or polymers. Conjugation significantly enhances the tissue uptake of AONs in mdx mice. However, so far only the vivo-morpholino (PMO conjugated with the octaguanidine dendrimer) has been tested in the dog model by local injection.⁹⁵ As expected, the vivo-morpholino resulted in robust, much persistent exon skipping.⁹⁵

An alternative approach to improve exon skipping is to deliver the AON with an AAV vector.⁹⁶ In this approach, the U7 promoter is used to drive the expression of an AON that is fused to the U7 small nuclear RNA (snRNA).^{97,98} The snRNA allows efficient targeting of the AON to the spliceosome in the nucleus. AAV allows efficient tissue penetration and continuous production of the AON. Two different studies evaluated the AAV U7 approach for skeletal muscle gene therapy in GRMD

dogs. Vulin et al. co-expressed two AONs (one for exon 6 skipping and the other for exon 8 skipping) in one vector using AAV-1 (AAV1-U7E6/8).⁹⁹ Exclusion of exons 6 and 8 results in an in-frame $\Delta 6-8$ transcript. Investigators performed local injection and forelimb perfusion in six 5–15-month-old dogs and multimuscle injection in the hindlimb of four 3-week-old puppies. Efficient dystrophin restoration (20–80% positive myofibers) was observed up to 10 months after injection. Treatment reduced the number of calcified myofibers and improved parameters of magnetic resonance imaging (MRI). Further, specific muscle force was enhanced in treated puppies.⁹⁹ Le Guiner et al. delivered the same set of AONs to fifteen 3–4-month-old juvenile GRMD dogs by forelimb perfusion.¹⁰⁰ Instead of AAV-1, the authors used AAV-8 (AAV8-U7E6/8). At 2–3.5 months after injection, the authors observed high-level dystrophin expression (10–80% positive myofibers), reduced regeneration and fibrosis (but no change in inflammation and calcification), and improvement in MRI parameters. In dogs with $\geq 40\%$ dystrophin-positive myofibers, treatment also prevented progressive force decline.¹⁰⁰

Two independent groups examined AAV-6-mediated exon skipping in the heart of GRMD dogs using exactly the same U7E6/8 construct developed by Vulin et al.^{99,101,102} AAV-6 U7E6/8 was delivered by multiple transendocardial injection in young adult dogs (5–13 months old). Both groups achieved expected exon skipping and dystrophin restoration. Bish et al. followed 5 dogs for 13 months and observed a clear reduction of myocardial fibrosis and an improvement of the peak circumferential strain in cardiac MRI.¹⁰² Barbash et al. followed 5 treated dogs for 3 months and demonstrated the stabilization of left ventricular ejection fraction by cardiac MRI.¹⁰¹

Besides exon skipping, gene repair therapy can also be used to correct the mutation itself. Oligonucleotide-mediated gene correction and nuclease-based gene editing are two primary approaches. However, these DNA-level gene repair strategies are largely unexplored in the canine model. So far, only one study tried a chimeric RNA/DNA oligonucleotide in one 6-week-old GRMD puppy.¹⁰³ The authors showed evidence of gene correction up to 1 year after therapy.

Dystrophin-independent gene therapy in the cDMD model

A number of cellular proteins have been shown to modify the dystrophic phenotype.¹⁰⁴ These include utrophin, $\alpha 7\beta 1$ -integrin, myostatin, insulin-like growth factor-1, cytotoxic T cell GalNAc transferase, sarcoplasmic reticulum calcium ATPase (SERCA), peroxisome proliferator-activated receptor gamma coactivator 1 α , osteopontin, and latent transforming growth factor- β (TGF- β) binding protein 4. Many of these have been confirmed in mouse studies by gene knock, transgenic overexpression, and AAV-mediated gene transfer. However, only two of the modifiers have been tested in the cDMD model.

Utrophin is a dystrophin homologous protein. It shares structural and functional similarity to dystrophin. A mini-utrophin has been developed based on the $\Delta 17-48$ *minidystrophin* gene. Cerletti et al. injected the miniutrophin adenoviral vector to 2-day-old GRMD puppies.¹⁰⁵ In immune-suppressed animals, they achieved a 15% transduction efficiency and significant reduction of fibrosis at 2 months after treatment. The highly abbreviated microutrophin

gene has also been generated recently.^{86,106} Therapeutic effect of microtrophin remains to be tested in affected dogs.

Myostatin is a TGF- β family muscle growth regulator.¹⁰⁷ Myostatin inhibition has been shown to increase muscle size and reduce myopathy in mdx mice.¹⁰⁸ Spontaneous mutation in the myostatin gene also leads to muscle hypertrophy in whippet dogs.¹⁰⁹ To determine whether myostatin inhibition can ameliorate muscle disease in GRMD dogs, Bish et al. expressed a secreted dominant negative myostatin peptide in the liver of four 9–10-month-old GRMD dogs using AAV-8.¹¹⁰ Thirteen months after injection, they observed the expected increase in muscle mass. Furthermore, treatment reduced the serum creatine kinase level and muscle fibrosis. More recently, Cotten et al. crossed GRMD dogs with the myostatin-deficient whippets.¹¹¹ The myostatin level was reduced in myostatin heterozygous GRMD dogs. Surprisingly, these dogs displayed a more severe phenotype. The discrepancy between Bish et al.'s study and Cotten et al.'s study remains to be explained. However, it should be pointed out

that an increase in the myofiber size could be counterproductive in dystrophin-null muscle because the higher surface-to-volume ratio may result in higher sarcolemmal stress during contraction.

SERCA overexpression protects heart and muscle in rodent models of muscular dystrophy.^{112,116} AAV-mediated SERCA expression has also improved cardiac function in various canine models of heart failure.^{117,118} Based on these results, it is possible that AAV SERCA therapy may also reduce muscle disease in cDMD dogs.

Systemic AAV Delivery in Dogs

DMD affects all muscles in the body. Only whole-body gene therapy can truly change the outcome of the disease. Systemic gene delivery has been established in mice using AAV-1, 6, 8, and 9 since 2004.^{72,119,120} However, only a few studies have evaluated intravascular AAV delivery in dogs. We demonstrated the first successful systemic gene

FIG. 3. Systemic AAV delivery in neonatal dogs. **(A)** Comparison of five different AAV variants in normal neonatal dogs. AAV-8 and tyrosine-engineered Y445/731F AAV-1 resulted in robust transduction in both skeletal muscle and the heart. AAV-9 and its variant Y731 AAV-9 effectively transduced only skeletal muscle. Y445F AAV-6 barely transduced striated muscle in neonatal dogs. **(B)** Evaluation of systemic AAV-9 gene transfer in neonatal affected dogs. An AAV-9 alkaline phosphatase reporter gene vector was injected to a 2-day-old affected puppy through the jugular vein. Efficient transduction was observed at 5 weeks postinfection. Transduction was still observed at the 11 weeks of age. However, expression appeared reduced.

transfer in newborn dogs in 2008 using AAV-9 (Fig. 3).⁴⁶ AAV-9 has been considered as a “cardiotropic” vector because of its efficient myocardial transduction in rodents.^{120,121} Unexpectedly, the dog heart was barely transduced by AAV-9.⁴⁶ Over the last few years, we tested additional AAV serotypes and identified AAV-8 and Y445/731F AAV-1 as the preferred vectors for whole-body (heart and skeletal muscle) gene transfer in neonatal dogs (Fig. 3A).^{122,123} We also tested AAV-6. Interestingly, little muscle transduction was observed.¹²³ This is in sharp contrast to what has been shown with direct AAV-6 injection in dog muscle.^{101,102,124} We believe that the difference is likely because of (1) the high-level preexisting AAV-6 neutralization antibody in the canine circulation,^{36,125} or (2) the presence of galectin 3 binding protein (G3BP) in dog serum.¹²⁶ It has been shown that G3BP can cause AAV-6 particle aggregation and compromise transduction.¹²⁶

Few studies have evaluated bodywide gene transfer in the cDMD model (Fig. 3B). Kornegay et al. delivered an AAV-9 microdystrophin vector to newborn GRMD puppies via the jugular vein.⁸⁴ Injection resulted in widespread transduction. Unfortunately, these puppies also developed a fulminant inflammatory response and had to be euthanized. We recently explored systemic AAV-9 injection in adolescent dystrophic dogs.¹²⁷ Gene transfer resulted in bodywide transduction in striated muscles without any untoward reaction.

Cellular Immune Response to AAV-Mediated Gene Transfer in the Canine Model

Host immune response is undoubtedly one of the greatest hurdles in gene therapy. It is influenced by the viral capsid, vector dose and purity, method of vector production and purification, delivery route, animal age and preexisting immunity, expression cassette (such as the promoter and microRNA142-3p-binding site), transgene product, regime of immune suppression, the species tested, and even the species origin of the transgene. Many studies have examined AAV immune reaction in normal and dystrophic dog muscle (Table 2). While it is generally thought that AAV can induce the cytotoxic T lymphocyte (CTL) response in canine muscle, there are important controversies that remain to be reconciled.

Vulin et al. found that intramuscular (3.5×10^{12} vg) or intravascular (1.4×10^{13} vg) injection of an AAV-1 vector that does not express a protein did not elicit the CTL response in GRMD dogs.⁹⁹ However, Wang et al. detected a strong T cell response to AAV-1 capsid in normal dog muscle after injection of a much lower dose (5×10^{11} vg) of a canine factor IX vector.¹²⁸

Two different groups reported cellular immune reaction to AAV-2 following direct muscle injection in normal dogs.^{44,45} Results of Yuasa et al. suggest that the immune response is against the transgene product (LacZ).⁴⁵ But Wang et al. showed that the immune response is independent of the transgene product.⁴⁴ Wang et al. observed robust mononuclear cell infiltration even with empty capsids.⁴⁴

AAV-6 has been shown to induce capsid-specific T cell infiltration in skeletal muscle of normal and affected dogs following direction injection.^{44,124} A similar capsid-specific immune response was detected when AAV-6 was injected to the heart of nondystrophic dogs.¹¹⁷ In support of these observations, transient immune suppression significantly pro-

longed transgene expression.^{37,117,124} Further, elimination of the contaminating AAV-6 capsid gene reduced the immune response.¹²⁹ Surprisingly, transendocardial injection of AAV-6 to normal or affected dog heart did not induce T cell reaction.^{101,102,130}

Initial study with AAV-8 revealed transient expression and T cell infiltration in dog skeletal muscle irrespective of muscular dystrophy.¹³¹ Interestingly, vascular delivery seemed less immunogenic and resulted in somewhat longer expression.¹³¹ Since then, several groups have tested AAV-8 in normal¹³² and affected dogs.^{100,133,134} In contrast to the initial report by Ohshima et al., these later studies did not detect cellular immune reaction. Koo et al. expressed a canine-microdystrophin in a 9-week-old affected dog without immune suppression.¹³³ Robust expression lasted for at least 2 months without the evidence of immune rejection. Three groups performed limb perfusion in normal,¹³² GRMD,¹⁰⁰ and myotubularin myopathy dogs¹³⁴ in the absence of immune suppressive drugs.¹⁰⁰ Persistent transduction was observed up to 1 year (the longest time point) without any signs of the CTL response.

It is currently unclear why the observed immune responses to AAV-8 are different between Ohshima et al.'s study and other studies. It is possible that the use of the tissue-specific promoter and the species-specific transgene may have played a role. Ohshima et al. used a ubiquitous promoter, while Childers et al. and Koo et al. used the muscle-specific promoter. Ohshima et al. expressed LacZ and human microdystrophin, while Childers et al., Koo et al., and Qiao et al. expressed canine proteins. In the Le Guiner et al.'s study, no protein product was expressed. Regarding the species specificity of the transgene, an AAV-6 study also reached the same conclusion.¹²⁴ Recently, several laboratories compared the immunity of AAV-8 to that of other AAV serotypes (AAV-1, 2, and 5 and rh32.33).^{124,131,135–140} Interestingly, all these studies suggest that AAV-8 is less immunogenic. Future mechanistic studies may reveal the molecular underpinning of the unique immune privilege demonstrated by AAV-8.

Direct injection of AAV-9 evokes a strong cellular immune response in adult dog muscle.⁴⁶ But this response is absent in neonatal dogs.⁴⁶ We recently found that local delivery of a CMV-driving canine microgene also induced CD4+ and CD8+ T cell infiltration in adult DMD dogs despite the use of transient immune suppression.⁴⁰ Nevertheless, we still observed robust expression for at least 2 months (the end of the study). So far, systemic AAV-9 injection has only been evaluated in newborn puppies. We achieved high-level persistent transduction in normal puppies.^{46,123} However, when the same technique was used in affected puppies, investigators observed a serious innate immune response that was so severe to require the termination of the study.⁸⁴ The reason for this unexpected reaction is not clear. One possibility could be the use of a ubiquitous promoter and the human microgene.

Summary and Perspectives

Mdx mice and cDMD dogs were discovered simultaneously 30 years ago. However, the research use of the canine model has significantly lagged behind that of mdx mice. Using “mdx, gene therapy” and “GRMD, gene therapy” as key words, 620 and 27 records are retrieved in PubMed. That is to say that, for every 100 studies performed

in mdx mice, there are only ~4–5 studies performed using the canine model. Besides the high cost in maintaining these severely disabled cDMD dogs, the lack of a comprehensive and accurate characterization of the model also hinders the use of the cDMD model. There are several issues in this regard. First, a large-size population study on the natural history of the cDMD model remains to be conducted. Since most colonies only have a limited number of dogs, collaborative efforts from different laboratories will be needed to establish a solid baseline. Second, we need to develop standardized assays to reliably determine the outcomes of experimental interventions. This is especially important for cross-colony comparison. Numerous tools and protocols exist to study mouse muscle force. However, there aren't many options for dog muscle function evaluation. In fact, until recently, there is even no physiological assay to measure the contractility of a single dog muscle.³⁸ Misinterpretation of the data has also been noticed because of insufficient understanding on dog-specific reagents (such as the antibody).^{141,142}

In terms of gene therapy, we believe that the cDMD model will provide critical insights on issues that are very difficult to address or cannot be addressed in mdx mice, for example, the amount of the AAV vector needed to achieve bodywide transduction in a large mammal. Another important issue is the minimum level of dystrophin expression needed to protect muscle in a large mammal. Three recent AAV exon skipping studies have offered some clues. Vulin et al. found that 20–50% dystrophin-positive cells might be sufficient to improve muscle force.⁹⁹ Le Guiner et al. reported that a correction of 33%, 35%, and 40% of myofibers might result in histopathology amelioration, MRI improvement, and muscle function preservation, respectively.¹⁰⁰ Bish et al. showed that a level of 15–20% of normal dystrophin might offer some heart protection.¹⁰² This value is quite close to what has been observed in mdx mice.^{143,144} However, in the case microdystrophin, the level seems different between mice and dogs. Takeda and colleagues found that microdystrophin expression in 20% myofibers could not protect dog muscle although the same level expression improved muscle function in mdx mice.^{79,131} Besides answering these basic gene therapy questions, the cDMD model will also be essential to determine whether strategies that are shown to protect mouse muscle can or cannot treat muscular dystrophy in large mammals. New technologies that have (such as the use of the dual-AAV system to express a 6–8 kb *minidystrophin* gene)^{145–147} or have not (such as the use of nuclease to correct *dystrophin* gene mutation *in vivo*)^{148–150} been tested in mice may ultimately require corroboration in the canine model.

As we move forward from treating mdx mice to treating affected large mammals, great caution should be taken to not overinterpret the data. Results from canine studies may inform the design of the clinical trial, but they cannot fully predict what will happen in human patients because of many species-related differences. Nevertheless, a large therapeutic margin in the cDMD model may more likely translate to DMD patients.

Acknowledgments

DMD research in the Duan lab is supported by the National Institutes of Health (AR-49419, HL-91883), De-

partment of Defense (MD130014), Muscular Dystrophy Association, Parent Project Muscular Dystrophy, Jesse's Journey—The Foundation for Gene and Cell Therapy, Hope for Javier, Kansas City Area Life Sciences Institute, and the University of Missouri.

Author Disclosure Statement

D.D. is a member of the scientific advisory board for Solid GT, a subsidiary of Solid Ventures.

References

- Hoffman EP, Brown RH Jr., Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 1987;51:919–928.
- Koenig M, Hoffman EP, Bertelson CJ, et al. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 1987;50:509–517.
- Mendell JR, Lloyd-Puryear M. Report of MDA muscle disease symposium on newborn screening for Duchenne muscular dystrophy. *Muscle Nerve* 2013;48:21–26.
- Emery AEH, Muntoni F. *Duchenne Muscular Dystrophy*, 3rd ed. (Oxford University Press, New York, NY). 2003.
- Kinnett K, Cripe LH. Transforming Duchenne care: meeting 25–26 June 2012, Ft. Lauderdale, Florida, USA. *Neuromuscul Disord* 2013;23:690–695.
- Bushby K, Finkel R, Birnkrant DJ, et al. Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. *Lancet Neurol* 2010;9:177–189.
- Rodino-Klapac LR, Chicoine LG, Kaspar BK, Mendell JR. Gene therapy for Duchenne muscular dystrophy: expectations and challenges. *Arch Neurol* 2007;64:1236–1241.
- McGreevy JW, Hakim CH, McIntosh MA, Duan D. Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. *Dis Model Mech* 2015. [Epub ahead of print]
- Duan D. Duchenne muscular dystrophy gene therapy: lost in translation? *Res Rep Biol* 2011;2:31–42.
- Foster H, Popplewell L, Dickson G. Genetic therapeutic approaches for Duchenne muscular dystrophy. *Hum Gene Ther* 2012;23:676–687.
- Chamberlain JS. Gene therapy of muscular dystrophy. *Hum Mol Genet* 2002;11:2355–2362.
- Banks GB, Chamberlain JS. The value of mammalian models for Duchenne muscular dystrophy in developing therapeutic strategies. *Curr Top Dev Biol* 2008;84:431–453.
- Sicinski P, Geng Y, Ryder-Cook AS, et al. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* 1989;244:1578–1580.
- Bulfield G, Siller WG, Wight PA, Moore KJ. X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc Natl Acad Sci USA* 1984;81:1189–1192.
- Cooper BJ, Winand NJ, Stedman H, et al. The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs. *Nature* 1988;334:154–156.
- Valentine BA, Cooper BJ, Cummings JF, deLahunta A. Progressive muscular dystrophy in a golden retriever dog: light microscope and ultrastructural features at 4 and 8 months. *Acta Neuropathol (Berl)* 1986;71:301–310.

17. Shimatsu Y, Yoshimura M, Yuasa K, et al. Major clinical and histopathological characteristics of canine X-linked muscular dystrophy in Japan, CXMDJ. *Acta Myol* 2005;24:145–154.
18. Valentine BA, Cooper BJ, de Lahunta A, et al. Canine X-linked muscular dystrophy—an animal model of Duchenne muscular dystrophy: clinical studies. *J Neurol Sci* 1988; 88:69–81.
19. Walmsley GL, Arechavala-Gomez V, Fernandez-Fuente M, et al. A Duchenne muscular dystrophy gene hot spot mutation in dystrophin-deficient cavalier king charles spaniels is amenable to exon 51 skipping. *PLoS One* 2010;5:e8647.
20. Schatzberg SJ, Olby NJ, Breen M, et al. Molecular analysis of a spontaneous dystrophin ‘knockout’ dog. *Neuromuscul Disord* 1999;9:289–295.
21. Kornegay JN, Tuler SM, Miller DM, Levesque DC. Muscular dystrophy in a litter of golden retriever dogs. *Muscle Nerve* 1988;11:1056–1064.
22. Klarenbeek S, Gerritzen-Bruning MJ, Rozemuller AJ, van der Lugt JJ. Canine X-linked muscular dystrophy in a family of Grand Basset Griffon Vendeen dogs. *J Comp Pathol* 2007;137:249–252.
23. Jones BR, Brennan S, Mooney CT, et al. Muscular dystrophy with truncated dystrophin in a family of Japanese Spitz dogs. *J Neurol Sci* 2004;217:143–149.
24. Bergman RL, Inzana KD, Monroe WE, et al. Dystrophin-deficient muscular dystrophy in a Labrador retriever. *J Am Anim Hosp Assoc* 2002;38:255–261.
25. Paola JP, Podell M, Shelton GD. Muscular dystrophy in a miniature Schnauzer. *Prog Vet Neurol* 1993;4:14–18.
26. Wieczorek LA, Garosi LS, Shelton GD. Dystrophin-deficient muscular dystrophy in an old English sheepdog. *Vet Rec* 2006;158:270–273.
27. Wetterman CA, Harkin KR, Cash WC, et al. Hypertrophic muscular dystrophy in a young dog. *J Am Vet Med Assoc* 2000;216:878–881.
28. Baltzer WI, Calise DV, Levine JM, et al. Dystrophin-deficient muscular dystrophy in a Weimaraner. *J Am Anim Hosp Assoc* 2007;43:227–232.
29. Baroncelli AB, Abellonio F, Pagano TB, et al. Muscular dystrophy in a dog resembling human Becker muscular dystrophy. *J Comp Pathol* 2014;150:429–433.
30. Ito D, Kitagawa M, Jeffery N, et al. Dystrophin-deficient muscular dystrophy in an Alaskan malamute. *Vet Rec* 2011;169:127.
31. Smith BF, Yue Y, Woods PR, et al. An intronic LINE-1 element insertion in the dystrophin gene aborts dystrophin expression and results in Duchenne-like muscular dystrophy in the corgi breed. *Lab Invest* 2011;91:216–231.
32. Smith BF, Kornegay JN, Duan D. Independent canine models of Duchenne muscular dystrophy due to intronic insertions of repetitive DNA. *Mol Ther* 2007;15:S51.
33. Beltran E, Shelton GD, Guo LT, et al. Dystrophin-deficient muscular dystrophy in a Norfolk terrier. *J Small Anim Pract* 2014. [Epub ahead of print]; doi:10.1111/jsap.12292.
34. Kornegay JN, Bogan JR, Bogan DJ, et al. Canine models of Duchenne muscular dystrophy and their use in therapeutic strategies. *Mam Genome* 2012;23:85–108.
35. Fine DM, Shin JH, Yue Y, et al. Age-matched comparison reveals early electrocardiography and echocardiography changes in dystrophin-deficient dogs. *Neuromuscul Disord* 2011;21:453–461.
36. Shin JH, Yue Y, Smith B, Duan D. Humoral immunity to AAV-6, 8, and 9 in normal and dystrophic dogs. *Hum Gene Ther* 2012;23:287–294.
37. Shin JH, Yue Y, Srivastava A, et al. A simplified immune suppression scheme leads to persistent micro-dystrophin expression in Duchenne muscular dystrophy dogs. *Hum Gene Ther* 2012;23:202–209.
38. Yang HT, Shin JH, Hakim CH, et al. Dystrophin deficiency compromises force production of the extensor carpi ulnaris muscle in the canine model of Duchenne muscular dystrophy. *PLoS One* 2012;7:e44438.
39. Shin JH, Greer B, Hakim CH, et al. Quantitative phenotyping of Duchenne muscular dystrophy dogs by comprehensive gait analysis and overnight activity monitoring. *PLoS One* 2013;8:e59875.
40. Shin JH, Pan X, Hakim CH, et al. Microdystrophin ameliorates muscular dystrophy in the canine model of Duchenne muscular dystrophy. *Mol Ther* 2013;21:750–757.
41. Basner-Tschakarjan E, Mingozzi F. Cell-mediated immunity to AAV vectors, evolving concepts and potential solutions. *Front Immunol* 2014;5:350.
42. Mendell JR, Rodino-Klapac L, Sahenk Z, et al. Gene therapy for muscular dystrophy: lessons learned and path forward. *Neurosci Lett* 2012;527:90–99.
43. Mendell JR, Campbell K, Rodino-Klapac L, et al. Dystrophin immunity in Duchenne’s muscular dystrophy. *N Engl J Med* 2010;363:1429–1437.
44. Wang Z, Allen JM, Riddell SR, et al. Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy. *Hum Gene Ther* 2007;18:18–26.
45. Yuasa K, Yoshimura M, Urasawa N, et al. Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products. *Gene Ther* 2007;14:1249–1260.
46. Yue Y, Ghosh A, Long C, et al. A single intravenous injection of adeno-associated virus serotype-9 leads to whole body skeletal muscle transduction in dogs. *Mol Ther* 2008;16:1944–1952.
47. Wright JF. Manufacturing and characterizing AAV-based vectors for use in clinical studies. *Gene Ther* 2008;15: 840–848.
48. Wilson JM. Progress in the commercial-scale production of adeno-associated viral vectors. *Hum Gene Ther* 2009; 20:695.
49. Kunkel LM. 2004 William Allan award address. cloning of the DMD gene. *Am J Hum Genet* 2005;76:205–214.
50. Howell JM, Fletcher S, O’Hara A, et al. Direct dystrophin and reporter gene transfer into dog muscle *in vivo*. *Muscle Nerve* 1998;21:159–165.
51. Thioudellet C, Blot S, Squiban P, et al. Current protocol of a research phase I clinical trial of full-length dystrophin plasmid DNA in Duchenne/Becker muscular dystrophies. Part I: rationale. *Neuromuscul Disord* 2002;12:S49–S51.
52. Duan D. Myodys, a full-length dystrophin plasmid vector for Duchenne and Becker muscular dystrophy gene therapy. *Curr Opin Mol Ther* 2008;10:86–94.
53. Pichavant C, Chapdelaine P, Cerri DG, Bizario JC, Tremblay JP. Electrotransfer of the full-length dog dystrophin into mouse and dystrophic dog muscles. *Hum Gene Ther* 2010;21:1591–1601.
54. Pichavant C, Chapdelaine P, Cerri DG, et al. Expression of dog microdystrophin in mouse and dog muscles by gene therapy. *Mol Ther* 2010;18:1002–1009.

55. Danthinne X, Imperiale MJ. Production of first generation adenovirus vectors: a review. *Gene Ther* 2000;7:1707–1714.
56. Ragot T, Vincent N, Chafey P, et al. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. *Nature* 1993;361:647–650.
57. England SB, Nicholson LV, Johnson MA, et al. Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* 1990;343:180–182.
58. Howell JM, Lochmuller H, O'Hara A, et al. High-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscle of dystrophic dogs: prolongation of expression with immunosuppression. *Hum Gene Ther* 1998;9:629–634.
59. O'Hara AJ, Howell JM, Taplin RH, et al. The spread of transgene expression at the site of gene construct injection. *Muscle Nerve* 2001;24:488–495.
60. Parks RJ, Graham FL. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol* 1997;71:3293–3298.
61. Gilbert R, Nalbantoglu J, Howell JM, et al. Dystrophin expression in muscle following gene transfer with a fully deleted (“guttled”) adenovirus is markedly improved by trans-acting adenoviral gene products. *Hum Gene Ther* 2001;12:1741–1755.
62. Mingozzi F, High KA. Therapeutic *in vivo* gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* 2011;12:341–355.
63. Lai Y, Yue Y, Duan D. Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. *Mol Ther* 2010;18:75–79.
64. Scott J, Li S, Harper S, et al. Viral vectors for gene transfer of micro-, mini-, or full-length dystrophin. *Neuromuscul Disord* 2002;12:S23.
65. Dickson G, Roberts M, Wells D, Fabb S. Recombinant micro-genes and dystrophin viral vectors. *Neuromuscul Disord* 2002;12:S40.
66. Arikawa-Hirasawa E, Koga R, Tsukahara T, et al. A severe muscular dystrophy patient with an internally deleted very short (110 kD) dystrophin: presence of the binding site for dystrophin-associated glycoprotein (DAG) may not be enough for physiological function of dystrophin. *Neuromuscul Disord* 1995;5:429–438.
67. Den Dunnen JT, Grootsholten PM, Bakker E, et al. Topography of the Duchenne muscular dystrophy (DMD) gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. *Am J Hum Genet* 1989;45:835–847.
68. Fanin M, Freda MP, Vitiello L, et al. Duchenne phenotype with in-frame deletion removing major portion of dystrophin rod: threshold effect for deletion size? *Muscle Nerve* 1996;19:1154–1160.
69. Koenig M, Beggs AH, Moyer M, et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am J Hum Genet* 1989;45:498–506.
70. Yue Y, Li Z, Harper SQ, et al. Microdystrophin gene therapy of cardiomyopathy restores dystrophin-glycoprotein complex and improves sarcolemma integrity in the mdx mouse heart. *Circulation* 2003;108:1626–1632.
71. Gregorevic P, Allen JM, Minami E, et al. rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. *Nat Med* 2006;12:787–789.
72. Gregorevic P, Blankinship MJ, Allen JM, et al. Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat Med* 2004;10:828–834.
73. Lai Y, Thomas GD, Yue Y, et al. Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J Clin Invest* 2009;119:624–635.
74. Gregorevic P, Blankinship MJ, Allen JM, Chamberlain JS. Systemic microdystrophin gene delivery improves skeletal muscle structure and function in old dystrophic mdx mice. *Mol Ther* 2008;16:657–664.
75. Bostick B, Yue Y, Lai Y, et al. Adeno-associated virus serotype-9 microdystrophin gene therapy ameliorates electrocardiographic abnormalities in mdx mice. *Hum Gene Ther* 2008;19:851–856.
76. Bostick B, Shin J-H, Yue Y, Duan D. AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. *Mol Ther* 2011;19:1826–1832.
77. Liu M, Yue Y, Harper SQ, et al. Adeno-associated virus-mediated microdystrophin expression protects young mdx muscle from contraction-induced injury. *Mol Ther* 2005;11:245–256.
78. Yue Y, Liu M, Duan D. C-terminal truncated microdystrophin recruits dystrobrevin and syntrophin to the dystrophin-associated glycoprotein complex and reduces muscular dystrophy in symptomatic utrophin/dystrophin double knock-out mice. *Mol Ther* 2006;14:79–87.
79. Yoshimura M, Sakamoto M, Ikemoto M, et al. AAV vector-mediated microdystrophin expression in a relatively small percentage of mdx myofibers improved the mdx phenotype. *Mol Ther* 2004;10:821–828.
80. Fabb SA, Wells DJ, Serpente P, Dickson G. Adeno-associated virus vector gene transfer and sarcolemmal expression of a 144 kDa micro-dystrophin effectively restores the dystrophin-associated protein complex and inhibits myofibre degeneration in nude/mdx mice. *Hum Mol Genet* 2002;11:733–741.
81. Wang B, Li J, Xiao X. Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. *Proc Natl Acad Sci USA* 2000;97:13714–13719.
82. Harper SQ, Hauser MA, DelloRusso C, et al. Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. *Nat Med* 2002;8:253–261.
83. Sampaioles M, Blot S, D'Antona G, et al. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* 2006;444:574–579.
84. Kornegay JN, Li J, Bogan JR, et al. Widespread muscle expression of an AAV9 human mini-dystrophin vector after intravenous injection in neonatal dystrophin-deficient dogs. *Mol Ther* 2010;18:1501–1508.
85. Banks GB, Judge LM, Allen JM, Chamberlain JS. The polyproline site in hinge 2 influences the functional capacity of truncated dystrophins. *PLoS Genet* 2010;6:e1000958.
86. Lai Y, Zhao J, Yue Y, Duan D. alpha2 and alpha3 helices of dystrophin R16 and R17 frame a microdomain in the alpha1 helix of dystrophin R17 for neuronal NOS binding. *Proc Natl Acad Sci USA* 2013;110:525–530.
87. Foster H, Sharp PS, Athanasopoulos T, et al. Codon and mRNA sequence optimization of microdystrophin trans-

- genes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. *Mol Ther* 2008;16:1825–1832.
88. Li D, Yue Y, Lai Y, et al. Nitrosative stress elicited by nNOSmu delocalization inhibits muscle force in dystrophin-null mice. *J Pathol* 2011;223:88–98.
 89. Monaco AP, Bertelson CJ, Liechti-Gallati S, et al. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 1988;2:90–95.
 90. Beggs AH, Hoffman EP, Snyder JR, et al. Exploring the molecular basis for variability among patients with Becker muscular dystrophy: dystrophin gene and protein studies. *Am J Hum Genet* 1991;49:54–67.
 91. McClorey G, Moulton HM, Iversen PL, et al. Antisense oligonucleotide-induced exon skipping restores dystrophin expression *in vitro* in a canine model of DMD. *Gene Ther* 2006;13:1373–1381.
 92. Yokota T, Lu QL, Partridge T, et al. Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann Neurol* 2009;65:667–676.
 93. Fletcher S, Ly T, Duff RM, et al. Cryptic splicing involving the splice site mutation in the canine model of Duchenne muscular dystrophy. *Neuromuscul Disord* 2001;11:239–243.
 94. Douglas AG, Wood MJ. Splicing therapy for neuromuscular disease. *Mol Cell Neurosci* 2013;56:169–185.
 95. Yokota T, Nakamura A, Nagata T, et al. Extensive and prolonged restoration of dystrophin expression with vivomorpholino-mediated multiple exon skipping in dystrophic dogs. *Nucleic Acid Ther* 2012;22:306–315.
 96. Goyenvallé A, Vulin A, Fougère F, et al. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* 2004;306:1796–1799.
 97. Gorman L, Suter D, Emerick V, et al. Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs. *Proc Natl Acad Sci USA* 1998;95:4929–4934.
 98. Suter D, Tomasini R, Reber U, et al. Double-target antisense U7 snRNAs promote efficient skipping of an aberrant exon in three human beta-thalassemic mutations. *Hum Mol Genet* 1999;8:2415–2423.
 99. Vulin A, Barthelemy I, Goyenvallé A, et al. Muscle function recovery in golden retriever muscular dystrophy after AAV1-U7 exon skipping. *Mol Ther* 2012;20:2120–2133.
 100. Le Guiner C, Montus M, Servais L, et al. Forelimb treatment in a large cohort of dystrophic dogs supports delivery of a recombinant AAV for exon skipping in Duchenne patients. *Mol Ther* 2014;22:1923–1935.
 101. Barbash IM, Cecchini S, Faranesh AZ, et al. MRI roadmap-guided transendocardial delivery of exon-skipping recombinant adeno-associated virus restores dystrophin expression in a canine model of Duchenne muscular dystrophy. *Gene Ther* 2013;20:274–282.
 102. Bish LT, Sleeper MM, Forbes SC, et al. Long-term restoration of cardiac dystrophin expression in golden retriever muscular dystrophy following rAAV6-mediated exon skipping. *Mol Ther* 2012;20:580–589.
 103. Bartlett RJ, Stockinger S, Denis MM, et al. *In vivo* targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide. *Nat Biotechnol* 2000;18:615–622.
 104. Swaggart KA, McNally EM. Modifiers of heart and muscle function: where genetics meets physiology. *Exp Physiol* 2014;99:621–626.
 105. Cerletti M, Negri T, Cozzi F, et al. Dystrophic phenotype of canine X-linked muscular dystrophy is mitigated by adenovirus-mediated utrophin gene transfer. *Gene Ther* 2003;10:750–757.
 106. Odom GL, Gregorevic P, Allen JM, et al. Microtrophin delivery through rAAV6 increases lifespan and improves muscle function in dystrophic dystrophin/utrophin-deficient mice. *Mol Ther* 2008;16:1539–1545.
 107. Lee SJ. Regulation of muscle mass by myostatin. *Annu Rev Cell Dev Biol* 2004;20:61–86.
 108. Bogdanovich S, Krag TO, Barton ER, et al. Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 2002;420:418–421.
 109. Mosher DS, Quignon P, Bustamante CD, et al. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. *PLoS Genet* 2007;3:e79.
 110. Bish LT, Sleeper MM, Forbes SC, et al. Long-term systemic myostatin inhibition via liver-targeted gene transfer in golden retriever muscular dystrophy. *Hum Gene Ther* 2011;22:1499–1509.
 111. Cotten SW, Kornegay JN, Bogan DJ, et al. Genetic myostatin decrease in the golden retriever muscular dystrophy model does not significantly affect the ubiquitin proteasome system despite enhancing the severity of disease. *Am J Transl Res* 2014;6:43–53.
 112. Shin JH, Bostick B, Yue Y, et al. SERCA2a gene transfer improves electrocardiographic performance in aged mdx mice. *J Transl Med* 2011;9:132.
 113. Ferretti R, Marques MJ, Pertille A, Santo Neto H. Sarcoplasmic-endoplasmic-reticulum Ca^{2+} -ATPase and calsequestrin are overexpressed in spared intrinsic laryngeal muscles of dystrophin-deficient mdx mice. *Muscle Nerve* 2009;39:609–615.
 114. Goonasekera SA, Lam CK, Millay DP, et al. Mitigation of muscular dystrophy in mice by SERCA overexpression in skeletal muscle. *J Clin Invest* 2011;121:1044–1052.
 115. Morine KJ, Sleeper MM, Barton ER, Sweeney HL. Overexpression of SERCA1a in the mdx diaphragm reduces susceptibility to contraction-induced damage. *Hum Gene Ther* 2010;21:1735–1739.
 116. Bouyon S, Roussel V, Fromes Y. SERCA2a gene therapy can improve symptomatic heart failure in delta-sarcoglycan-deficient animals. *Hum Gene Ther* 2014;25:694–704.
 117. Zhu X, McTiernan CF, Rajagopalan N, et al. Immunosuppression decreases inflammation and increases AAV6-hSERCA2a-mediated SERCA2a expression. *Hum Gene Ther* 2012;23:722–732.
 118. Mi YF, Li XY, Tang LJ, et al. Improvement in cardiac function after sarcoplasmic reticulum Ca^{2+} -ATPase gene transfer in a beagle heart failure model. *Chin Med J* 2009;122:1423–1428.
 119. Wang Z, Zhu T, Qiao C, et al. Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* 2005;23:321–328.
 120. Bostick B, Ghosh A, Yue Y, et al. Systemic AAV-9 transduction in mice is influenced by animal age but not by the route of administration. *Gene Ther* 2007;14:1605–1609.
 121. Pacak CA, Mah CS, Thattaliyath BD, et al. Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction *in vivo*. *Circ Res* 2006;99:e3–9.
 122. Pan X, Yue Y, Zhang K, et al. Long-term robust myocardial transduction of the dog heart from a peripheral

- vein by adeno-associated virus serotype-8. *Hum Gene Ther* 2013;24:584–594.
123. Hakim CH, Yue Y, Shin JH, et al. Systemic gene transfer reveals distinctive muscle transduction profile of tyrosine mutant AAV-1, -6, and -9 in neonatal dogs. *Mol Ther Methods Clin Dev* 2014;1:14002.
 124. Wang Z, Kuhr CS, Allen JM, et al. Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. *Mol Ther* 2007;15:1160–1166.
 125. Rapti K, Louis-Jeune V, Kohlbrenner E, et al. Neutralizing antibodies against AAV serotypes 1, 2, 6, and 9 in sera of commonly used animal models. *Mol Ther* 2011;20:73–83.
 126. Denard J, Beley C, Kotin R, et al. Human galectin 3 binding protein interacts with recombinant adeno-associated virus type 6. *J Virol* 2012;86:6620–6631.
 127. Yue Y, Pan X, Hakim CH, et al. Safe and bodywide muscle gene transfer in young adult Duchenne muscular dystrophy dogs. *New Directions in Biology and Disease of Skeletal Muscle*, 2014, Chicago, Illinois, June 29–July 22, 2014.
 128. Wang Z, Storb R, Lee D, et al. Immune responses to AAV in canine muscle monitored by cellular assays and non-invasive imaging. *Mol Ther* 2010;18:617–624.
 129. Wang Z, Halbert CL, Lee D, et al. Elimination of contaminating cap genes in AAV vector virions reduces immune responses and improves transgene expression in a canine gene therapy model. *Gene Ther* 2014;21:363–370.
 130. Bish LT, Sleeper MM, Brainard B, et al. Percutaneous transendocardial delivery of self-complementary adeno-associated virus 6 achieves global cardiac gene transfer in canines. *Mol Ther* 2008;16:1953–1959.
 131. Ohshima S, Shin JH, Yuasa K, et al. Transduction efficiency and immune response associated with the administration of AAV8 vector into dog skeletal muscle. *Mol Ther* 2009;17:73–80.
 132. Qiao C, Li J, Zheng H, et al. Hydrodynamic limb vein injection of AAV8 canine myostatin propeptide gene in normal dogs enhances muscle growth. *Hum Gene Ther* 2009;20:1–10.
 133. Koo T, Okada T, Athanasopoulos T, et al. Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic CXMDj dog. *J Gene Med* 2011;13:497–506.
 134. Childers MK, Joubert R, Poulard K, et al. Gene therapy prolongs survival and restores function in murine and canine models of myotubular myopathy. *Sci Transl Med* 2014;6:220ra210.
 135. Vandenberghe LH, Wang L, Somanathan S, et al. Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. *Nat Med* 2006;12:967–971.
 136. Lu Y, Song S. Distinct immune responses to transgene products from rAAV1 and rAAV8 vectors. *Proc Natl Acad Sci USA* 2009;106:17158–17162.
 137. Wang L, Figueredo J, Calcedo R, et al. Cross-presentation of adeno-associated virus serotype 2 capsids activates cytotoxic T cells but does not render hepatocytes effective cytolytic targets. *Hum Gene Ther* 2007;18:185–194.
 138. Xin KQ, Mizukami H, Urabe M, et al. Induction of robust immune responses against human immunodeficiency virus is supported by the inherent tropism of adeno-associated virus type 5 for dendritic cells. *J Virol* 2006;80:11899–11910.
 139. Mays LE, Vandenberghe LH, Xiao R, et al. Adeno-associated virus capsid structure drives CD4-dependent CD8+ T cell response to vector encoded proteins. *J Immunol* 2009;182:6051–6060.
 140. Faust SM, Bell P, Cutler BJ, et al. CpG-depleted adeno-associated virus vectors evade immune detection. *J Clin Invest* 2013;123:2994–3001.
 141. Rozkalne A, Adkin C, Meng J, et al. Mouse regenerating myofibers detected as false-positive donor myofibers with anti-human spectrin. *Hum Gene Ther* 2014;25:73–81.
 142. Kodippili K, Vince L, Shin JH, et al. Characterization of 65 epitope-specific dystrophin monoclonal antibodies in canine and murine models of Duchenne muscular dystrophy by immunostaining and western blot. *PLoS One* 2014;9:e88280.
 143. Wells DJ, Wells KE, Asante EA, et al. Expression of human full-length and minidystrophin in transgenic mdx mice: implications for gene therapy of Duchenne muscular dystrophy. *Hum Mol Genet* 1995;4:1245–1250.
 144. Phelps SF, Hauser MA, Cole NM, et al. Expression of full-length and truncated dystrophin mini-genes in transgenic mdx mice. *Hum Mol Genet* 1995;4:1251–1258.
 145. Odom GL, Gregorevic P, Allen JM, Chamberlain JS. Gene therapy of mdx mice with large truncated dystrophins generated by recombination using rAAV6. *Mol Ther* 2011;19:36–45.
 146. Zhang Y, Duan D. Novel mini-dystrophin gene dual adeno-associated virus vectors restore neuronal nitric oxide synthase expression at the sarcolemma. *Hum Gene Ther* 2012;23:98–103.
 147. Zhang Y, Yue Y, Li L, et al. Dual AAV therapy ameliorates exercise-induced muscle injury and functional ischemia in murine models of Duchenne muscular dystrophy. *Hum Mol Genet* 2013;22:3720–3729.
 148. Ousterout DG, Kabadi AM, Thakore PI, et al. Correction of dystrophin expression in cells from Duchenne muscular dystrophy patients through genomic excision of exon 51 by zinc finger nucleases. *Mol Ther* 2014. [Epub ahead of print]; doi: 10.1038/mt.2014.234.
 149. Ousterout DG, Perez-Pinera P, Thakore PI, et al. Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients. *Mol Ther* 2013;21:1718–1726.
 150. Long C, McAnally JR, Shelton JM, et al. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* 2014;345:1184–1188.

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Received for publication January 12, 2015;

accepted January 13, 2015.

Published online: January 14, 2015.