



Published in final edited form as:

*Res Rep Biol.* 2011 March ; 2011(2): 31–42. doi:10.2147/RRB.S13463.

## Duchenne muscular dystrophy gene therapy: Lost in translation?

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### Abstract

A milestone of molecular medicine is the identification of dystrophin gene mutation as the cause of Duchenne muscular dystrophy (DMD). Over the last 2 decades, major advances in dystrophin biology and gene delivery technology have created an opportunity to treat DMD with gene therapy. Remarkable success has been achieved in treating dystrophic mice. Several gene therapy strategies, including plasmid transfer, exon skipping, and adeno-associated virus-mediated microdystrophin therapy, have entered clinical trials. However, therapeutic benefit has not been realized in DMD patients. Bridging the gap between mice and humans is no doubt the most pressing issue facing DMD gene therapy now. In contrast to mice, dystrophin-deficient dogs are genetically and phenotypically similar to human patients. Preliminary gene therapy studies in the canine model may offer critical insights that cannot be obtained from murine studies. It is clear that the canine DMD model may represent an important link between mice and humans. Unfortunately, our current knowledge of dystrophic dogs is limited, and the full picture of disease progression remains to be clearly defined. We also lack rigorous outcome measures (such as in situ force measurement) to monitor therapeutic efficacy in dystrophic dogs. Undoubtedly, maintaining a dystrophic dog colony is technically demanding, and the cost of dog studies cannot be underestimated. A carefully coordinated effort from the entire DMD community is needed to make the best use of the precious dog resource. Successful DMD gene therapy may depend on valid translational studies in dystrophin-deficient dogs.

### Keywords

Duchenne muscular dystrophy; gene therapy; dystrophin; adeno-associated virus; exon-skipping; canine model

### Introduction

Duchenne muscular dystrophy (DMD) is the most common lethal muscle disease caused by dystrophin gene mutation.<sup>1–3</sup> It affects 1–3 boys per 10,000 male birth worldwide.<sup>4,5</sup> Patients start to lose their mobility around 2–6 years of age and are often wheelchair bound by their early teenage years. Life expectancy is shortened to one-third to half of normal as a

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#### Disclosure

The author reports no conflicts of interest in this work.

consequence of respiratory insufficiency and/or heart failure. DMD remains an incurable disease today.

In 1987, the coding sequence of the dystrophin gene was discovered and deciphered.<sup>3</sup> Dystrophin is a subcellular cytoskeletal protein. It scaffolds a series of transmembrane and cytosolic proteins (including dystroglycans, sarcoglycans, sarcospan, neuronal nitric oxide synthase (nNOS), syntrophin, and dystrobrevin) into a dystrophin-associated glycoprotein complex (DGC). The DGC plays the important mechanical and signaling roles in muscle cells. The cloning of the dystrophin gene led to the recognition that the loss of dystrophin expression underlies clinical manifestations of DMD.<sup>1</sup> The discovery of the dystrophin gene has revolutionized DMD diagnosis and created hope for a cure with gene therapy. The reasoning is straightforward. If a method can be invented to deliver a functional dystrophin gene into the diseased muscle cells, the problem will be solved. Transgenic studies confirmed this hypothesis, and, it was also found that dystrophin overexpression was not toxic.<sup>6,7</sup> Meanwhile, exciting advances were made in dystrophin biology and muscle gene transfer,<sup>8-15</sup> heralding a new era of muscle gene therapy. To many, a possible cure of DMD by gene therapy appeared just around the corner.

### Genes that can be used to treat DMD

The discovery of the dystrophin gene has also raised a seemingly insurmountable challenges to the nascent field of DMD gene therapy. The gene itself is huge. The 2.5-mega base (mb) gene contains 79 exons, and it transcribes into a 14-kb cDNA.<sup>2</sup> A vehicle that is capable of carrying the full-length dystrophin gene is beyond the reach of the current technology. A few viral vectors that may have the packaging capacity for the full-length dystrophin coding sequence (such as gutted adenovirus and herpes virus) are very inefficient in transducing whole-body muscle cells. The hurdle of gene size was partially overcome by genotype-phenotype analysis in patient populations and by spectacular case reports of individual patients.<sup>16-19</sup> These studies suggest that deletion of a fairly large region of the dystrophin gene may not cause significant clinical consequences as long as the remaining parts are in frame and the most critical regions are retained.<sup>20-22</sup> This reading frame theory constitutes the foundation of current DMD gene therapy. Briefly, two distinctive roads are taken. One approach attempts to generate a synthetic, minimized dystrophin gene that expresses these key components (Figure 1).<sup>23</sup> Examples of these synthetic genes include the highly functional minidystrophin genes (6-8 kb) and the highly abbreviated microdystrophin genes (<4 kb).<sup>24</sup> The other approach is to restore the reading frame using antisense oligonucleotide (AON)-mediated exon skipping.<sup>25</sup>

One obvious problem associated with dystrophin replacement is the potential immune rejection of the newly introduced protein. The use of an existing gene may minimize this risk. Although intensive research is ongoing to reestablish dystrophin expression, creative means have also been developed using the alternative self-genes.<sup>26</sup>

Soon after the discovery of the dystrophin gene, the utrophin gene was identified.<sup>27-29</sup> Utrophin shares structural and functional similarity to dystrophin (Figure 1). Although utrophin cannot carry out all the functions of dystrophin, it still provides substantial benefit to dystrophic muscle.<sup>30,31</sup> In addition to utrophin, increased expression of several functionally relevant proteins (such as laminin, sarcoglycan, sarcospan, integrin, and nNOS) has also been shown to reduce muscular dystrophy in the mouse model.<sup>32-36</sup>

Another promising field for alternative gene therapy is the identification of genetic modifiers. Several highly promising candidate genes have emerged. Upregulation of follistatin, insulin-like growth factor 1, ADAM12, cytidine monophosphate-sialic acid hydroxylase (CMAH), sarcoplasmic reticulum calcium ATPase, or downregulation of

myostatin, osteopontin, cyclophilin D, latent transforming growth factor- $\beta$  binding protein 4, vascular endothelial growth factor receptor 1, and histone deacetylase have been shown to reduce dystrophinopathy in animal models.<sup>37–48</sup> Targeting these alternative genes will likely complement dystrophin gene replacement/repair therapies.

## We cured a DMD mouse

Many dystrophin-deficient mice have been generated, such as naturally occurring mdx; chemically induced mdx2cv, mdx3cv, mdx4cv, and mdx5cv; and exon 52 knockout mdx (mdx52) mice (Figure 2).<sup>49–51</sup> Unlike human patients, dystrophin-null mice exhibit very mild symptoms until they get very old (Figure 2).<sup>52,53</sup> To create a phenotypic model that more closely mimics human disease, a great variety of double knockout (dKO) mice were made. These include utrophin/ dystrophin dKO, myoD/dystrophin dKO, integrin/dystrophin dKO,  $\delta$ -sarcoglycan/dystrophin dKO, CMAH mdx, and mdx/ mTR mice (Figure 2).<sup>32,48,54–59</sup> Although there are genetic and/ or phenotypic differences between mice and humans, these mouse models, nevertheless, provide a great opportunity to test experimental DMD gene therapy in a live animal.

Early gene therapy studies were performed on a single-limb muscle in mdx mice. The primary end points of these studies were dystrophin expression, DGC restoration, myofiber degeneration/regeneration, sarcolemmal integrity, and muscle force. Long-term robust dystrophin expression was first demonstrated using vectors based on adeno-associated virus (AAV).<sup>24,60</sup> AAV is a single-stranded DNA virus. In an AAV vector, all the wild-type viral genes are removed, and a therapeutic/marker gene expression cassette serves as the vector genome. The naturally occurring AAV serotypes and the molecularly engineered AAV capsids have offered essentially unlimited options for gene delivery.<sup>61,62</sup> The major drawback of AAV is its 5 kb packaging capacity.<sup>63–65</sup> Only the massively truncated microgenes can fit into a single AAV vector. Although a microgene only carries ~30% of the dystrophin coding sequence, local AAV microgene injection has ameliorated muscle pathology in mdx mice.<sup>24,60</sup> Subsequent studies suggest that AAV microdystrophin vectors also improved muscle force and prevented eccentric contraction-induced injury in dystrophin-deficient mice.<sup>66,67</sup>

There remain several limitations of the AAV microgene vector. First, it cannot anchor nNOS to the sarcolemma. In normal muscle, dystrophin helps recruit nNOS to the membrane.<sup>31,68–70</sup> In DMD, the loss of membrane-associated nNOS results in muscle ischemia.<sup>69,71,72</sup> Further, nitrosative stress induced by delocalized nNOS inhibits muscle force generation.<sup>70</sup> Second, the microgene cannot fully restore muscle strength to the normal level.<sup>24</sup> The larger minigene is needed for better force recovery.<sup>24</sup> Third, the configuration of the earlier versions of the microgene may not be ideal.<sup>24,60</sup> In this regard, it has been suggested that the inclusion of hinge 2 and/or hinge 3 may compromise the function.<sup>73,74</sup> The first issue was addressed recently by identification of dystrophin spectrin-like repeats 16/17 (R16–17) as the nNOS-binding domain.<sup>69</sup> Incorporation of this domain in the microgene results in the R16–17/ $\Delta$ C microgene that normalizes nNOS localization (Figure 1).<sup>69,70</sup> To address the second issue, innovative strategies are needed to expand the AAV packaging capacity. This is achieved using various dual-vector systems, including the trans-splicing, overlapping, and hybrid vectors.<sup>75–77</sup> The trans-splicing vectors are based on the head-to-tail concatamerization of the AAV inverted terminal repeats. The overlapping vectors are based on homologous recombination of the transgene. Newly developed hybrid vectors integrate the advantages of these two approaches and may result in the most efficient reconstitution of a split gene.<sup>78,79</sup> Promising minidystrophin expression has been achieved with all three dual-vector systems.<sup>78,80,81</sup> To address the third issue, investigators have

developed newer versions of microdystrophins that do not carry hinges 2 and 3. An example is the  $\Delta R2-15/\Delta R18-23/\Delta C$  microgene (Figure 1).

Although local intramuscular gene delivery is important for proof of principle, it cannot meet the need of treating all affected muscles in the body. A cure for DMD requires systemic gene transfer. Whole-body delivery was first demonstrated with AAV serotype 6 in mdx mice by intravascular injection.<sup>82</sup> Subsequently, it was reported that several other AAV serotypes (such as AAV-8 and AAV-9) also mediate robust body-wide gene transfer.<sup>83-87</sup> Successful systemic dual AAV vector transduction was also achieved.<sup>80,88-90</sup> With these great tools in hand, it did not take long to prove that we can effectively treat a dystrophic mouse with AAV microdystrophin vectors.<sup>91,92</sup> The histopathology was ameliorated, muscle function improved, and life span prolonged.

Initial studies with exon skipping were performed with 2-*O*-methylated phosphorothioated (2-OMePS) AON. These studies showed efficient local restoration of dystrophin expression.<sup>93,94</sup> More effective exon skipping has been achieved with recently developed phosphorodiamidate morpholino oligomers (PMOs), peptide-tagged PMO, and nonpeptide polymer-tagged PMO.<sup>95-99</sup> Collectively, these studies suggest that repeated intravascular or intraperitoneal AON injection is sufficient to ameliorate muscular dystrophy in dystrophin-deficient mice. Additionally, a combination of AAV gene transfer and AON-mediated exon skipping may yield more persistent dystrophin expression.<sup>100</sup> In summary, curing a dystrophin-deficient mouse is no longer beyond the reach of the current technology.

## The status of DMD clinical trials

A total of three DMD gene therapy approaches have entered clinical trials. These include full-length dystrophin replacement with a plasmid vector, AON-mediated exon skipping, and AAV-mediated microgene therapy.

The first DMD gene therapy clinical trial was performed with a plasmid vector via direct muscle injection.<sup>101,102</sup> Three 17- to 21-year-old DMD patients and six 30- to 50-year-old Becker muscular dystrophy (a mild form of DMD) patients received 200–1200  $\mu$ g dystrophin plasmid in the extensor radialis muscle.<sup>102</sup> This phase I study showed low and variable expression at 21 days after injection.<sup>103,104</sup> The efficiency is clearly below the therapeutic threshold.<sup>102</sup> Unless there is a revolutionary breakthrough in the transduction efficiency, it seems unlikely the DMD patients will benefit from this seemingly simple and straightforward gene transfer technology in the near future.

There is no doubt that exon-skipping trials have yielded unprecedented success in terms of restoring dystrophin expression.<sup>105,106</sup> Two trials have been reported. Both trials aimed at restoring the open-reading frame by skipping exon 51. Four 8- to 16-year-old DMD patients received 800  $\mu$ g (in a total volume of 800  $\mu$ L) of 2-OMePS AON along a 1.5-cm long line in the tibialis anterior muscle.<sup>106</sup> Biopsy at day 28 showed 64%–97% of dystrophin-positive fibers at the intensity of 17%–35% of control. In another trial, two DMD patients (13- and 16-year-old) received 90  $\mu$ g and five DMD patients (10 to 15 years old) received 900  $\mu$ g (all in the volume of 900  $\mu$ L/patient) of PMO AON in the extensor digitorum brevis muscle.<sup>105</sup> Minimal expression was detected at the low dose. However, significant dystrophin expression was detected in the high-dose group between 21 and 28 days. Myofibers in the range of 44%–79% showed increased dystrophin expression at the intensity of 22%–32% of healthy muscle. No adverse response was detected in either trial. Currently, both trials have moved to repeated systemic administration.<sup>25</sup>

Although the results of these initial exon-skipping trials are highly promising, we should be cautious. Dystrophin contains four domains, including the N-terminal, rod, cysteine-rich

(CR), and the C-terminal domain (Figure 1). The rod domain can be further divided into 24 spectrin-like repeats and four hinges. The N-terminal domain and a specific region of the rod domain (repeats 11–17) provide two independent binding sites for the cytoskeleton. The CR domain mediates connection to the extracellular matrix. These domains are essential for dystrophin function. A patient with mutations in the CR domain is unlikely to benefit from a treatment that removes the CR domain. By definition, exon skipping only produces an internally truncated, but not necessarily an optimized protein.<sup>74</sup> It is quite clear that the function of the dystrophin protein is more complex than previously thought.<sup>107</sup> For example, exons 42–45 encode R16–17, the nNOS-binding domain.<sup>69</sup> Skipping these exons may compromise nNOS anchoring. As a matter of fact, many patients who carry in-frame deletion in this region are still afflicted by the disease.<sup>16,108–111</sup> Perhaps, the biggest challenge for exon skipping is the need to design the patient-specific AON. Due to the difference in the mutation location, one has to use individualized AON to skip specific exon(s) in order to restore the open reading frame. The AON tailored to one type of mutation in one patient may not be applicable to another mutation in a different patient. Since every AON has its unique composition, it will be a challenge for the regulatory authority to approve all AONs based on the success of a specific AON.<sup>112</sup>

The highly anticipated AAV microgene trial has some interesting revelations.<sup>113</sup> Six DMD boys received 0.2 to  $1 \times 10^{11}$  viral genome particles/kg body weight of an AAV serotype 2.5  $\Delta R3-19/\Delta R20-21/\Delta C$  microdystrophin vector in their biceps (Figure 1). The ubiquitous cytomegalo-ovirus promoter was used to control microgene expression. Previous studies with similar vectors have yielded a great success in the mouse model.<sup>60</sup> Unexpectedly, biopsy at 42 and 90 days showed essentially no microdystrophin expression despite detection of the AAV genome. Further investigation revealed a potential T-cell immune response to the dystrophin epitopes that were either presented or not presented in the AAV vector.<sup>113</sup> This result was totally unpredicted by the mouse studies.

## DMD dogs, a bridge between mice and humans

A model that better resembles human patients may bridge the gap between mice and humans. In this regard, a dystrophin-deficient dog represents an ideal intermediate model. Unlike mice, dogs have a body size similar to that of affected boys. More importantly, it has been shown that the absence of dystrophin indeed causes severe muscular dystrophy in dogs (reviewed in various studies<sup>114–118</sup>). The affected pups are stunted and weaker. This soon progresses into dysphagia, fatigue, abnormal gait, joint contracture, severe muscle wasting, and premature death (Figure 2). Recent studies suggest that the phenotypic differences in mice, dogs, and humans may be rooted in the difference of cell surface sialic acids.<sup>48</sup> Dogs and humans share similar glycol modification, but mice have a different type of glycol modification.<sup>48,119</sup> In summary, dystrophin-deficient dogs are genetically (in terms of mutated dystrophin gene) and phenotypically (in terms of clinical manifestation) similar to human patients. They represent superior models for DMD research.

Duchenne-like muscular dystrophy has been reported in many different dog breeds (Table 1).<sup>120–138</sup> However, few have been characterized. Currently, experimental dog colonies have only been established in Beagle, golden retriever muscular dystrophy (GRMD), and Corgi models. The GRMD model is the best studied (Figure 2).<sup>126,127</sup> Beagle and GRMD models share the same mutation.<sup>120</sup> In these dogs, an A-to-G transition near the end of intron 6 disrupts the normal splicing acceptor signal. Exon 7 is lost in the resulting transcript. Jumping from exon 6 to exon 8 introduces frameshift mutation and a premature stop codon.<sup>137,138</sup> Mutations in several other canine DMD models have also been identified. In the case of German short-haired pointer, a 2.7-mb deletion in the X chromosome removes the entire dystrophin gene.<sup>125</sup> In the case of Cavalier King Charles spaniels, a G-to-T

transversion at the beginning of intron 50 results in exon 50 deletion and subsequent frameshift and premature termination.<sup>124</sup> Smith et al have recently reported a Corgi DMD model.<sup>139</sup> In this model, insertion of a repetitive element in intron 13 aborts dystrophin translation.<sup>139</sup>

Plasmid injection, exon-skipping, and AAV microgene therapies have all been tested in the canine model. So far, all the reported studies were performed in either GRMD or Beagle dystrophic dogs. The result of plasmid injection has been briefly mentioned in several articles, but a comprehensive report is lacking. It was suggested that plasmid injection efficiency was very poor. In general, <1% myofibers were transduced.<sup>102,104,140,141</sup> Exon skipping was initially tested in cultured GRMD muscle cells in vitro.<sup>142</sup> However, recent studies suggest that such an in vitro assay cannot faithfully predict the in vivo outcome.<sup>143,144</sup> Instead of a single AON, it appears that a cocktail of several different AONs is needed to achieve efficient skipping in the Beagle dystrophy dogs.<sup>144</sup> AAV microgene therapy has been tested in adult GRMD dogs by local injection and in newborn GRMD dogs by systemic injection.<sup>145–148</sup> Transient immune suppression is required to achieve persistent expression following direct muscle injection in adult dystrophic dogs.<sup>147</sup> Intravascular delivery of AAV serotype-9  $\Delta R3-19/\Delta R20-21/\Delta C$  microdystrophin (this microgene contains hinge 3) vector resulted in widespread expression in newborn dogs<sup>145,146</sup> (Figure 1). Paradoxically, this therapy did not lead to expected disease amelioration. In contrast, the treated dogs showed growth delay, pelvic limb muscle atrophy, and contracture.<sup>146</sup> The exact reason behind this peculiar finding is not clear. However, a clinical study suggests that in-frame deletion of hinge 3 is associated with a milder disease.<sup>74</sup> From this point, a microgene without hinge 3 may represent a better option. Collectively, the preliminary results of the canine studies have probably revealed a more realistic picture of the challenges facing DMD gene therapy. Yes, we can now cure a dystrophic mouse. However, we are still far from curing a DMD boy.

### To-do list in the canine model

How can we fill the gap between mice and humans? Considering the limitations of the murine models and also considering the genetic and clinical similarities between dystrophic dogs and DMD patients, a logical next step would be to test DMD gene therapy in the dog model. Our limited experience in dystrophic dogs has already offered critical insight. The low efficiency of plasmid therapy was confirmed in human trials.<sup>104</sup> The canine studies also raised the need for applying transient immune suppression in AAV-mediated delivery.<sup>145,147,148</sup>

Unfortunately, there are more limitations to the canine studies than the murine studies. The cost of housing, breeding, and raising dystrophic dogs greatly exceeds that of dystrophic mice. A specialized team consisting of veterinary doctors of different disciplines, experienced technicians, and basic scientists are needed to maintain a dystrophic dog colony for translational research.<sup>149</sup> Another critical issue is the experimental scale. Unlike mice, dogs only go in heat twice a year, and dystrophic dogs are usually not suitable for natural breeding. This significantly limits the number of affected dogs one can obtain for experiments. Considering the pronounced variations of disease progression among individual dogs (Fine D, Shin J-H, Duan D. Unpublished data),<sup>150,151</sup> great caution should be taken in interpreting the data from a few dogs.<sup>152</sup> Lack of canine-specific reagents constitutes another barrier. More than 100 epitope-specific dystrophin antibodies have been developed (see <http://glennmorris.org.uk/monopubs.htm>).<sup>153</sup> However few have been characterized for canine muscle applications (Figure 3). Perhaps, the most constraining hurdle is the lack of rigorous physiological parameters to evaluate the therapeutic outcome in dogs. Standard protocols are available to diagnose dystrophin gene mutation and to

evaluate dystrophin expression at the mRNA and protein levels. A wide array of in vitro and in vivo physiological assays has also been established to monitor muscle strength changes in mice.<sup>154,155</sup> These include force measurement in a single, intact muscle, such as in vitro assay in the extensor digitorum longus muscle and in situ assay in the tibialis anterior muscle.<sup>66,70,156</sup> Other assays include forelimb or hind limb grip strength and treadmill exercise. However, similar assays are either not developed or the baseline values are not available for dystrophic and normal control dogs. Further, clinical relevance and reliability remain to be validated.

There are many unanswered questions. With limited resources, how can we take maximal advantage of the canine model? To prioritize our effort, it is important to point out that the core of current DMD gene therapies (such as exon-skipping therapy and AAV microgene therapy) is based on minimized dystrophin genes. Yet the reading frame theory does not always hold true for every single patient. Severe cases have been reported in patients harboring an in-frame deletion (reviewed in Yokota et al<sup>157</sup>). The gene size is particularly relevant to the microgene approach. All the mildly affected patients reported so far carry at least 50% of the dystrophin coding sequence.<sup>16,17,19–22,158,159</sup> However, none of the microgenes contain more than 40% of the coding sequence. A patient who carried a fairly large rod-domain in-frame deletion actually developed severe DMD.<sup>160</sup> The size of the truncated dystrophin in this severely affected patient is ~160 kDa, a size similar to that of microdystrophins.<sup>160</sup> Although it is possible that a rationally designed microgene may be functionally superior, this clinical report indeed raises the importance of rigorously testing the synthetic microgenes prior to human trials.

Although individual investigator-initiated studies should be encouraged, focused studies in a few carefully weighed experimental therapies could be more productive for the entire field. In this regard, a platform is needed to convincingly test the therapeutic efficacy in a single, intact dog muscle at the molecular and physiological levels. Such an approach may lead to some immediate benefits in terms of improving the quality of life. It will also lay the foundation for whole-body therapy. On the other hand, we should continue our effort to expand the available colonies for statistically meaningful large sample-size studies. Further characterization of the existing dystrophic dog models and the development of new canine DMD models are also important measures for preclinical investigations.

## What else?

Our current effort is mainly focused on skeletal muscle disease. However, we cannot and should not ignore other aspects of DMD. Although DMD is often referred to as a muscle disease, it actually affects multiple organ systems. Cardiac complications and central nerve system involvement are also highly relevant to the health of DMD patients.<sup>161,162</sup> A therapy for skeletal muscle may not effectively treat other complications.<sup>163</sup> Recent studies suggest that very old female mdx mice may model dilated cardiomyopathy in DMD patients.<sup>84,164</sup> However, none of the current gene therapy strategies have been evaluated in this model. Reports on the cardiac changes of dystrophin-deficient dogs are rare. Age-matched electrocardiogram and echocardiography examination between normal and affected dogs may provide a valuable baseline for cardiac outcome measurement.

Most current studies are aimed at restoring dystrophin expression (yes, this is still on the very top of our list). The rapidly expanding library of the disease-modifying genes may also offer new opportunities. Considering the immense variance in clinical manifestations among certain patients who carry the same gene mutation,<sup>165</sup> investigation in disease-modifying genes may yield novel alternative gene therapies to treat DMD.

## Perspective

Tremendous progress has been achieved over the last two decades in developing novel genetic therapies for treating DMD. Our success in treating dystrophic mice suggests that gene therapy may be a successful modality. Clinical trials have been initiated but they have yet to produce convincing benefit in DMD patients. Dystrophic dogs represent an important translational bridge between mice and humans. Unfortunately, we know much less about dystrophic dogs than we do about dystrophin-deficient mice and DMD patients. There is an urgent need to expand our investigations in the canine model. This investment will allow us to perfect gene therapy protocols and minimize unnecessary detours in human trials. To make this emphasis shift requires commitment and support from the entire DMD community, including the researchers, funding agencies, and patients and their families and friends.

## Acknowledgments

DMD research in the author's lab is supported by National Institutes of Health (AR-49419, AR-57209, HL-91883 and NS-62934), Muscular Dystrophy Association, Parent Project Muscular Dystrophy and Jesse's Journey, The Foundation for Gene and Cell Therapy. I thank Dr Robert J McDonald for critical reading of the manuscript.

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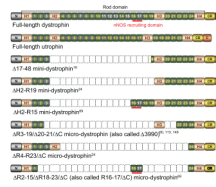
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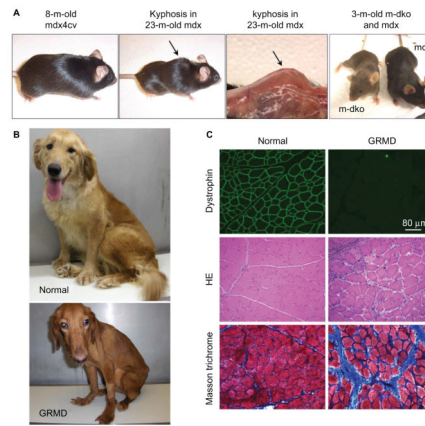
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**Figure 1.** Schematic illustration of full-length dystrophin, utrophin, and representative minidystrophin and microdystrophin.

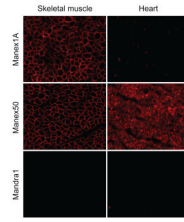
**Abbreviations:** N, the N-terminal domain of the dystrophin protein; H1–4, hinges 1–4 in the rod domain of the dystrophin protein; numeric numbers, spectrin-like repeats in the dystrophin rod domain. Positively charged repeats are in white color. Repeats 11–17 represent the second actin-binding domain. CR, the cysteine-rich domain in the dystrophin protein; C, the C-terminal domain of the dystrophin protein; nNOS, neuronal nitric oxide synthase. Empty boxes denote the regions that are deleted in each respective minidystrophin and microdystrophin construct. Among three microgenes listed here, the  $\Delta R3-19/\Delta 20-21/\Delta C$  microdystrophin has been tested in affected dogs and human patients. However, these trials have failed to demonstrate a therapeutic efficacy. The hinge 2 region in  $\Delta R4-R23/\Delta C$  microdystrophin has been shown to compromise function. The potentially deleterious hinges 2 and 3 are removed in the  $\Delta R2-15/\Delta R18-23/\Delta C$  microgene. Further, this microgene carries the nNOS localization domain.



**Figure 2.**

Mouse and dog models of DMD. **A**) Representative pictures of an 8-month-old dystrophin-deficient mdx4cv mouse (left panel), a 23-month-old mdx mouse (middle two panels), and a 3-month-old myoD/dystrophin double knockout (m-dKO) mouse and a 3-month-old mdx mouse (right panel). Adult dystrophin-null mice do not display clinical symptoms (left panel). The middle two panels show body emaciation and mouse in aged dystrophin-null mouse. (Arrow points to kyphosis seen in aged mdx mice. The left panel is a full-body view. The right-side panel represents a close view of kyphosis after the skin is removed.) The difference in the genetic background results in different fur color in m-dKO (brown) and mdx (black) mice (right panel). Findings from dKO mice may be biased by their mixed genetic background. **B**) Representative pictures of a 2-year-old normal golden retriever (top) and an affected GRMD (bottom) dog. The affected dog shows body and limb muscle atrophy, drooling, and ulnocarpal joints (forelimbs) dropped to the ground level. **C**) Representative muscle immunofluorescence staining and histopathology staining in normal (left panels) and GRMD (right panels) dogs. Dystrophin is detected by an antibody (Dys-1) specific for spectrin-like repeats 6–8 (top row). Muscle pathology is revealed by hematoxylin–eosin (HE) staining (middle row) and Masson’s trichrome staining (bottom row). HE staining shows inflammatory cell infiltration, central nucleation, and variable myofiber size. Masson’s trichrome staining shows fibrosis. Blue color in Masson’s trichrome staining images represents fibrotic tissue.

**Abbreviations:** DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy.



**Figure 3.**

Detection of canine dystrophin with monoclonal antibodies. Monoclonal dystrophin antibodies were used to detect dystrophin expression in a normal dog skeletal muscle and the heart. Representative immunostaining photomicrographs are shown for antibodies Manex1 A, Manex50, and Mandra1. Manex1A recognizes an epitope encoded by exon 1 (N-terminal domain). Interestingly, it only reacts with dystrophin in dog skeletal muscle. Manex50 maps to an epitope encoded by exon 50 (rod domain). This antibody can detect dog dystrophin in both skeletal and cardiac muscles. Mandra1 is supposed to react with an epitope encoded by exon 70 (C-terminal domain). However, it fails to reveal canine dystrophin in either skeletal muscle or the heart.

**Table 1**

## Dystrophin-deficient dogs

<b>Breed</b>	<b>Mutation</b>	<b>Comment</b>	<b>References</b>
Beagle	Intron 6 point mutation (same as in GRMD)	Colony established. Exon skipping tested	120,121
Belgian Groenendaeler shepherds	Unknown		122
Brittany spaniels	Unknown		123
Cavalier King Charles spaniels	Intron 50 point mutation	Exon skipping tested in cultured myoblasts	124
Corgi	Repetitive element insertion in intron 13	Colony established, AAV microgene ongoing	139
German short-haired pointer	Unknown		125
Golden retriever (GRMD)	Intron 6 point mutation	Colony established in several places. Plasmid, exon skipping, and AAV microgene tested	121,126,127
Grand Basset Griffon Vendéen	Unknown		128
Irish terriers	Unknown		129
Japanese spitz	Unknown		130
Labrador retriever <sup>a</sup>	Unknown		131
Labrador retriever <sup>a</sup>	Repetitive element insertion in intron		166
Miniature schnauzer	Unknown		132
Old english sheepdog	Unknown		133
Rat terrier	Unknown		134
Rottweiler	Point mutation in exon 52		167
Samoyed	Unknown		135
Weimaraner	Unknown		136

**Note:**

<sup>a</sup>It is not clear whether the two Labrador retrievers share the same mutation.

**Abbreviations:** GRMD, golden retriever muscular dystrophy; AAV, adeno-associated virus.