What is the level of dystrophin expression required for effective therapy of Duchenne muscular dystrophy?

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Abstract

Duchenne muscular dystrophy (DMD) is a fatal X-linked muscle wasting disease. The disease is due to mutations in the DMD gene that encodes for a large intracellular protein called dystrophin. Dystrophin plays a critical role in linking the internal cytoskeleton of the striated muscle cell with the extracellular matrix as well as having cell signalling functions. In its absence muscle contraction is associated with cycles of damage, repair, inflammation and fibrosis with eventual loss of muscle and replacement with fat. Experiments in animal models of DMD have generated a number of different approaches to the induction of dystrophin including viral vector mediated delivery of a recombinant dystrophin gene, antisense oligonucleotide mediated exon-skipping to restore the open reading frame in the dystrophin mRNA, read-through of premature stop mutations, genome modification using CRISPR-Cas9 or cell based transfer of a functional dystrophin gene.

In all cases, it will be important to understand how much dystrophin expression is required for a clinically effective therapy and this review examines the data from humans and animal models to estimate the percentage of endogenous dystrophin that is likely to have significant clinical benefit. While there are a number of important caveats to consider, including the appropriate outcome measures, this review suggests that approximately 20% of endogenous levels uniformly distributed within the skeletal muscles and the heart may be sufficient to largely prevent disease progression.

Keywords: animal models; *mdx* mouse; GRMD; muscle physiology; eccentric contractions

Introduction to Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD) is a fatal X-linked muscle wasting disorder characterised by repeated rounds of muscle degeneration, inflammation and repair. Over time, the inflammation leads to fibrosis, which in turn, possibly associated with satellite cell senescence, leads to a failure of regeneration and replacement of muscle with fat. Clinical signs include delayed motor milestones and using the arms to get up from the floor and to straighten the back (Gower's manoeuvre). The gait becomes progressively stiffer and most boys become wheelchair dependent between 8 and 12 years old. With good medical management, the average longevity is into the late 20's although some patients live into their 40s. The most common causes of death are respiratory complications and cardiomyopathy, as also the heart is affected. DMD occurs in 1 in 5,000 male births (Mendell et al., 2012; Mah et al., 2014; Gatheridge et al., 2016) and is due to mutations in the DMD gene at position Xp21. A few unlucky girls with substantially skewed X-inactivation have clinical symptoms with the degree of skewedness correlating with disease severity (Pegoraro et al., 1995; Azofeifa et al., 1995). The DMD gene, isolated in 1986 (Koenig et al., 1987), is the largest known mammalian gene at 2.4million base pairs long and encodes a 427kDa protein called dystrophin (Hoffman et al., 1987). Mutations associated with DMD come in all forms, deletions, duplications, nonsense and missense mutations but in almost all cases they lead to loss of the open reading frame and a premature stop codon. The gene has a high rate of spontaneous mutation and this accounts for over a third of cases. Consequently, there is a pressing unmet clinical need for novel therapies for this disease.

There is an allelic condition, called Becker muscular dystrophy (BMD), where the mutation in the DMD gene does not disrupt the open reading frame. BMD patients produce an internally truncated dystrophin with intact amino and carboxyl termini. In general, BMD is a milder disease than DMD and in some cases BMD patients are asymptomatic (e.g. Melis et al., 1998; Ferreiro et al., 2009; Zimowski et al., 2017).

Dystrophin has an amino terminal portion, which contains an actin-binding domain, 24 spectrin like repeat domains, four hinge regions, a cysteine rich domain and a carboxyl terminal portion. Dystrophin forms part of a link between the internal cytoskeleton of the striated muscle and the extracellular matrix. The amino domain binds to filamentous actin, whereas the cysteine rich domain binds to beta-dystroglycan, a membrane association protein that is bound to the highly glycosylated alpha dystroglycan that in turn binds to proteins in the extracellular matrix including the laminin alpha 2 chain of laminin 211. The carboxyl terminus of dystrophin interacts with a number of intracellular molecules including dystrobrevin and syntrophins that interact with cell signalling molecules such as neuronal nitric oxide synthase (nNOS). Dystrophin is essential for maintaining the

integrity of striated muscle. When dystrophin is missing, the muscle is damaged during contractile activity.

A wide range of potential therapies have been considered for DMD. They fall broadly into three categories. First is the induction of dystrophin in the striated muscles. The second is upregulation of other genes to replace dystrophin, focussed primarily on the autosomal homologue utrophin. The third strategy is to deal with the downstream consequences of the dystrophin deficiency: unstable membranes, accumulation of intracellular calcium, poor vascular perfusion, oxidative stress, nitrosylation of proteins, inflammation and fibrosis. This review focusses on the induction of dystrophin in striated muscle.

Animal models of DMD

A wide range of animal models have been discovered or generated for DMD. The best known is the dystrophic *mdx* mouse (Bulfield et al., 1984) that has a premature stop mutation in exon 23 of the murine DMD gene (Sicinski et al., 1989). Consequently, the *mdx* fails to produce dystrophin except in a small subset of fibres where idiosyncratic splicing leads to restoration of the open reading frame (revertant fibres). The *mdx* mouse has been the most widely used model of DMD with more than 2,800 papers published and it is on the C57Bl10ScSn background. However, it is getting increasingly hard to access congenic control mice and so many investigators have used C57Bl6 controls. The *mdx* mouse is a good biochemical model of DMD but does not exhibit clear clinical signs of disease. Lifespan is only moderately reduced, the limb muscles show a hypertrophic response to muscle damage and only the diaphragm shows substantial fibrosis although other muscles show increased fibrosis in old age (e.g. Chamberlain et al., 2007).

The original *mdx* has been backcrossed onto a variety of genetic backgrounds including BALB/cJ, C57BL/6J and FVB/Nj genetic backgrounds and these appear very similar to the original *mdx* mouse (McGreevy et al., 2015; Wasala et al., 2015). A backcross onto the DBA/2J appears to worsen the phenotype of the dystrophic mouse such as lower hind limb muscle weight, fewer myofibres, increased fibrosis and fat accumulation, and marked muscle weakness that may be the consequence of reduced regeneration following muscle damage (Fukada et al., 2010; Coley et al., 2016; Rodrigues et al., 2016; van Putten et al., 2019). Initial studies suggested that this cross also caused earlier cardiomyopathy but this pathology was observed also in the DBA/2J wild-type controls (Hakim et al., 2017).

A series of variants with mutations in different parts of the murine DMD gene were developed using N-ethylnitrosourea treatment of male mice and were termed *mdx*^{2cv}, *mdx*^{3cv}, and *mdx*^{4cv}. (Chapman et al., 1989). Subsequently, the *mdx*^{5cv} was also identified (Danko et al., 1992). These mice have all been backcrossed onto the C57Bl6 background. The *mdx*^{4cv} and *mdx*^{5cv} dystrophic mice have approximately 10-fold fewer revertants than the *mdx* mouse whereas the *mdx*^{3cv} has a low level of full-length dystrophin expression (Danko et al., 1992). Another mouse mutant, lacking many of the smaller isoforms of dystrophin, has been generated by gene targeting, the *mdx*^{52} mouse (Araki et al., 1997).

In dystrophic mice utrophin, an autosomal homologue of dystrophin that preceeds dystrophin during skeletal muscle development (Helliwell et al., 1992), is upregulated in mature myofibres. Therefore, *mdx* mice were crossed with utrophin knockout mice to produce a mouse commonly referred to as the double knockout mouse, which has a more severe phenotype than the *mdx* mouse (Deconinck et al., 1997; Grady et al., 1997). While this mouse is arguably a better phenocopy of DMD, patients do not lack both dystrophin and utrophin. The *mdx* or *mdx*^{cv} variants have also been crossed with a variety of other genetically manipulated mice to exacerbate the pathology. Because these mice show continued good regeneration, the *mdx*^{4cv} has been crossed with a knockout for telomerase to produce the *mdx*^{4cv}/*mTR*$^{-/-}$ mouse. This is a complex model as the loss of telomerase results in rapid ageing that increases with each generation. Second generation *mdx*^{4cv}/*mTR*$^{-/-}$ mice show a skeletal muscle phenotype closer to DMD with a defect in muscle regenerative response (Sacco et al., 2010). Sialic acids are a class of cell-surface glycans that are involved in extracellular signalling. Mammals have two forms of sialic acids, Nacetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). However, humans completely lack Neu5Gc. Thus, to "humanise" the *mdx* mouse, they were crossed with a mouse knockout for the enzyme that catalyzes the synthesis of Neu5Gc (*Cmah*$^{-/-}$). *Mdx/Cmah*$^{-/-}$ mice were reported to show enhancement of DMD pathophysiology (Chandrasekharan et al., 2010) and show earlier cardiac pathophysiology than the standard mdx mice, although skeletal muscle physiology defects appeared less severe in a separate study (Betts et al., 2019). These and additional models are reviewed in more detail by Yucel and colleagues (2018).

Dog models are also available, the most commonly used being a Golden Retriever with Muscular Dystrophy (GRMD), also known as CXMD, which has a splice site mutation that leads to the loss of exon 7 and thus a failure to produce dystrophin (Cooper et al., 1988; Sharp et al., 1992). Another dog model, this time with a splice site mutation leading to the loss of exon 50 is undergoing a natural history study at the Royal Veterinary College (London, UK) based on an index case identified in 2009 (Walmsley et al., 2010). This latter dog model has been used to assess dystrophin levels arising from

skipping of exon 51 using a CRISPR-Cas9 single cut strategy (Amoasii et al., 2018). New spontaneous mutations in the DMD gene continue to be recorded in dogs on a regular basis (e.g. Mata López et al., 2018; Nghiem et al., 2017).

A number of other animal models have been developed but have not yet been used in published examples of dystrophin induction. These include dystrophic rats (Larcher et al., 2014; Nakamura et al., 2014), dystrophic cats (Gaschen et al., 1992), dystrophic rabbits (Sui et al., 2018) and dystrophic pigs (Klymiuk et al., 2013). The main features of these different animal models of DMD have been reviewed by Wells (2018).

Induction of dystrophin

Therapeutic induction of dystrophin expression potentially can be achieved by a number of different methods including viral vector mediated delivery of a recombinant dystrophin gene, antisense oligonucleotide mediated exon-skipping to restore the open reading frame in the dystrophin mRNA, read-through of premature stop mutations, genome modification using CRISPR-Cas9 or cell based transfer of a functional dystrophin gene. In all cases, it will be important to understand how much dystrophin expression is required for a clinically effective therapy.

The use of viral vectors for gene therapy of DMD has been considered ever since the DMD gene was first reported. A number of systems were developed ranging from a heavily engineered version of adenovirus capable of accommodating the full coding sequence of the 11.5 kb dystrophin cDNA (Kochanek et al., 1996) through to small adeno-associated virus (AAV) only capable of accommodating 4.8 kb of exogenous DNA, thus requiring a compact promoter and a highly recombinant micro-dystrophin (Wang, Li and Xiao, 2000). Variants of the latter are currently in three human clinical trials (NCT03362502, NCT03368742 and NCT03375164). There is an excellent recent review by Duan (2018) of the development and use of AAV vectors to introduce recombinant microdystrophins into dystrophic muscle. In addition, a very recent paper by Ramos and colleagues (2019) has described problems with some of the existing microdystrophin constructs. The paper also suggests solutions for some of these problems.

Another approach to induction of dystrophin is to use an antisense oligonucleotide (AO) to modulate splicing of the primary RNA transcript. The AO is designed to either target exon recognition sequences or splice site sequences to prevent binding of the splicesosome and thus exclude one or more exons to restore the open reading frame in the dystrophin mRNA (exon-skipping). This approach, first published by the Dickson laboratory (Dunckley et al., 1998) and subsequently by

other groups, was shown to be effective by inducing expression of dystrophin in murine and human cells in culture and in dystrophic mouse and dog models. Consequently, this approach was taken into human clinical trials using two different oligonucleotide chemistries. Both sets of trials targeted skipping of exon 51, as this would treat approximately 13% of DMD patients. One set of trials used a phosphorodiamidate morpholino oligomer (PMO) – eteplirsen - delivered via the intravenous route (Sarepta) and the other used a 2'OMethyl modified phosporothioate oligonucleotide (2OmePS) – drisapersen - delivered by the subcutaneous route (Prosensa / Biomarin). Drisapersen was rejected by the FDA on the basis of toxicity whereas eteplirsen was given accelerated approval in 2016 and is marketed as ExonDys51. In contrast, the European Medicines Agency did not approve eteplirsen.

Approximately 13-15% of DMD patients have nonsense mutations that form a premature stop mutation. Aminoglycoside antibiotics have the ability to cause read-through these mutations, and this was demonstrated in the *mdx* mouse using gentamicin (Barton-Davies et al., 1999). Unfortunately, this was only achieved at high doses that have the potential to cause ototoxicity and nephrotoxicity and, although several small short clinical trials in DMD were subsequently undertaken, this was not a realistic long-term treatment for DMD. However, this observation inspired the search for small molecule drugs that could achieve the same effect with less toxicity and resulted in the identification of PTC124, also known as ataluren (Welch et al., 2007). The efficacy and safety of ataluren were assessed in two randomised, double-blind, placebo-controlled, trials. Pre-specified subset analysis was considered sufficient for a conditional authorisation by the European Medicines Agency in 2014 and the drug is now marketed as Translarna. In contrast, the FDA did not approve Translarna.

Another approach to inducing dystrophin is to perform genome editing, in most cases by using CRISPR-Cas9 technology. A series of papers were published in Science in 2016 reporting in vivo gene editing in the *mdx* mouse (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). Subsequently, issues relating to dose have been examined in long-term studies in the *mdx* mouse (Hakim et al., 2018) and one group has reported initial studies in a dystrophic dog model (Amoasii et al., 2018). The latter showed the potential for highly efficient gene editing but clinical application will require a greater understanding of the potential off-target effects with this technology (Nelson et al., 2019).

The final approach to induction of dystrophin utilises the ability of myoblasts to fuse with existing muscle fibres in both growth and repair. This enables cell based transfer of a functional dystrophin gene and was demonstrated by transferring congenic wild-type C57Bl10 myoblasts into the *mdx* mouse which resulted in good local levels of dystrophin (Partridge et al., 1989). This led to human

clinical trials that were unsuccessful. Considerable effort was expended in testing potential stem cell therapies acting via myoblast fusion and one of the most promising, the use of mesoangioblasts (pericytes), led to a clinical trial which unfortunately failed to confirm the promising results in dystrophic mice and dogs (Cossu et al., 2015).

Quantification of dystrophin

In order to be able to predict the amount of dystrophin required for a clinically effective therapy it clearly will be important to understand the advantages and disadvantages of different methods of assessing the quantity of dystrophin protein. It is also important to understand whether the expression of dystrophin is patchy or uniform, as this may also influence the clinical effectiveness of any therapy that induces expression of dystrophin. Patchy expression can best be assessed by immunostaining, ideally calibrated against another membrane associated protein that does not change significantly with muscle pathology. Spectrin is commonly used when examining transverse sections of skeletal muscle, although it should be noted that spectrin expression is increased in dystrophic muscle. Whether this is the result of an increased number of small fibres or a genuine upregulation is unclear but clearly spectrin should be used with caution. A number of investigators have developed methods of quantifying the level of dystrophin expression in individual fibres in a section.

The variation between quantitative immunohistochemistry and western blotting and between laboratories has been examined in a five laboratory study of a blinded sample set from BMD and DMD patients. After careful standardisation of the protocols, this study showed very similar quantification with minimal inter- and intra-laboratory variability (Anthony et al., 2014). This was particularly true of the quantitative immunohistochemistry and results from the quantitative immunohistochemistry closely matched those from western blot. Quantitative immunohistochemistry should cover the whole section and not just a region of interest. It is recommended that several different methods of quantification are used to increase confidence in the quantity of dystrophin detected. It is important that the same antibody is used to allow comparison of levels of dystrophin as different antibodies will have different affinities for dystrophin.

Since then, additional refinements and novel technologies have been developed to improve the quantification of dystrophin. These include a new high throughput semi quantitative fluorescent immunofluorescence method for quantifying dystrophin expression in the whole of a transverse

sections of skeletal muscle (Sardone et al., 2018) and the ProteinSimple capillary immunoassay (Wes) method, a gel- and blot-free method (Beekman et al., 2018). Another approach is to use mass spectroscopy of trypsin digests to quantify dystrophin. This approach was reported as having a lower detection limit of 5% of endogenous (Brown et al., 2012) but methods are in development with a lower detection limit of about 1% (see the following workshop report).

Most recently, representatives of academia, patient organisations, industry and the United States Food and Drug Administration met in March 2018 to discuss the potential and problems of techniques currently used in translational research (western blot and immunofluorescence) and emerging techniques (mass spectrometry and capillary western immunoassay). The workshop concluded that it is now clear that dystrophin expression levels can vary considerably between healthy individuals and so there is a need for a primary reference standard for human clinical trials (Aartsma-Rus et al., 2019).

Samples from patients

Studies of patients with Duchenne and Becker muscular dystrophy have been used to assess the effects of low levels of dystrophin expression on clinical outcome. Early studies suggested that a delay in the loss of ambulation in DMD patients was due to low (trace) levels of dystrophin in otherwise dystrophic muscles (Nicholson et al., 1993) although this was in the absence of our current understanding about disease modifying genes in DMD (see for example Bello et al., 2016; Weiss et al., 2018). Dystrophin levels as low as 30% were reported as sufficient to prevent the development of muscular dystrophy in man (Neri et al., 2007). There are also several case reports of individual patients with no significant muscle impairment and low levels of dystrophin. For example, Nakamura and colleagues (2016) report a patient with a deletion of exons 3-9 and dystrophin protein expression 15% that of control level who had no muscle involvement at the age of 27 years old. While the above reports suggest that even low levels of dystrophin might be beneficial for patients, the quantification was based on muscle biopsies of a single muscle and so may not be representative of the body wide level of dystrophin in different muscles, yet clinical assessment is generally performed at the whole body level.

The issue of problems quantifying the effects of treatment from single muscle biopsies is clearly outlined by studies in dystrophic mice. For example, both Alter and colleagues (2006) and Vila and co-workers (2015) have clearly shown that induction of dystrophin varies between muscles in mice systemically treated with antisense morpholino oligomers.

Another caveat about patient studies is that internally truncated forms of the dystrophin protein, as seen in patients with Becker muscular dystrophy, may be substantially less functional that the normal full length isoform of dystrophin. A study by Anthony and colleagues (2011) examined 17 BMD patients with a confirmed in-frame exon deletions equivalent to skipping of exons 51, 53 or 45–55 multi-exon skipping in Duchenne muscular dystrophy. Patients in the model 51 group were either asymptomatic (three patients), or mildly affected (five patients). In the model 53 group, three patients were classified as mild; one asymptomatic and one severe; all four patients in the model 45-55 group were classified as mild. All had levels of dystrophin 50% or more compared to normal controls. The authors concluded that "all varieties of internally deleted dystrophin assessed in this study have the functional capability to provide a substantial clinical benefit to patients with Duchenne muscular dystrophy". However, modelling of different deletions has predicted substantial differences in the extent of disorganisation of the central rod domain of dystrophin (Delalande et al., 2018) that might cause important differences in clinical benefit.

Assessing the effects of different levels of dystrophin in animal models:

A number of different outcome measures have been used to assess the response to treatment in animal models of muscular dystrophy. In order to ensure a degree of comparison between laboratories, a number of standard operating procedures (SOPs) have been developed with the animal model community (Grounds et al., 2008). These SOPs are currently available at the Treat-NMD website (http://www.treat-nmd.eu/resources/research-resources/dmd-sops/).

1. Histological and gene expression assays in the mouse

Historically much has been made of changes in muscle pathology and gene expression. However, these are not in themselves a direct assessment of the functional result of dystrophin induction, but rather are an indirect assessment that can be misleading. SOPs exist on the above webpage for: Quantitative determination of muscle fibre diameter (minimal Feret's diameter) and percentage of centralized nuclei (Treat-NMD SOP DMD_M.1.2.001); Quantification of histopathology in Haemotoxylin and Eosin stained muscle sections (Treat-NMD SOP DMD_M.1.2.007); and Serum Creatine Kinase analysis in mouse models of muscular dystrophy (Treat-NMD SOP MD_M.2.2.001).

Changes in gene expression have been used to infer a functional benefit but some quantitative RT-PCR studies are marred by the wrong choice of genes used to normalise the data. A comprehensive analysis of suitable housekeeping genes in normal and dystrophic mice of different ages and different muscles has recently been published (Hildyard et al., 2019).

2. Conscious Functional assays in the mouse

In order to assess the functional effects of treatment, a number of assays of the locomotor system have been developed for use in conscious mice. SOPs exist on the above webpage for: Behavioural and Locomotor Measurements Using Open Field Animal Activity Monitoring System (Treat-NMD SOP DMD_M.2.1.002); Use of treadmill and wheel exercise to assess dystrophic state (Treat-NMD SOP DMD_M.2.1.003); The use of hanging wire tests to monitor muscle strength and condition over time (Treat-NMD SOP DMD_M.2.1.004); Use of grip strength meter to assess the limb strength of mdx mice (Treat-NMD SOP DMD_M.2.2.001); and Whole body tension measurements (Treat-NMD SOP DMD_M.2.2.006). A potential problem with all of the above is that the performance of treated mice may vary with the central nervous system effects of the treatment. Activation of CNS centres or side-effects such as nausea may give a misleading impression of the magnitude of the functional improvement.

SOPs also exist for the assessment in conscious mice of the respiratory system (Treat-NMD SOP DMD_M.2.2.002) and the cardiovascular system (Treat-NMD SOP DMD_M.2.2.003). These are potentially less problematic than the locomotor system tests. There is also an SOP for cardiac assessment in anaesthetised mice (Treat-NMD SOP DMD_M.2.2.004).

3. Muscle physiology in the mouse

As an alternative to functional assessments in the conscious mouse, muscle physiology in the anaesthetised mouse or ex-vivo, measures changes in performance without the potential confounding effects of motivational state. Dystrophic muscle generates a lower specific force and is more vulnerable to eccentric (lengthening) contractions that wild-type muscle. An SOP has been developed for in situ measurement of muscle physiology in the lower hindlimb muscles (Treat-NMD SOP DMD_M.2.2.005). Alternatively, muscle physiology can be performed ex-vivo provided the muscles are small enough to ensure adequate perfusion to supply oxygen and substrate and to remove waste metabolites. Thus, this approach is suitable for small limb muscles such as the soleus

and extensor digitorum longus muscles and for strips from the diaphragm. Again, an SOP is available (Treat-NMD SOP DMD_M.1.2.002).

4. Assessments in the dystrophic dog

As the dystrophic dogs show clear clinical signs of muscular dystrophy, in contrast to dystrophic mice, there are a number of functional tests that can be applied in the conscious animal, such as gait measures. It is also possible to do similar muscle physiology tests but as non-terminal anaesthetised procedures with needle electrodes and measuring torque using a pedal (Childers et al., 2002; Treat-NMD SOP DMD_D.2.2.001). The response to eccentric exercise can also be measured using a similar approach (Treat-NMD SOP DMD_D.2.2.002).

Effects of different levels of dystrophin in mouse models.

The *mdx*^{3cv} mouse shows low levels of full-length dystrophin, estimated at 5% of wild-type levels, and this is associated with higher muscle forces, and a reduced force drop associated with eccentric exercise, despite dystrophic pathology similar to the *mdx*^{4cv} mouse (Li et al., 2008). The same strain showed a reduced muscle stiffness compared to the *mdx*^{4cv} mouse (Hakim and Duan, 2012). Similarly, the same low level uniform expression of dystrophin in the mdx^{3cv} mouse also partially preserved heart function compared to the *mdx*^{4cv} mouse (Wasala et al., 2017). The same level of dystrophin also ameliorated the pathology and increased the lifespan in the double knockout mouse when the utrophin knockout mouse was crossed with the mdx^{3cv} mouse (Li et al., 2010).

A number of studies have reported the effects of restoring different levels of dystrophin in the *mdx* mouse. 20% of normal levels of dystrophin induced by treatment with high doses of gentamicin significantly reduced the force deficit associated with eccentric exercise when compared to untreated mdx mice (Barton-Davis et al., 1999).

Sharp and colleagues (2011) examined the functional effects of exon-skipping arising from increasing intramuscular doses of the M23D phosphorodiamidate morpholino oligomer (PMO) sequence that was originally developed by Gebski and colleagues (2003). They showed a good correlation between the percentage of dystrophin positive fibres and the induction of dystrophin and that this also correlated with the reduction in force drop following eccentric exercise. They concluded that a minimum of 20% of dystrophin-positive fibers is required for any meaningful improvement in muscle physiology.

Godfrey and colleagues (2015) examined the functional consequences of inducing dystrophin expression using a cell penetrating peptide (Pip6a) coupled to the M23D PMO. They concluded that 15% of normal levels of dystrophin were sufficient to prevent the force drop associated with eccentric exercise in the tibialis anterior muscle of terminally anaesthetised *mdx* mice. Further studies performed in the Wells laboratory (in preparation) using a lower dose of Pip6aPMO confirm that expression of 15% of endogenous levels of dystrophin following chronic dosing studies is indeed sufficient to prevent the force drop associated with eccentric contractions and that lower levels offer some reduction in the force drop.

The Aartsma-Rus laboratory have developed a mouse model expressing low dystrophin levels, based on non-random X-inactivation. They crossed the *mdx* mouse with a transgenic mouse that shows a skewed X-inactivation (*Xist*$^{\Delta hs}$) that generates mice with a range of different levels of dystrophin (van Putten et al., 2012). They have used these mice to examine the effects of different levels of dystrophin on locomotor performance and response to chronic exercise. The 2012 study concluded that "while even dystrophin levels below 15% can improve pathology and performance, levels of >20% are needed to fully protect muscle fibers from exercise-induced damage". They have subsequently used the same model system to look at the effects of low levels of dystrophin on slowing the development of heart failure in the *mdx-Xist*$^{\Delta hs}$ mice (van Putten et al., 2014) but failing to normalize the neuromuscular synaptic abnormalities of *mdx-Xist*$^{\Delta hs}$ mice (van der Pijl et al., 2018). The same strategy was used in the dystrophin/utrophin double knockout mice with low levels of dystrophin increasing survival and improving muscle pathology and function (van Putten et al., 2013). However, it is not possible to directly compare the results from the *mdx-Xist*$^{\Delta hs}$ studies with those in the *mdx*^{3CV} mice and the Pip6aPMO studies as in the latter two examples there was fairly uniform expression of dystrophin between muscle fibres whereas the *mdx-Xist*$^{\Delta hs}$ mice show a mosaic pattern of dystrophin expression within and between muscle fibres. In contrast, the patchy expression in the mdx-Xist□hs mice may be a good model of a less than optimal response to gene therapy. It should also be noted that the *mdx-Xist*$^{\Delta hs}$ and the *mdx*^{3CV} mice have expressed dystrophin prior to the onset of the 3 week necrotic phase seen in the *mdx* mouse, whereas the Pip6aPMO studies were conducted in adult *mdx* mice that had already developed muscular dystrophy.

Effects of different levels of dystrophin in dog models.

There are relatively few studies of induced dystrophin expression in dystrophic dogs. While recent studies such as (Yue et al., 2015; Le Guiner et al., 2017) show the effectiveness of systemic delivery in the GRMD model, they do not allow a quantification of the minimum levels of dystrophin required

for clinical benefit. Le Guiner and colleagues (2014) used rAAV8-U7snRNA to promote permanent exon-skipping by locoregional delivery to the forelimb in GRMD. They determined a minimum threshold of dystrophin expressing fibres (>33% for structural measures and >40% for strength) under which there was no clear-cut therapeutic effect. Gentil and colleagues (2016) examined some of the same samples and compared the percentage of dystrophin-positive fibres with western blots of key proteins such as neuronal nitric oxide synthase mu (nNOSμ), inducible nitric oxide synthase (iNOS), and ryanodine receptor-calcium release channel type 1 (RyR1). They concluded that 40% of the fibres need to be dystrophin positive for normalisation. Neither of the two locoregional perfusion studies measured the quantity of dystrophin in the positive fibres. Thus, to date we really have no measure of the minimum amount of dystrophin required to be therapeutic in the dog.

Conclusions

A number of different strategies have been developed to restore dystrophin expression in skeletal and cardiac muscles and a wide range of animal models of DMD are available for testing these strategies. To date, the majority of such studies have been conducted in dystrophic mice and dogs. Data from man and animal models shows that any increase in dystrophin is likely to have some benefit and the more dystrophin the better. It is more difficult to assign a minimal level of dystrophin expression required for effective therapy of DMD. The most promising of the current strategies for induction of dystrophin result in an internally truncated dystrophin protein, which may have different properties from the normal full-length isoform. There are likely to be differences in the amount of dystrophin required to prevent the development of muscular dystrophy compared to that required to address existing disease. In the case of existing disease, there may well be differences between animal models and man, particularly in the case of the *mdx* mouse that manifests a relatively mild version of the disease and shows limited fibrosis compared to humans with DMD. The amount of dystrophin required is likely to vary with the stage of disease progression. Finally, the longevity of the treatment effect is also likely to be dependent on the level of dystrophin expression achieved and the uniformity of this expression both within and between individual myofibres or cardiomyocytes.

As a working hypothesis, it would appear that about 20% of normal levels of a functional version of recombinant dystrophin with a uniform expression is likely to be sufficient to essentially stop disease progression and thus offer major clinical benefit for DMD patients.

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