Pharmacologic Management of Duchenne Muscular Dystrophy: Target Identification and Preclinical Trials

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked human disorder in which absence of the protein dystrophin causes degeneration of skeletal and cardiac muscle. For the sake of treatment development, over and above definitive genetic and cell-based therapies, there is considerable interest in drugs that target downstream disease mechanisms. Drug candidates have typically been chosen based on the nature of pathologic lesions and presumed underlying mechanisms and then tested in animal models. Mammalian dystrophinopathies have been characterized in mice (mdx mouse) and dogs (golden retriever muscular dystrophy [GRMD]). Despite promising results in the mdx mouse, some therapies have not shown efficacy in DMD. Although the GRMD model offers a higher hurdle for translation, dogs have primarily been used to test genetic and cellular therapies where there is greater risk. Failed translation of animal studies to DMD raises questions about the propriety of methods and models used to identify drug targets and test efficacy of pharmacologic intervention. The mdx mouse and GRMD dog are genetically homologous to DMD but not necessarily analogous. Subcellular species differences are undoubtedly magnified at the whole-body level in clinical trials. This problem is compounded by disparate cultures in clinical trials and preclinical studies, pointing to a need for greater rigor and transparency in animal experiments. Molecular assays such as mRNA arrays and genome-wide association studies allow identification of genetic drug targets more closely tied to disease pathogenesis. Genes in which polymorphisms have been directly linked to DMD disease progression, as with osteopontin, are particularly attractive targets.

Key Words: animal models; drug development; Duchenne muscular dystrophy; golden retriever muscular dystrophy; genome wide association studies; mRNA arrays; mdx mouse; preclinical studies

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder that affects approximately 1 in 5000 newborn human males (Cowan et al. 1980) in whom absence of the protein dystrophin causes degeneration of skeletal and cardiac muscle (Hoffman et al. 1987). A less severe form, with in-frame *DMD* gene mutations, is termed Becker muscular dystrophy (BMD) (Malhotra et al. 1988). For sake of treatment development, considerable focus has appropriately been placed on introducing the *DMD* gene or correcting the underlying mutation (Foster et al. 2012; Konieczny et al. 2013). Some of these therapies involve pharmacologic approaches, as with compounds that promote stop codon read-through or correct frame-shift mutations. Drugs also have been developed to upregulate surrogate proteins that could take the place of dystrophin at the muscle cell membrane (Malik et al. 2012). Still others that target downstream mechanisms of disease and associated lesions, such as muscle necrosis, inflammation, fibrosis, or regeneration, have been studied (Malik et al. 2012; Ruegg 2013).

Animal models have been used extensively to identify targets for drug therapy and assess therapeutic efficacy. Spontaneous mammalian forms of X-linked muscular dystrophy due to dystrophin deficiency have been identified in mice (Bulfield et al. 1984; Gillis 1999), cats (Carpenter et al. 1989; Gaschen et al. 1992), pigs (Hollinger et al. 2013a), and multiple dog breeds (Figure 1) (Cooper et al. 1988; Kornegay et al. 1988, 2012a; Smith et al. 2011; Walmsley et al. 2010). The disease

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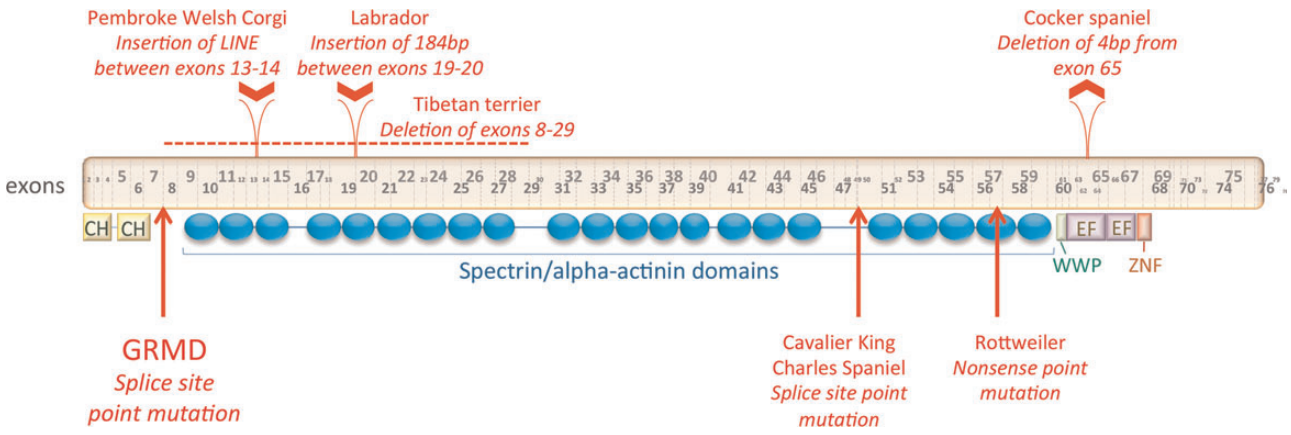

Figure 1 The canine dystrophin protein (Ensembl protein ID ENSCAFP00000031637), along with mutation information for seven dog breeds known to exhibit *DMD*-linked muscular dystrophy. CH indicates calponin homology domains, which are actin-binding. WWP indicates the WW domain, which binds proline-rich polypeptides and is the primary interaction site for dystrophin and dystroglycan. EF indicates members of the EF-hand family; this domain stabilizes the dystrophin-dystroglycan complex. ZNF represents a putative zinc-binding domain, ZnF_ZZ, which is present in dystrophin-like proteins and may bind to calmodulin. All 79 exons are represented. Exons and protein domains are depicted approximately to scale. Insertion and deletion mutations are shown above the exons. Arrows at the bottom of the figure indicate point mutations. Reprinted by permission from Bentham Science Publishers: *Current Genomics*. Comparative genomics of X-linked muscular dystrophies: The golden retriever model, 14:330–342, © 2013.

phenotype in dystrophic dogs is more severe than in mice, suggesting that canine studies might better translate to humans. Dogs with golden retriever muscular dystrophy (GRMD) have been used increasingly to define disease mechanisms and assess potential treatments (Kornegay et al. 2012a). In the context of these studies, marked phenotypic variation has been seen at the level of individual animals and different muscles. Such variability confounds statistical analysis in preclinical trials but also provides a platform to define disease pathogenesis.

This paper reviews the use of animals in developing pharmacologic therapies in a general sense and for DMD in particular. Emphasis is placed on drugs that either promote dystrophin or surrogate gene/protein expression or delay downstream pathogenetic events. Published preclinical studies targeting pathologic lesions or disease mechanisms are reviewed, highlighting the frequent failure of animal experiments to translate to human trials. We subsequently emphasize the potential for genetic assays, like mRNA expression profiles and genome-wide association studies (GWAS), to better identify drug targets, especially when results are correlated with phenotypic data.

Current Status and Therapeutic Goals

Glucocorticoids are the current standard of care for DMD (Manzur et al. 2008; Moxley et al. 2010). Beneficial effects of prednisone are poorly understood and cannot be explained by antiinflammatory properties alone (Kissel et al. 1993; Weller et al. 1991) (see treatments targeting *Muscle Inflammation* below). Despite beneficial effects of prolonged ambulation and improved cardiopulmonary function, glucocorticoids are often discontinued due to side effects ranging from weight gain to pathologic bone fractures (Connolly et al. 2002). This has prompted use of many different protocols and a search for alternative treatments, as with steroid analogues and other agents that target disease mechanisms. In this context, the ultimate goal is to identify a therapy that achieves at least the benefit of steroids, with fewer side effects.

Drug Development Regulatory Process

The Food and Drug Administration is governed by the Federal Food, Drug, and Cosmetic Act, which defines drugs as "articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals" and "articles (other than food) intended to affect the structure or any function of the body of man or other animals" (http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/default.htm). Therapeutic biologics (monoclonal antibodies and small protein molecules) are included in the definition of drugs (Roberts and McCune, 2008). The FDA generally becomes involved in the drug discovery process after the drug's sponsor (usually the manufacturer or potential marketer) has screened the new molecule for pharmacological activity and acute toxicity potential in animals and wishes to test its diagnostic or therapeutic potential in humans. The process for human use entails filing an Investigational New Drug Application and testing through a series of clinical trials (Phases 1–4), extending from smaller populations to establish safety (Phase 1) to large diverse clinical groups to determine efficacy (Phase 4) (Lesko et al. 2000). There are currently 121 DMD studies of diagnostic tests and treatment trials in various stages of initial enrollment to termination on the NIH Clinical Trials.gov website (http://www.clinicaltrials.gov/ct2/results?term=Duchenne+Muscular+Dystrophy&Search=Search). An analogous agency, the European Medicines Agency (http://www.ema.europa.eu/ema/index.jsp?curl=pages/home/Home_Page.jsp&mid=), regulates drug development in Europe (Lis et al. 2012).

There is high risk in drug development. Only 16% of drugs originating collectively from the 50 largest pharmaceutical companies in the United States between 1993 and 2004 were approved (DiMasi et al. 2010). The top two reasons for failed drug development in pharmaceutical companies in the United Kingdom from 1964 to 1985 were pharmacokinetics (PK) (39%) and efficacy (29%). With regard to PK data, it is especially important to pay close attention to differences among species (Lin 1995; Tibbitts 2003) and, in the case of dogs, even breeds (Fleischer et al. 2008). With more careful analysis of the PK properties of drugs and development of tools to better predict drug absorption and clearance, drug–drug interactions, and scaling of PK parameters from animals to man (Walker 2004), PK issues accounted for only 10% of drug failures in 2000 (Kola and Landis 2004). Beyond issues related to drug PK, problems with drug targeting and efficacy have persisted. A more recent study found that success rates for Phase II human clinical trials for drugs originating from 16 companies, representing 60% of global research and development spending, had fallen from 28% in 2006–2007 to 18% for 2008–2009 (Arrowsmith 2011). Alarmingly, this same review noted that 51% of 108 reported Phase II failures occurred due to insufficient efficacy, despite the fact that most drugs proceeded through standard validation methods, with assessment in animal models (Plenge et al. 2013). Paralleling the low success rate for pharmaceutical industry drug approval, only 28 of 76 (37%) highly cited studies that investigated a preventive or therapeutic intervention in an in vivo animal model over the 1980 to 2000 period were replicated in human randomized trials (Hackam and Redelmeier 2006). Overall, most of these animal efficacy studies included dose-response gradients, clinically relevant outcomes, and long-term endpoints, but few incorporated random animal allocation, adjustment for multiple hypotheses testing, or blinded outcome assessment. Animal studies incorporating dose-response gradients were more likely to translate to humans. A follow-up study provided 55 different recommendations to improve translation of preclinical research, including power calculation to determine sample size, randomized treatment allocation, and characterization of disease phenotype in the animal model prior to experimentation (Henderson et al. 2013). Additional papers have highlighted inconsistencies in experimental design and statistical analysis, with associated challenges in reproducing results, for a broad range of preclinical studies directed at neurological diseases (Benatar 2007; Sena et al. 2007; Steward et al. 2012). This led National Institute of Neurological Disorders and Stroke (NINDS) Director Story Landis and colleagues (Landis et al. 2012) to call for greater transparency in preclinical studies and more rigorous experimental design, to include random assignment of animals to treatment groups, blinding of data assessment, greater attention to power analysis of outcome parameters used to establish benefit, and increased stringency in data handling.

The "lost in translation" problem for preclinical research has been attributed to three issues, identified as the "Butterfly Effect" (chaotic behavior whereby small differences in the animal model lead to substantial differences in clinical results); the "Princess and the Pea" problem, based in variability of effect size when progressing from biochemical findings through tissue culture and animal and human studies (the pea does not indent the mattress to the same degree as the princess); and the "Two Cultures" problem in preclinical and clinical research, as evidenced by the data collection issues summarized above (Ergorul and Levin 2013). Some guidance for increasing the rigor of preclinical studies could be drawn from the "Animal Rule" put in place by the FDA to guide drug approval in instances of chemical, biologic, radiologic, and nuclear threats "when adequate and well-controlled clinical studies in humans cannot be ethically conducted and field efficacy studies are not feasible." In such cases, drugs may be approved for human use when four criteria are fulfilled: (1) data define pathophysiological mechanisms for the product; (2) the product effect is demonstrated in more than one species; (3) the animal study endpoint is clearly related to the desired benefit in humans; and (4) PK data in animals and humans are sufficiently well understood to allow selection of an effective dose in humans (Roberts and McCune 2008).

Animal Models

The National Research Council has defined a biomedical model as "a surrogate for a human being, or a human biologic system, that can be used to understand normal and abnormal function from gene to phenotype and to provide a basis for preventive or therapeutic intervention in human diseases" (National Research Council 1998). This same report emphasized that biomedical models can be "of many types – from animal models of human diseases to animal, *in vitro*, or modeling systems for studying any aspect of human biology or disease" and that "a model need not be an exact replica of a human disease or condition" (here, specifically citing the mdx mouse model of DMD/BMD). An earlier National Research Council study, conducted in 1985 before the discovery of the *DMD* gene, emphasized that biologic models can be divided into two broad classes, depending on whether the modelling is based on analogy or homology. Modelling by analogy implies a point-by-point relationship between one structure or process to another (National Research Council 1985). From a mathematical perspective, this is termed mapping. By definition, modelling by analogy requires that there are similarities between the structures and processes being compared in the modeling relationship. On the other hand, modelling by homology implies a shared evolutionary history and matching DNA makeup between the two structures or processes. Importantly, for models by homology to be functionally useful, they must also be good models by analogy for the phenomenon being studied. Unfortunately, as discussed further below in the context of DMD, homolog animal models are not always good analog models because of physiologic adaptions that may have occurred over time. Surrogate models may also be classified as either one-to-one, as with a disease state in humans and a particular species that share the same

clinical features (mdx or GRMD compared with DMD), or many-to-many, where findings from more than one species or organ system may model particular features of the underlying process or state (multiple features of DMD potentially being modeled by different available animal models or systems) (National Research Council 1985). The many-to-many approach is probably best suited to address questions raised by complex genetic diseases such as DMD.

The regulatory process discussed above highlights the critical role that animal studies play in essentially all phases of preclinical drug development, extending from the original target identification; determination of the drug's PK, absorption, distribution, metabolism, and excretion; potential toxicity to the treated animal or person; and ultimate efficacy against the intended disease (Roberts and McCune 2008). Efficacy studies have often been completed in symptomatic, experimental animal models, where a drug's efficacy is tested on a clinical effect, as with high blood pressure, versus the underlying disease itself. With the advent of the genomic era, specific genetic disease models have become more prevalent (Hunter 2011).

Genetic models may either occur naturally (spontaneously) or be produced through genetic manipulation (genetically modified [transgenic or gene knock-down/out] animals). For sake of DMD, both spontaneous and genetically modified animal models have been used to define pathogenetic mechanisms and establish efficacy of experimental therapies. Nonmammalian models, occurring either by natural *DMD* gene mutation or gene knock-down/out, have been characterized in the zebrafish (Gibbs et al. 2013), *Drosophila* fly (Shcherbata et al. 2007), and *Caenorhabditis elegans* nematode (Bessou et al. 1998). These models offer tremendous value in studying disease pathogenesis and developmental therapeutics.

Spontaneous mammalian models of DMD have been identified in the mouse (Bulfield et al. 1984; Gillis 1999), cat (Carpenter et al. 1989; Gaschen et al. 1992), pig (Hollinger et al. 2013a), and multiple dog breeds (Cooper et al. 1988; Kornegay et al. 1988, 2012a; Smith et al. 2011; Walmsley et al. 2010). Cats with *DMD* gene mutations express a curious debilitating hypertrophic myopathy and are predisposed to a syndrome similar to malignant hyperthermia during stress or anesthesia (Gaschen et al. 1992, 1998). These features limit the feline condition's value as a model. The malignant hyperthermia-like syndrome also occurs in DMD (Gurnaney et al. 2009) and occasional GRMD dogs (JN Kornegay, unpublished data). Pigs with a spontaneous *DMD* gene missense mutation and an analogous stress syndrome were recently described (Hollinger et al. 2013a; Nonneman et al. 2012). In addition, transgenic/knock-out gene technology can be applied to produce pigs with specific *DMD* gene mutations (Klymiuk et al. 2013), providing other valuable models (see the mdx52 model below).

From an investigational standpoint, drug discovery typically starts by developing and testing hypotheses using in vitro systems, including cell culture and ex vivo explants, and, increasingly, as discussed below, genetic assays. The DMD research field is fortunate to have nonmammalian and both small (mdx mouse) and large (GRMD; potentially pigs) mammal models available for follow-up mechanistic and preclinical studies. Once hypotheses have been substantiated in vitro or in nonmammalian models, treatments should ideally first be tested in the mdx mouse and, if efficacy is shown, moved to GRMD dogs before initiating DMD trials. But, as reflected by this review, because of expense, lack of familiarity, and relative scarcity, dystrophic dogs have not been widely used in pharmacologic testing. This deficiency could be partially countered by using the GRMD model in tandem with the mdx mouse in genetic assays to better identify relevant therapeutic targets and then more consistently testing drug efficacy first in mdx mice and then dystrophic dogs, especially if compounds carry substantial risk to humans.

DMD

To understand whether an animal disease truly models its counterpart in people, one must first appreciate the nuances of the human condition. For sake of preclinical drug efficacy studies, care should be taken in identifying the age over which treatment is administered and the natural history of outcome parameters during this period. Ideally, the time course for treatment and outcome parameters used to assess efficacy should approximate those of DMD. Patients with DMD have delayed milestones, typically not walking until approximately 18 months of age or later (Bushby et al. 1999, 2010). The diagnosis is generally not made until around 5 years of age. Other early symptoms that may be seen prior to diagnosis include frequent falls and difficulty in running and climbing stairs. By definition, affected boys are wheelchair bound by 12 to 13 years, although mild and severe forms of the disease may deviate from this trajectory (Nicholson et al. 1993c). In contrast, BMD patients walk beyond 16 years of age (Bushby et al. 2010). There is variation among DMD patients in other outcome parameters, including measures of strength and joint angles/contractures (Brooke et al. 1983; McDonald et al. 1995; Ziter et al. 1977). The rate of functional decline may also vary. Brooke et al. (1983) noted that children walk and climb stairs fairly easily until 8 years, when function declines rapidly. McDonald et al. (1995) found a relatively uniform decline of muscle strength from 5 to 13 years. Results from joint angle measurements, reflecting the degree of contracture, and strength testing tend to correlate (Brooke et al. 1983). Vignos et al. (1963) indicated that quadriceps strength was the single best predictor of time to wheelchair. The 6-minute walk test (6MWT) has become the principal outcome measure used in DMD clinical trials (Mazzone et al. 2013; McDonald et al. 2013a, 2013b). Results of the 6MWT correlate with wheelchair status (Mazzone et al. 2013; McDonald et al. 2013a) and knee extensor strength (McDonald et al. 2013b). Most affected boys die due to respiratory or cardiac disease by their late teens or early twenties, although life has been extended with more aggressive

supportive care (Eagle et al. 2007; Finsterer and Stöllberger 2003; Inkley et al. 1974).

Individual patients, muscles, and myofiber types are differentially affected in DMD (Ciciliot et al. 2013; McDonald et al. 1995). Variable involvement at the patient level suggests the possibility of genetic influences, as with modifier genes (see discussion below under *Pharmacogenomic Drug Targeting*). With regard to individual muscles, DMD is a proximal myopathy, with muscles like the quadriceps being selectively affected. In contrast, certain other muscles, such as those of the eye, are relatively spared (Karpati et al. 1988). Some muscles may even undergo paradoxical hypertrophy, as with classical calf pseudohypertrophy (Cros et al. 1989; Jones et al. 1983). Fast twitch (type IIb) myofibers are selectively affected, leading to characteristic slow twitch (type 1) predominance (Webster et al. 1988).

As discussed above and further below, GRMD dogs also demonstrate dramatic phenotypic variation. Moreover, differential muscle involvement is a prominent feature of both the GRMD and mdx models (reviewed in Kornegay et al. 2012b). Selective muscle involvement appears to occur because of mechanical factors at play during different stages of the disease. Eccentric (lengthening) contraction injury contributes to selective quadriceps involvement in DMD (Edwards et al. 1984). High usage and attendant necrosis of muscles, as with the tongue and certain flexors, early in life seems to result in a disordered regenerative response and hypertrophy. Data from the GRMD cranial sartorius (CS) muscle suggests that initial enlargement results from increased muscle mass (true hypertrophy) followed by deposition of connective tissue and fat (pseudohypertrophy) (Kornegay et al. 2003, 2012b). Individual patient/animal and muscle phenotypic variation must be considered when targeting DMD lesions or disease mechanisms.

Mdx Mouse

The mdx mouse has a spontaneous nonsense point mutation in exon 23 (Sicinski et al. 1989). Although genetically homologous to DMD, this model is not analogous, with affected mice having a relatively mild phenotype. After undergoing an acute wave of muscle necrosis at 3 to 5 weeks of age (Bulfield et al. 1984; Gillis 1999), mice largely recover and do not experience substantial clinical dysfunction until approximately 18 months of age (Lefaucheur et al. 1995). However, affected mice have progressive histopathologic changes and an approximately 20% reduction in life span (Chamberlain et al. 2007).

In keeping with this sequential disease course, Grounds (2008) has proposed a so-called "two-tier hypothesis" to explain disease progression in DMD and the mdx mouse. As discussed further below under recommendations to ensure consistency among mdx preclinical trials, emphasis has been placed on the need to develop different therapeutic strategies for the acute and chronic phases. Drawing parallels to DMD, the ages in mice and humans have been compared, with 3, 4, 6, and 8 weeks of age in mice roughly corresponding to about 6 months, 10 years, 16 to 18 years, and 20 years for humans, respectively (Grounds et al. 2008). In this sense, the acute muscle damage in mice at 3 to 5 weeks would correspond to a much more extended period in DMD boys.

With the mdx mouse and other murine models, transgenic/gene knock-out technology can be used to further modify the genotype and associated phenotype (Willmann et al. 2009). Most notably, the mdx phenotype is exaggerated when the utrophin gene is knocked-out to produce so-called double knock-out (dko) mice (Deconinck et al. 1997). Other genes can be knocked-out or silenced to study their role in disease pathogenesis. Although double mutants typically have a more severe phenotype that better models symptoms of DMD, the second mutation introduces a biochemical or biologic difference that could affect the disease course independent of the absence of dystrophin. Knock-out technology also allows specific *DMD* gene mutations to be produced, as with targeting exon 52, to create models to better explore therapeutic strategies like exon skipping (Aoki et al. 2012; Araki et al. 1997).

Reasons for the relatively mild mdx phenotype are not well defined. Bodor and McDonald (2013) have speculated that signs are more severe in larger species and individuals because of added stress placed on higher caliber muscle fibers. Grounds (2008) stressed that growth exacerbates necrosis in muscles lacking dystrophin and suggested that the proportionally longer life spans of humans and dogs could allow for additional bouts of necrosis and fibrosis and a more severe phenotype. Another factor might be the shorter telomere length and lower telomerase activity and associated reduced muscle regenerative capacity in humans and dogs versus mice (see mechanisms contributing to *Muscle Atrophy* below). Regardless of the exact cause(s), the relatively mild mdx phenotype has caused concerns about the degree to which findings will translate to humans. Additional questions have been raised because of the mouse's size and whether variables influenced by scale, such as cell migration or drug diffusion, can be appropriately modeled (Partridge 2013). As discussed further below, despite these reservations, international consensus has established the mdx mouse as the model of choice for preclinical and proof-of-concept studies, because they have the exact monogenic biochemical defect present in DMD (Willmann et al. 2009).

To increase consistency of mdx preclinical studies and improve translation to humans, efforts have been made to standardize selection of mice to be used (genetic background, gender, etc); husbandry practices (diet, stress, etc); experimental design (age at onset and route and duration of drug administration); and outcome parameters (De Luca 2012; Grounds et al. 2008; Nagaraju et al. 2009; Spurney et al. 2009; Willmann et al. 2012). A recent comprehensive review of the mdx mouse as a preclinical model (Grounds et al. 2008) put forth two major recommendations to ensure more consistent results: (1) Employ basic standard experimental regimes for preclinical testing. Building on the "two-tier hypothesis" discussed above, two potential regimes were recommended. In regime A, treatments would be started at 14 to 17 days of age before the acute wave of necrosis, with

Table 1 Outcome parameters that most effectively distinguish drug-induced changes in mdx mice

Test	Age (wk)	Wild-type (C57BL/10) mice[a]	Mdx mice[a]	Significance (*p* value)
Body weight (g)	10–12	22.17 ± 0.96	23.64 ± 1.33	0.0023
	38–40	26.93 ± 2.06	27.30 ± 1.50	NS
Grip strength (front) (normalized KGF/kg)	10–12	5.506 ± 0.274	4.409 ± 0.346	< 0.0001
	38–40	5.430 ± 0.722	4.054 ± 0.485	< 0.0001
Grip strength (hind) (normalized KGF/kg)	10–12	7.273 ± 0.380	6.244 ± 0.466	< 0.0001
	38–40	9.189 ± 1.207	8.455 ± 1.067	NS
Horizontal activity	10–12	1650 ± 270.75	1014.10 ± 230.03	< 0.0001
	38–40	1394.91 ± 344.50	1326.62 ± 416.72	NS
Rest time(s)	10–12	562.22 ± 8.45	576.50 ± 12.15	0.0012
	38–40	569.05 ± 13.17	569.63 ± 20.00	NS
LVID (d)	38–40	3.90 ± 0.14	3.70 ± 0.19	0.0077
LV%FS	38–40	30.63 ± 2.58	27.89 ± 1.86	0.0091
Systolic BP	38–40	79.15 ± 3.08	74.10 ± 8.62	NS
Diastolic BP	38–40	68.54 ± 6.50	50.60 ± 8.36	< 0.0001
Mean BP	38–40	71.85 ± 4.83	58.20 ± 8.08	0.0001
Peripheral nuclei/fiber	38–40	1.33 ± 0.11	1.142 ± 0.151	0.0211
Central nuclei/fiber	38–40	0.010 ± 0.007	0.633 ± 0.152	< 0.001
Total nuclei/fiber	38–40	1.34 ± 0.10	1.77 ± 0.28	0.0029
Serum CK (U/L)	38–40	85.20 ± 74.66	6439.66 ± 2506.59	< 0.001

From Spurney CF, et al. (2009).
[a]Data expressed as mean ± SD.
Abbreviations: BP, blood pressure; CK, creatine kinase; LVID (d), left ventricular diameter in diastole; LV%FS, left ventricular per cent shortening fraction; normalized, kg of force divided by body weight in kg; NS, not significant.

tissues sampled at 28 days and, potentially, 12 weeks or later. Regime B would utilize older (> 4 weeks) mice in which histopathologic changes have stabilized. For sake of these studies, mice would be exercised to further exacerbate phenotypic effects. (2) Employ basic methods to analyse the preclinical experiments. Longitudinal measurements of whole body function, ex vivo individual muscle strength, serum creatine kinase, and standardized histopathologic lesions should be completed. Willmann et al. (2012) made somewhat analgous recommendations in a subsequent review. To provide context for the anticipated benefit of an experimental therapeutic, they also suggested calculating a "recovery score" by comparing the differential between treated and untreated mdx and wild-type mice.

In assessing potential biomarkers to be used in the mdx mouse, Spurney et al. (2009) found that body weight, normalized grip strength, horizontal activity, rest time, cardiac function measurements, blood pressure, total central/peripheral nuclei per fiber, and serum creatine kinase were most effective for detecting drug-induced changes (Table 1). Given the mild phenotype of mdx mice, eccentric contraction and exercise are often used to exaggerate clinical signs. A forced treadmill protocol to accentuate the phenotype and increase likelihood of detecting drug effects over a 3- to 6-month trial period has been recommended (Spurney et al. 2009). Mdx and dko mice develop cardiomyopathy (Duan 2006; Spurney et al. 2011a) and respiratory disease (Huang et al. 2011), allowing for preclinical testing of drugs that target the heart (Spurney et al. 2011b, 2011c) and improve diaphragmatic function (Percival et al. 2012).

GRMD

Numerous canine breeds with dystrophin-deficient muscular dystrophy have been clinically characterized, but few have been studied at the molecular level (see Figure 1 above). We have conducted extensive studies in a dystrophin-deficient form of muscular dystrophy originally characterized in golden retrievers (GRMD) (Kornegay et al. 2012a). An mRNA processing error in GRMD dogs results from a single base change in the 3′ consensus splice site of intron 6 (Sharp et al. 1992). Exon 7 is consequently skipped during mRNA processing. The resulting transcript predicts termination of

Figure 2 Homozygous female GRMD dog (*Jelly*) at 6 years of age. Note the characteristic plantigrade stance, most noticeably carpal hyperextension and glossal hypertrophy.

the dystrophin reading frame within its N-terminal domain in exon 8. Compared with mdx mice, dystrophic dogs more closely mimic the DMD phenotype, with signs occurring early and progressing. With this said, even some dogs stabilize and live well into adulthood (Ambrosio et al. 2008).

Dystrophic pups are often ineffectual sucklers and exhibit stunted growth. Some have a particularly fulminant form in which severe dyspnea can lead to death or necessitate euthanasia during the neonatal period. By 6 weeks of age, the pelvic limbs may be simultaneously advanced and trismus is noted. Subsequently, dogs develop stilted gait; atrophy of particularly the truncal, temporalis, and certain extensor muscles; a plantigrade stance due to hyperextension of the carpal joints and flexion at the tibiotarsal (TTJ) joints; excessive drooling, suggesting pharyngeal muscle involvement; and initial lumbar kyphosis that progresses to lordosis (Figure 2). Whereas most muscles atrophy, some, such as the CS and tongue, hypertrophy (Kornegay et al. 2003, 2012b). Respiratory and cardiac involvement occurs and can be objectively assessed (DeVanna et al. 2014; Fine et al. 2011; Su et al. 2012). To partially explain variable disease involvement, Valentine et al. (1988) suggested that homozygous females and smaller dogs might have milder signs. Analogous moderation of signs in female mdx mice has been attributed to estrogen effects (Salimena et al. 2004). Bodor and McDonald (2013) cited Valentine's work, as well as a tendency for smaller beagle crosses with the GRMD mutation to have a less severe phenotype (Shimatsu et al. 2003; Yugeta et al. 2006), in making a case that size is a major contributor to disease severity in DMD and the animal models. Nonetheless, in assessing outcome parameters in our own GRMD preclinical studies, we did not find differential disease involvement in homozygous females and larger dogs (Kornegay et al. 2012a). Still, efforts should be made to balance gender and size in treatment groups.

The experimental design, outcome parameters, and sample size must be carefully considered to achieve significance in GRMD preclinical studies, especially in light of phenotypic variation. Clinical signs of GRMD progress particularly rapidly between the ages of 3 and 6 months. Thus, somewhat akin to the mdx mouse, the natural history of GRMD provides a relatively short window over which therapies can be assessed. Comparative longevity studies for dogs and humans suggest that the first year of a golden retriever's life roughly equates to 20 years of a human (Patronek et al. 1997). By extrapolation, the 3- to 6-month age in a GRMD dog would correspond to 5 to 10 years of a DMD boy, a period over which symptoms also progress markedly (Brooke et al. 1983; McDonald et al. 1995). A variety of outcome parameters, in many cases modeled after analogous procedures used to assess DMD patients and mdx mice, has been developed (Table 2) (Kornegay et al. 2012a). Efforts have been made to standardize methods used for these procedures in dogs in parallel with those for mice (Nagaraju et al. 2009). For our own natural history studies, we have generally collected data at 3 and 6 months of age to be in sync with the period used for preclinical studies. To counter the effects of phenotypic variation on data analysis, baseline (pretreatment) values should first be established at 3 months and compared

Table 2 Outcome parameters in GRMD dogs

Test	Age (mo)	Normal dogs	GRMD dogs	Significance (p value)	Reference
Body weight (kg)	3	10.65 ± 1.75	7.47 ± 1.21	< 0.01	Kornegay et al. 1999
	6	20.24 ± 2.30	12.86 ± 3.08	< 0.01	
	12	23.17 ± 1.70	18.23 ± 3.22	< 0.01	
TTJ tetanic flexion (normalized N/kg)	3	0.486 ± 0.142	0.200 ± 0.094	< 0.01	Kornegay et al. 1999
	6	0.825 ± 0.256	0.469 ± 0.183	< 0.01	
	12	1.10 ± 0.27	0.550 ± 0.200	< 0.01	
TTJ tetanic extension (normalized N/kg)	3	2.55 ± 0.28	1.32 ± 0.43	< 0.01	Kornegay et al. 1999
	6	2.95 ± 0.53	0.965 ± 0.506	< 0.01	
	12	2.98 ± 0.28	1.34 ± 0.58	< 0.01	
Speed (m/sec)	2	1.77 ± 0.29	1.02 ± 0.24	0.001	Barthélémy et al. 2011
	9	2.61 ± 0.18	0.88 ± 0.46	<0.0001	
Stride length/height at withers	2	1.97 ± 0.26	1.35 ± 0.27	0.0001	Barthélémy et al. 2011
	9	1.99 ± 0.06	0.92 ± 0.31	<0.0001	
LVID (d) (cm)[a]	3	3.0 ± 0.0	2.5 ± 0.2	< 0.01	Fine et al. 2011
	6	3.6 ± 0.1	2.9 ± 0.1	< 0.01	
	12	4.1 ± 0.2	3.2 ± 0.1	< 0.01	
Fraction shortening (%)[a]	3	36.9 ± 2.5	35.0 ± 1.2	NS	Fine et al. 2011
	6	33.7 ± 0.5	39.3 ± 2.6	NS	
	12	32.3 ± 1.7	38.8 ± 6.5	NS	
EKG lead 2 Q/R ratio[a]	3	0.2 ± 0.0	0.4 ± 0.1	< 0.01	Fine et al. 2011
	6	0.3 ± 0.0	0.7 ± 0.0	< 0.01	
	12	0.3 ± 0.0	0.6 ± 0.1	< 0.01	
Serum CK[b]	2 days	700 (200–800)[c]	18,900 (2,300–39,500)	ND	Valentine et al. 1988
	6 wk	300 (200–500)	8,200 (6,500–162,100)	ND	
	6	400 (400–400)	32,400 (30,300–42,100)	ND	

Listed tests were assessed longitudinally and are at least somewhat analogous to those of Mdx mice in Table 1.
Abbreviations: CK, creatine kinase; LVID (d), left ventricular diameter at diastole; ND, not determined; N/kg, Newtons/kg; TTJ, tibiotarsal joint; NS, not significant.
[a]Cardiac data were from GRMD dogs crossbred with Labrador retrievers carrying a different DMD gene mutation.
[b]CK results for CXMD (GRMD) dogs were from male "small breed" dogs.
[c]Median and range for CK values.

with those at the end of treatment. In this way, the relative longitudinal effect of systemic treatments can be established. For local (intramuscular) or regional (single limb) approaches, the opposite untreated limb can serve as a control. Although independent control groups for each preclinical trial should ideally be assessed, budgetary and animal number limitations may necessitate the use of natural history controls.

Loss of ambulation is the defining clinical feature of DMD and could logically be used to characterize disease phenotype in GRMD. However, although occasional severely affected dogs lose the ability to walk, most remain ambulatory. We have used tetanic torque/force generated by TTJ flexion/extension as a key outcome parameter in GRMD dogs (Kornegay et al. 1999). Our original natural history study suggested that measurement of flexion torque would be most useful to document therapeutic benefit in GRMD dogs. Groups of 15 and 5 were necessary to demonstrate differences of 0.2 and 0.4 in the means of treated and untreated GRMD dogs at 6 months of age, with associated powers of 0.824 and 0.856, respectively. The use of flexion as an outcome parameter is complicated by a relative recovery of strength between 3 and 6 months. Extension values in this initial study varied more markedly, necessitating larger group sizes to establish significance. Since this original study, we have more carefully monitored inbreeding coefficient in selecting sire-dam pairs. As a result, the overall phenotype has been less severe and not as variable. In a subsequent trial of prednisone in GRMD dogs, we demonstrated therapeutic benefit of approximately 60% ($p < 0.05$) for extension force using a group size of six GRMD dogs (Figure 3). Interestingly, there was a paradoxical decrease in flexion force, which we attributed to reduced early necrosis that would otherwise lead to functional flexor hypertrophy. Consistent with this interpretation, there were reduced numbers of fetal myosin positive myofibers, indicating reduced regeneration. Another study of GRMD dogs treated with prednisone and

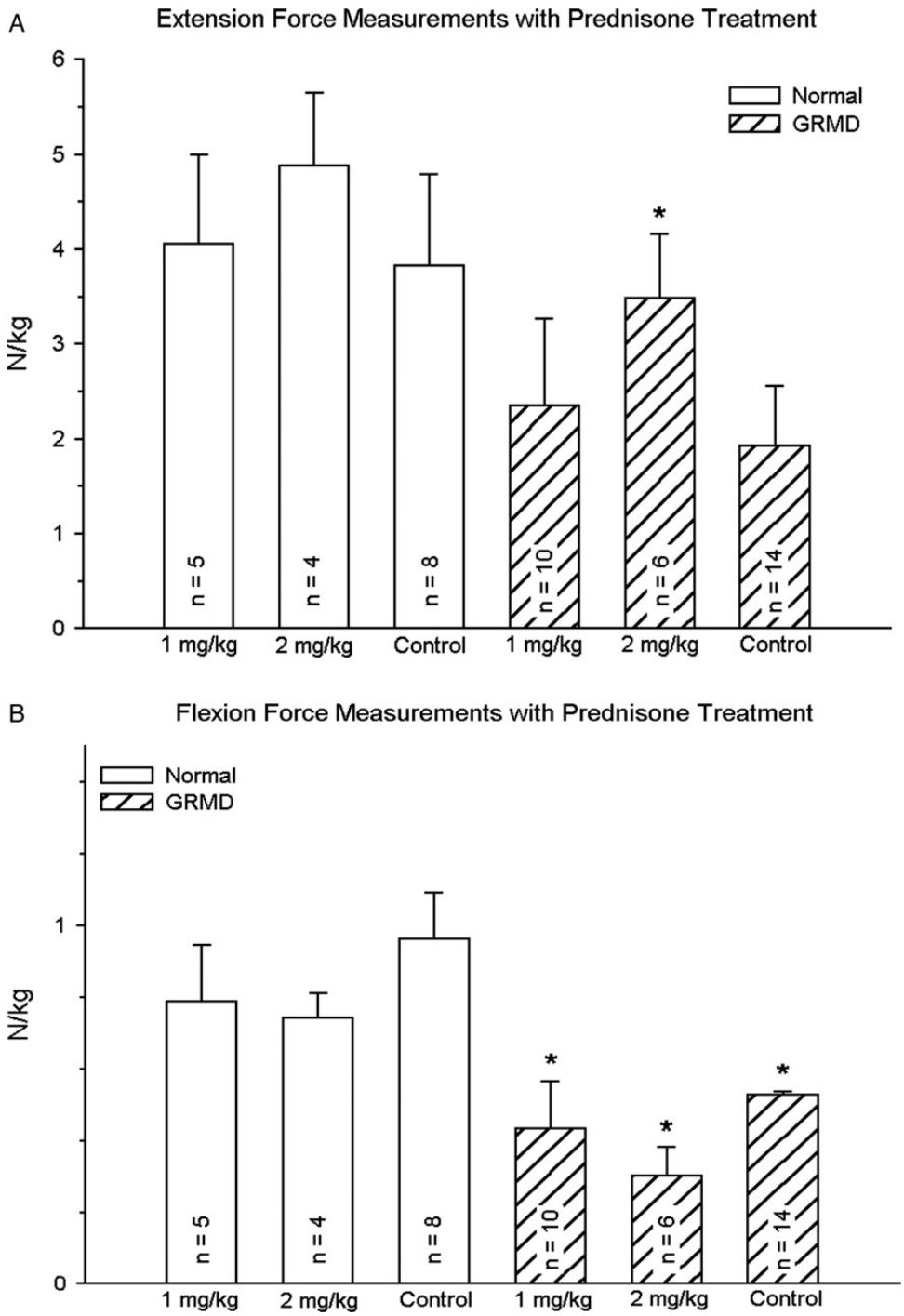

Figure 3 Tetanic force in prednisone-treated GRMD dogs. Note the reverse pattern for extension and flexion. For extension (A), a trend for increased force at 1 mg/kg becomes significant at 2 mg/kg. Flexion is similar (B) but values are decreased. *$p < 0.05$ compared with normal. Data are from Liu et al. 2004.

cyclosporine found a similar decrease in flexion force (Barthélémy et al. 2012).

As with DMD, results of GRMD functional tests track and correlate with each other (Kornegay et al. 2011, 2012a; Nghiem et al. 2013). Dating to our early phenotypic studies, we have been intrigued with the relationship between the TTJ angle and torque generated by extension and flexion of this joint. These values have also been placed in the context of CS muscle hypertrophy. Mildly affected dogs have proportionally larger TTJ angles and tetanic extension torques and smaller CS circumferences and tetanic flexion torques at 6 months of age. The opposite pattern is seen in severely affected dogs. Given the prominent role that the 6MWT now plays in DMD clinical trials, we have begun assessing this metric in GRMD dogs and correlating results with other functional outcome parameters. Providing precedent for applying

the 6MWT to canine studies, abnormalities have already been shown in dogs with heart (Boddy et al. 2004) and respiratory (Swimmer and Rozanski 2011) disease. In our experiments, dogs are first conditioned/trained for several sessions beginning at approximately 8 wks of age and then evaluated at 2-week intervals. Our preliminary data indicate that affected dogs walk shorter distances than normal/carrier dogs.

The GRMD cardiomyopathy has a later clinical onset than the skeletal muscle phenotype, with, as an example, echocardiographic changes not being seen until at least 6 months of age and often considerably later (Kornegay et al. 2012a). Thus, ideally, for determining drug effects on the dystrophic heart, dogs should be older when entered into preclinical trials, or the cardiac phenotype should be exaggerated by stressing the heart, as with dobutamine (Maruo et al. 2007; McEntee et al. 1998). A membrane-sealing poloxamer was shown to be beneficial using group sizes of four each treated and untreated 13- to 18-month–old GRMD dogs that had been challenged with dobutamine (Townsend et al. 2010). The differential between poloxamer- and saline-treated dogs varied from approximately 50% to 100% for the physiologic indices tested in these dogs. Another study of the peptide, bradykinin, showed normalization of most cardiac indices using four and five untreated and treated 11-month–old GRMD dogs (Su et al. 2012) (see *Muscle Fibrosis* below).

Optimization of Animal Model Use for DMD Preclinical Trials

As discussed above and in greater detail below for treatments targeting DMD, preclinical studies in animal models often have failed to translate to humans. To optimize translation of findings from the mdx mouse and GRMD models to DMD patients, the following points should be considered:

- Design powered studies with sample sizes sufficient to detect drug effects with the outcome paramaters used.
- Follow randomization and blinding procedures, including who is blinded and when.
- Provide details of the statistical methods used for data analysis and report all the results for each analysis.
- Develop reliable and sensitive primary and secondary endpoints for the animal model used.
- Independently validate drug efficacy results in another laboratory.
- Validate drug efficacy in two species (e.g., mdx mice and GRMD dogs for DMD) whenever possible and especially with treatments that carry substantial risk.

DMD Treatment Development

Potential treatments for DMD may be broadly categorized as genetic, cellular, or pharmacologic (Foster et al. 2012; Konieczny et al. 2013; Malik et al. 2012; Ruegg 2013). In the context of pharmacologic approaches, some drugs promote stop codon read-through, correct frame-shift mutations, or upregulate surrogate proteins. Others target specific pathogenetic mechanisms that contribute to the dystrophic phenotype.

Genetic Therapies

Genetic and cell-based therapies, in principle, offer the possibility of cure. Genetic strategies have included adeno-associated virus (AAV)-mediated insertion of truncated dystrophin transgenes, antisense oligonucleotides to induce exon skipping and reestablish the dystrophin reading frame, agents to read-through stop codon mutations, and replacement of dystrophin at the sarcolemma with surrogates such as utrophin. Animal models have been important to establish potential efficacy and safety. In the case of AAV-mediated mini-/micro-dystrophin transgene therapy, foundational studies in the mdx mouse (Wang et al. 2000; Yuasa et al. 1998) were extended to the GRMD (CXMD) model (Kornegay et al. 2010; Wang et al. 2007; Yuasa et al. 2007). Canine experiments have demonstrated long-term dystrophin protein expression and highlighted the potential for immunologic rejection of viral capsid antigens and/or dystrophin acting as a neoantigen (reviewed in Kornegay et al. 2012a).

Antisense Oligonucleotide-Induced Exon Skipping and Chimeraplasty

Although viral-mediated gene replacement (augmentation) is a logical approach to therapy, other methods rely on innate systems intended to repair mRNA or remove introns to form the transcript. The basis for applying antisense therapies to DMD resides in the observation that BMD patients have in-frame mutations that allow production of truncated, partially functional dystrophin proteins (Bushby et al. 1993; Malhotra et al. 1988). Pioneering work by Louise Nicholson demonstrated that truncated isoforms of dystrophin, apparently produced through transcript alternative splicing, occur in DMD patients (Nicholson 1993a; Nicholson et al. 1993b). Alternatively spliced dystrophin isoforms have also been demonstrated in mdx mice (Chamberlain et al. 1993) and GRMD dogs (Schatzberg et al. 1998). As a corollary, so-called revertant fibers, which express dystrophin, occur spontaneously in DMD (Arechavala-Gomeza et al. 2010) and both animal models (Kornegay et al. 2003; Pigozzo et al. 2013).

Antisense oligonucleotides are designed to complement specific pre-mRNA sequences so that targeted exons are removed (spliced, skipped) at the level of the spliceosome, reestablishing the reading frame (Chen and Cheng 2012; Summerton and Weller 1997). Strategies were developed principally in the mdx mouse (Mann et al. 2001) and then applied to dystrophic dogs (Bish et al. 2012; Vulin et al. 2012; Yokota et al. 2009, 2012) before moving to DMD. Efficacy of different oligomer sequences and chemical backbones was first established in each species using cultured muscle cells (Arechavala-Gomeza et al. 2007; Mann et al. 2001; Vulin et al. 2012; Yokota et al. 2009). Trials in DMD patients

have relied on two principal antisense oligomer chemistries, 2′O-methylribooligonucleoside-phoshophorothioate and phosphorodiamidate morpholino oligomers (morpholinos), marketed, respectively, by Glaxo-Smith-Kline/Prosensa (drisapersen) (Goemans et al. 2011) and Serepta (eteplirsen) (Cirak et al. 2012). Each oligomer targets exon 51, a notable *DMD* gene hot spot involved in approximately 13% of cases. Despite promising initial results in DMD patients, the drisapersen trial was recently stopped because of concerns about efficacy (Hoffman and Connor 2013). The eteplirsen trial continues. Methods to improve delivery of the antisense peptides and, therefore, achieve wider skeletal muscle and cardiac effects, as well as increase the duration of efficacy, are needed (Benedetti et al. 2013; Betts and Wood 2013; Partridge 2010).

An analogous method, termed chimeraplasty, employs chimeric oligonucleotides to induce normal host cell mismatch repair mechanisms to correct single nucleotide point mutations (Graham and Dickson 2002). Chimeric oligonucleotides injected intramuscularly into a single dystrophic dog achieved correction of the GRMD mutation and expression of a normal-sized dystrophin protein for 48 months (Bartlett et al. 2000). Chimeraplast-mediated exon skipping also was demonstrated in the mdx mouse (Bertoni et al. 2003). Despite these successes, inconsistencies in chimeraplasty studies for other diseases have dampened enthusiasm for this approach (de Semir and Aran 2006).

Stop-Codon Read-Through

An alternative genetic strategy for DMD employs drugs, such as gentamicin, that selectively promote translational readthrough of premature stop codons (Hirawat et al. 2007; Nudelman et al. 2009). This therapeutic strategy applies to the approximately 15% of DMD patients who have nonsense mutations (Dent et al. 2005) resulting from single nucleotide DNA polymorphisms that give rise to in-frame UAA, UAG, or UGA codons in messenger RNA coding regions. These stop codons lead to premature termination of protein translation, with resultant truncated, nonfunctional proteins. Gentamicin was initially shown to increase dystrophin readthrough in the mdx mouse, first using cultured myotubes and then moving to in vivo studies, where treated mice expressed dystrophin in muscle and had functional improvement (Barton-Davis et al. 1999). Subsequent gentamicin treatment of DMD patients harboring stop codons provided mixed results, with one study failing to show full-length dystrophin protein or functional improvement (Wagner et al. 2001) and another demonstrating dystrophin protein expression (Politano et al. 2003).

Because of concerns regarding potential toxicity of higher dose or longer duration gentamicin regimens, a high-throughput, screening tool was developed to identify chemical compounds with equal efficacy and high safety profiles. This process led to the discovery of PTC124, subsequently called ataluren, which is chemically distinct from aminoglycosides. Ataluren suppressed *DMD* gene nonsense mutations in mdx muscle cell culture and led to dystrophin expression and functional improvement in mice treated systemically (Welch et al. 2007). These encouraging results prompted DMD trials with ataluren, starting with Phase 1 studies in healthy volunteers that showed no toxicity and extending to Phase 2 trials in DMD patients. The DMD trials have generated inconclusive results. Whereas the initial Phase 2a study demonstrated dystrophin expression in about one-third of the treated patients, the larger 2b study showed only marginal 6MWT benefit and did not include dystrophin protein expression data (Hoffman and Connor 2013; Peltz et al. 2013).

Dystrophin Surrogates (Utrophin)

A number of proteins colocalize with dystrophin at the sarcolemma and could serve as surrogates (Nghiem et al. 2013). As examples, upregulation of GalNAc transferase (Martin et al. 2009) and sarcospan (Peter et al. 2008) ameliorated the mdx phenotype. Particular interest has focused on utrophin (dystrophin-related protein), the autosomal isoform of dystrophin (Fairclough et al. 2011; Miura and Jasmin, 2006), with a number of factors suggesting that it could have therapeutic benefit in DMD. Normally expressed chiefly at the neuromuscular synapse and myotendinous junction (Khurana et al. 1991; Nguyen et al. 1991), utrophin moves extrajunctionally towards the muscle belly in the absence of dystrophin (Karpati et al. 1993; Nguyen et al. 1991) and is upregulated in DMD (Karpati et al. 1993; Mizuno et al. 1993), the mdx mouse (Law et al. 1994), and the GRMD (Nghiem et al. 2013; Wilson et al. 1994) and German shorthaired pointer (Schatzberg et al. 1999) dog models. Mdx mice, in which utrophin is knocked out (dko mice), have a more severe phenotype (Deconinck et al. 1997). Upregulation of utrophin, either genetically (Tinsley et al. 1996) or through treatment with the small molecule SMTC1100 (Tinsley et al. 2011), improves the mdx phenotype. High-throughput, cell-based screens have been used to identify drugs that upregulate utrophin (Moorwood et al. 2013). Treatment of mdx mice with recombinant human biglycan, an extracellular matrix protein associated with the dystrophin-glycoprotein complex, recruits utrophin to the sarcolemma and reduces histopathologic lesions and the degree of eccentric contraction decrement (Amenta et al. 2011). Adenovirus-mediated utrophin therapy reduced histopathologic lesions in GRMD dogs (Cerletti et al. 2003). However, other canine studies have not validated a role for utrophin in improving phenotype. Utrophin was not differentially expressed in mildly and severely affected GRMD dogs (Zucconi et al. 2010) or in the less-severely affected German shorthaired pointer model (Schatzberg et al. 1999). Although utrophin was increased in the spared/hypertrophied GRMD CS muscle, levels did not correlate with the degree of muscle hypertrophy (Nghiem et al. 2013).

Cell Therapies

As with genetic approaches, cell based-therapies provide an opportunity to replace dystrophin, achieving a relative cure.

In light of the loss of muscle mass with chronic disease, cell replacement also offers the potential to reverse muscle atrophy (Meregalli et al. 2013). Studies of myoblasts and their parent satellite cells (Briggs and Morgan 2013) have been complemented by experiments utilizing stem cell populations in both the mdx (Benedetti et al. 2013) and GRMD (Rouger et al. 2011; Sampaolesi et al. 2006) models, with some approaches moving to human trials (Benedetti et al. 2013).

Drug Target Identification

There are numerous hurdles with gene and cell-based therapies. As examples, viral vector constructs or cell populations may be rejected immunologically, and older affected individuals have chronic, essentially irreversible muscle loss. Therefore, although these approaches will remain a major focus of research, additional emphasis has been placed on development of therapeutic strategies that target downstream disease mechanisms. In the early days of DMD treatment development, there were three main hypotheses for disease pathogenesis. Over and above the membrane theory that was ultimately substantiated, considerable support existed for neurogenic and vascular mechanisms (Rowland 1976). Interest in these other two theories was driven, in large part, by the nature of histopathologic changes, namely myofiber atrophy and central nuclei in keeping with denervation (McComas et al. 1970, 1988) and small group myofiber necrosis typical of muscle ischemia (Mendell et al. 1971). From these early days and extending to the present, pharmacologic targets principally have been identified based on the nature of pathologic lesions (Figure 4) and underlying pathogenetic mechanisms. Here, we discuss treatment strategies in the context of the progression of DMD lesions, extending from acute necrosis to fibrosis with atrophy.

Myofiber Necrosis

Membrane lesions in DMD are generally thought to allow leakage of calcium, with activation of proteases like calpain, leading to necrosis (accidental cell death) (Miller and Girgenrath 2006). Apoptosis (programmed cell death) mediated through the ubiquitin proteasome system (UPS) also may play a role in DMD pathogenesis (Tidball et al. 1995). Accordingly, pathways associated with necrosis and apoptosis are potential drug targets in DMD (De Paepe and De Bleecker 2013) (see discussion of calpain and UPS inhibitors as potential therapies under *Muscle Atrophy* below). Indeed, dystrophin surrogates and poloxamer-based therapies, which have been efficacious in both the mdx mouse (Ng et al. 2008; Spurney et al. 2011c) and GRMD dog (Townsend et al. 2010), are intended to repair membrane injury.

The membrane theory for DMD pathogenesis derived, in part, from the overlapping nature of histopathologic changes like hyaline fiber necrosis (Figure 4B) seen in both DMD and nutritional myopathies resulting from reduced levels of the antioxidants vitamin E and selenium (Clark 1984; Kakulas 1975; Serafin et al. 1987). However, muscle lesions are not typically seen in vitamin E-deficient humans (Binder et al. 1965),

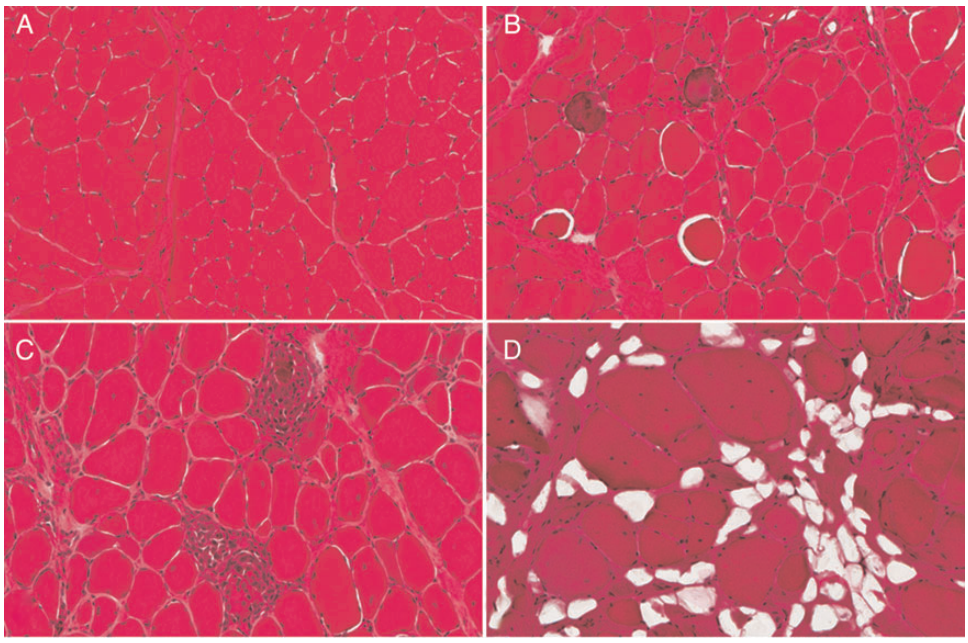

Figure 4 The histologic appearance of normal canine muscle (A) is contrasted with characteristic progressive histopathologic lesions of GRMD (B–D). (B) Several myofibers are swollen/hypercontracted (hyalin necrosis), and two myofibers are mineralized (calcified). The endomysial space is relatively normal. (C) Two small groups of myofibers have undergone necrosis and there is an associated inflammatory cell infiltrate. The endomysial space is mildly expanded. (D) The endomysial space is markedly expanded due to both fibrosis and fatty deposition. Individual myofibers are enlarged and many have central nuclei. H&E stain and 20 × original magnification for all.

and antioxidant treatment has not been beneficial in DMD patients (Bäckman et al. 1988; Gamstorb et al. 1986). Moreover, vitamin E supplementation did not substantially improve the mdx mouse phenotype (Hübner et al. 1996). With this said, there is continued interest in the role of oxidants in DMD pathogenesis, and oxidative stress remains a target for therapeutic intervention (Arthur et al. 2008; Lawler 2011). Pentoxifylline (Burdi et al. 2009), N-acetylcysteine (Whitehead et al. 2008), green tea extract (Nakae et al. 2012; Evans et al. 2010), and idebenone (Buyse et al. 2009) are examples of compounds with antioxidant properties shown to benefit mdx mice (see further the role that some of these compounds have in inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway under *Muscle Inflammation* below). In a subsequent clinical trial of idebenone, DMD patients had improved indices of respiratory (expiratory) function and a trend toward increased cardiac contractility (Buyse et al. 2011). Angiotensin-converting-enzyme (ACE) inhibitors have several potentially beneficial effects in muscle, with one being reduction of oxygen radicals (Cozzoli et al. 2011), and are a current standard of care for DMD cardiomyopathy (see further under *Muscle Fibrosis* below).

Mitochondrial dysfunction and associated failed energy metabolism have been implicated in DMD pathogenesis, acting in concert with reactive oxygen species and altered calcium homeostasis (Godin et al. 2012). The mitochondrial permeability transition pore (PTP), whose induction can lead to mitochondrial swelling and cell death by apoptosis, has become a key therapeutic target (Rasola and Bernardi 2007). In particular, treatments have targeted cyclophilin D, which regulates PTP opening in response to calcium and reactive oxygen species (Giorgio et al. 2010). Treatment of mdx mice with the cyclophilin inhibitor, Debio-025, reduced mitochondrial swelling and light microscopic histopathologic lesions (Millay et al. 2008). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) is another target because of its role in activities beneficial to mitochondrial function, including reduced PTP opening, increased mitochondrial mass, and improved calcium handling (Godin et al. 2012), as well as upregulation of utrophin (Angus et al. 2005). Injection of an AAV6-PGC1α construct in mdx mice led to upregulation of utrophin and improved functional and histopathological indices (Hollinger et al. 2013b). Treatment of mdx mice with two agents, AICAR and GW50156, that increase expression or are activated by PGC1α, also improved mitochondrial function and phenotype (Jahnke et al. 2012).

An additional therapeutic approach to reduce muscle necrosis harkens back to the vascular theory of DMD pathogenesis and relates to the loss of neuronal nitric oxide synthase (nNOS) due to the absence of dystrophin and instability of its associated membrane complex. Without nNOS, muscle blood flow is reduced (Chang et al. 1996; Thomas et al. 1998), likely contributing to the characteristic grouped myofiber necrosis of both ischemia and DMD. Pharmacological enhancement of nitric oxide–cyclic guanosine monophosphate signaling pathways with phosphodiesterase 5 inhibitors, as with sildenafil, enhanced nNOS activity and improved the mdx phenotype (Percival et al. 2012). Despite these promising results, a clinical trial in which sildenafil was assessed for its potential benefits in DMD and BMD cardiomyopathy has suspended patient recruitment (http://clinicaltrials.gov/ct2/show/NCT01168908?term=sildenafil&rank=32).

Myofiber Calcification

Numerous studies have focused on the role of calcium homeostasis in DMD pathogenesis. This is a logical extension of studies of mitochondrial function and oxygen reactive species given the interrelated role that these factors have in cell function. Although calcium has a nonspecific role in numerous cell injury pathways, histopathologic studies have shown that levels are increased in muscle biopsies from DMD patients more so than other myopathies (Bodensteiner and Engel 1978). Muscle injury in the mdx mouse is associated with increased entry of calcium through channels activated by reactive oxygen species (Allen et al. 2010), providing potential targets for therapy. An analogous increase in calcium is seen pathologically in GRMD muscle (Figure 4B) (Valentine et al. 1989). Creatine supplementation of mdx muscle cell cultures improved calcium handling and promoted formation and survival of myotubes (Pulido et al. 1998). Benefits of the tricyclic antidepressants, imipramine and amitriptyline, in mdx mice were explained, in part, by effects on calcium homeostasis in skeletal muscle (Carre-Pierrat et al. 2011). On the other hand, mdx mice treated with dantrolene, which inhibits the release of calcium from the sarcoplasmic reticulum, did not show benefit (Quinn et al. 2013). Perhaps most importantly, a Cochrane review of DMD clinical trials found calcium antagonists had no useful effect (Phillips and Quinlivan 2008).

Muscle Inflammation

Myofiber necrosis in DMD (McDouall et al. 1990), the mdx mouse (Carnwath et al. 1987), and GRMD dog (Figure 4C) (Kornegay et al. 1988; Valentine et al. 1990) elicits a mixed inflammatory cell response, with macrophages and lymphocytes predominating. Macrophages were originally thought to play principally a phagocytic role. They are now seen as having a dual purpose, with M1 macrophages occurring acutely to phagocytize cell debris, followed closely by M2 macrophages to drive myofiber regeneration (Kharraz et al. 2013; Tidball and Villalta 2010). A complex set of cytokines (tumor necrosis factor, interkeukins, transforming growth factor beta [TGFβ], and osteopontin [OPN]) (De Paepe and De Bleecker 2013; Evans et al. 2009) and transcription factors (NF-κB, E2F1) (Blanchet et al. 2012; Chen et al. 2005; Peterson et al. 2011) are involved in DMD pathogenesis.

The cytokine OPN is particularly intriguing in that mRNA levels are increased in DMD (Chen et al. 2000) and mdx mouse (Porter et al. 2003) muscle (also see mRNA arrays discussion below). OPN is secreted by inflammatory cells and myoblasts and serves multiple, at times seemingly contradictory, functions

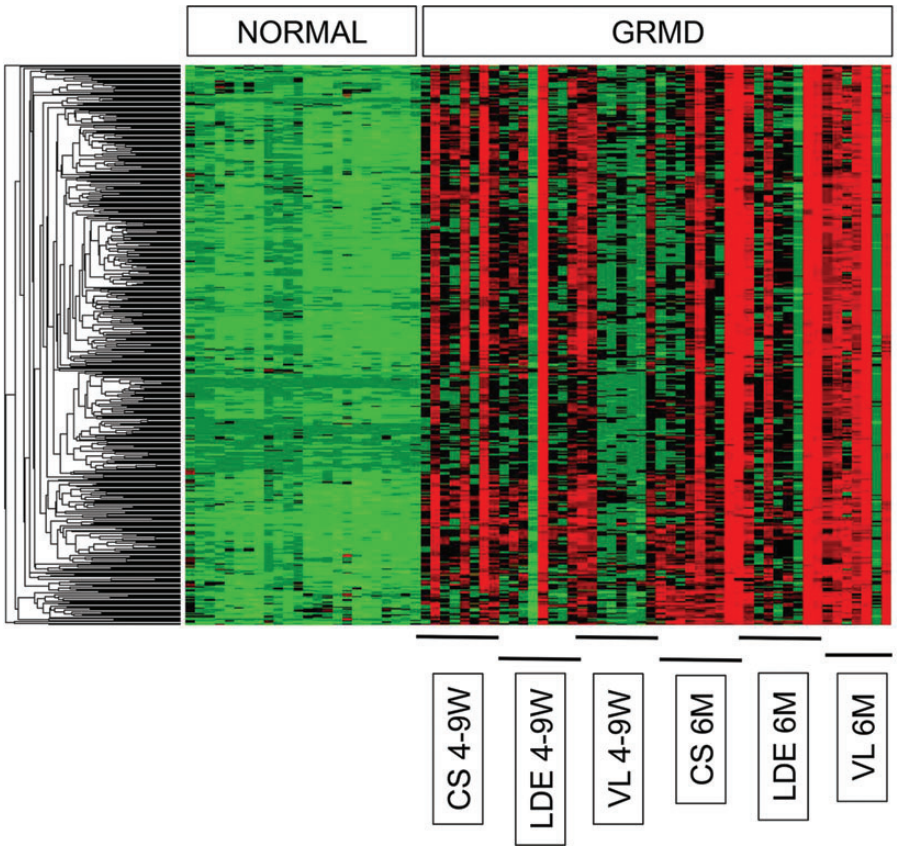

Figure 5 Heat map depicting supervised hierarchical clustering in GRMD dogs. A total of 485 genes correlated with OPN expression in 72 mRNA expression profiles from normal and GRMD CS, long digital extensor (LDE), and vastus lateralis (VL) muscles (Nghiem et al. 2013) at 4 to 9 weeks and 6 months. All mRNA profiles were correlated with OPN mRNA expression, and the top correlated genes ($r \geq 0.9$; $p \leq 0.001$; 485 genes) are depicted here. Note that the OPN-correlated genes (1) have minimal to no expression in normal muscle profiles, (2) increase in expression with age in GRMD profiles, (3) increase in expression in the more affected GRMD muscles at the respective time point, and (4) are variably expressed even within muscles.

in diseased muscle. Expression has been linked to increased fibrosis in the mdx mouse (Vetrone et al. 2009) and enhanced muscle regeneration in a murine injury model (Uaesoontrachoon et al. 2013). Moreover, the same OPN promoter polymorphism is associated with greater disease severity in DMD (Bello et al. 2012; Pegoraro et al. 2011) and enhanced muscle mass in normal human females (Hoffman et al. 2013). Working with the Hoffman laboratory, we have demonstrated that OPN is also dramatically increased in muscle from GRMD dogs (Figure 5), with levels negatively correlating with CS muscle size (PP Nghiem, JN Kornegay, K Uaesoontrachoon, L Bello, LW Fisher, Y Yin, Z Wang, A Kesari, P Mittal, SJ Schatzberg, NH Lee, EP Hoffman, unpublished data). Multiple factors, including stage of disease and isoform types, probably contribute to these differing effects of OPN.

With regard to transcription factors, research has focused on the NF-κB pathway, which exacerbates muscle lesions and dysfunction in DMD and the mdx mouse (Acharyya et al. 2007; Monici et al. 2003). Treatments targeting NF-κB are of particular interest in DMD, because prednisone blocks this pathway (Auphan et al. 1995). A number of compounds that inhibit the NF-κB pathway have been shown to benefit the mdx mouse (Figure 6) (Table 3). As an example, inhibiting NF-κB signaling with Nemo Binding Domain (NBD) peptide alleviates dystrophic histopathologic lesions and improves muscle function in DMD mouse models (Delfin et al. 2011; Peterson et al. 2011). We have collaborated with the Guttridge laboratory at The Ohio State University on a study of NBD in GRMD dogs, employing a previously performed treatment protocol and biomarker analysis (Liu et al. 2004). Consistent with observations in mice, NBD treatment in GRMD dogs exhibited an efficacious response, providing further support for its potential use as a DMD therapeutic (JN Kornegay, DC Guttridge, unpublished data). Importantly, studies have linked NF-κB and OPN in a number of disease processes in which inflammation and fibrosis occur. For instance, OPN mediates activation of the NF-κB pathway in hepatic fibrosis (Urtasun et al. 2012) and relapsing episodes of multiple sclerosis (Steinman 2009). On the other hand, NF-κB induction of OPN may contribute to fibrosis in myocardial infarction (Zhang et al. 2010). Both molecules were increased, and levels correlated positively, in synovial fluid of osteoarthritis patients (Qin et al. 2013). These findings suggest that NF-κB and OPN may be interrelated through a

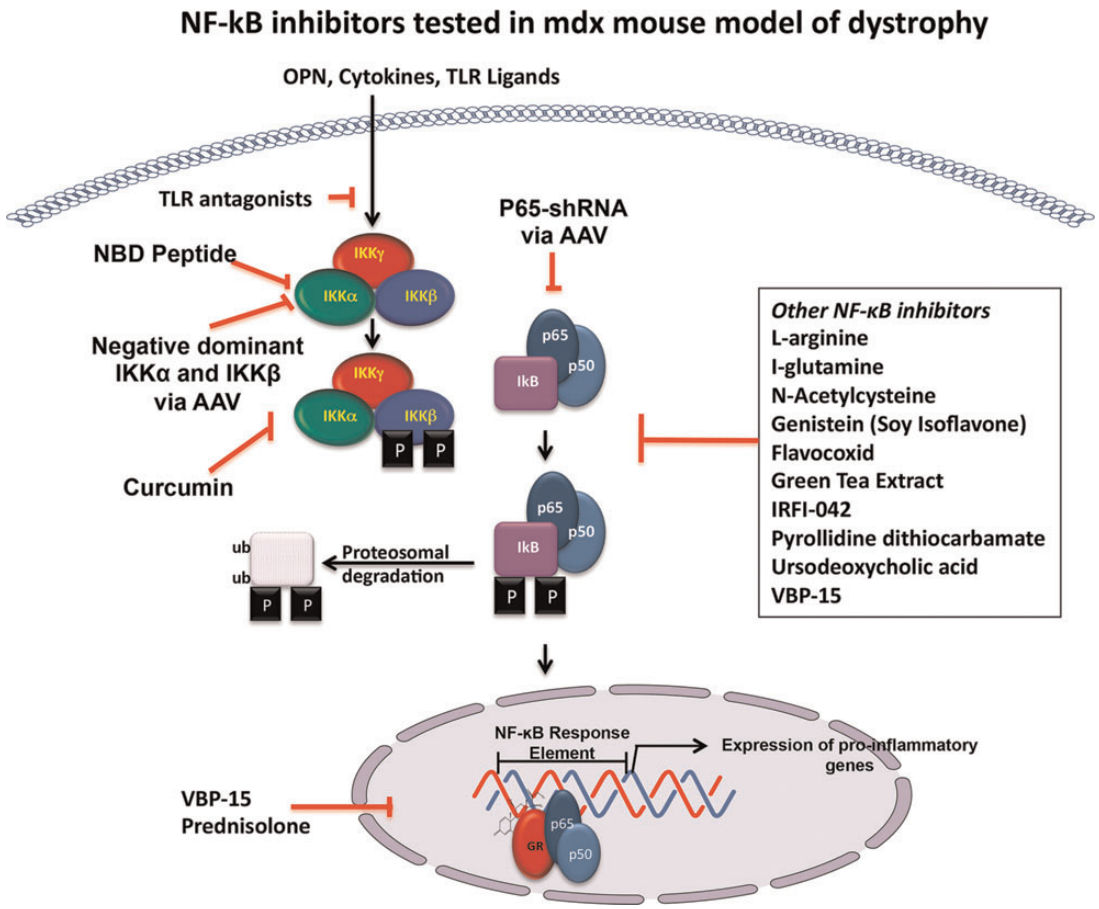

Figure 6 NF-κB inhibitors tested in mdx mice. Inhibition of the NF-κB pathway, utilizing mechanisms illustrated here, has shown phenotypic benefit in the mdx mouse (also see Table 3).

feedback loop (Zhang et al. 2010). Accordingly, treatments inhibiting the NF-κB pathway (Figure 6, Table 3) likely exert their effect, in part, through reduced OPN expression. Other downstream OPN effectors would also be potential therapeutic targets (Jain et al. 2007). The statin drug, simvastatin, reduced expression of NF-κB, OPN, and collagen I in a rat model of myocardial infarction and could have therapeutic value in DMD (also see discussion of *Muscle Fibrosis* below). More specific OPN targeting using humanized antibodies directed against OPN isoforms involved in particular diseases also has shown promise (Dai et al. 2010; Fan et al. 2008, 2011). Further definition of OPN isoforms involved in DMD and subsequent antibody targeting warrants further investigation.

Aside from macrophages, other inflammatory and immune cells play important roles in the pathogenesis of DMD and the mdx and GRMD models (Evans et al. 2009; Iannitti et al. 2010; Tidball and Villalta 2010). Mast cells are associated with areas of myofiber necrosis in all three conditions, suggesting that released proteases could contribute to cell injury (Gorospe et al. 1994). DMD patients and GRMD dogs had more persistent degranulation, perhaps allowing prolonged release of mediators (heparan sulfate, tryptase) that would enhance fibrosis. Cromolyn administration to block mast cell degranulation reduced myofiber necrosis in mdx mice (Radley and Grounds 2006). Populations of CD4 and CD8-positive lymphocytes have also been seen in DMD (Engel and Arahata 1986), mdx mice (Spencer et al. 2001), and GRMD dogs (Barthélémy et al. 2012), further highlighting involvement of the immune system in disease pathogenesis. Of particular note, dystrophin, serving as a neoantigen in revertant fibers, may induce a lymphocytic response in DMD (Flanigan et al. 2013). Depleting immune cell populations and general immunosuppressant regimens have improved the mdx phenotype (Evans et al. 2009; Iannitti et al. 2010; Vetrone et al. 2009). Recent studies by Nagaraju's laboratory demonstrated that proinflammatory innate immune receptors such as toll-like receptors (TLRs) on muscle and immune cells play an important role in dystrophic lesions in mdx mice (Henriques-Pons et al. 2014). Knockout of the central TLR adaptor protein, myd88, in mdx mice improved skeletal and cardiac muscle function. Likewise, preclinical trials in young mdx mice with a TLR7/9 antagonist significantly reduced skeletal muscle inflammation and increased muscle force, demonstrating that inhibiting this pathway may have therapeutic potential for DMD.

Given the role of inflammation in disease pathogenesis, it is tempting to speculate that prednisone's benefit in DMD and somewhat mixed effects in the mdx mouse (Guerron et al.

Table 3 NF-κB therapeutic approaches tested in the mdx mouse

Intervention	Pathology	Function	Reference
AAV-p65-shRNA	Yes	No	Yang et al. 2012
Nemo Binding Domain Peptide	Yes	Yes	Delfin et al. 2011; Peterson et al. 2011
Nemo Binding Domain Peptide	Yes	Yes	Reay et al. 2011
AAV-IKKa-dn or -IKKbdn	Yes	No	Tang et al. 2010
Pyrollidine Dithiocarbamate	Yes	No	Carlson et al. 2005; Graham et al. 2010; Messina et al. 2006a
Ursodeoxycholic Acid (UDCA)	Yes	Yes	Siegel et al. 2009, 2011
Curcumin	Yes	Yes	Pan et al. 2008
Curcumin	No	Yes	Durham et al. 2006
Flavocoxid	Yes	Yes	Messina et al. 2009
IRFI-042	Yes	Yes	Messina et al. 2006b
Genistein	Yes	Yes	Messina et al. 2011
Green Tea Extract	Yes	No	Evans et al. 2010
L-Arginine	Yes	No	Hnia et al. 2008
L-Glutamine	Yes	No	Mok et al. 2008
N-Acetylcysteine	Yes	No	Whitehead et al. 2008
VBP-15	Yes	Yes	Heier et al. 2013
TLR-7/8 Antagonist	Yes	Yes	Henriques-Pons et al. 2014

2010; Sali et al. 2012) and GRMD dog (Barthélémy et al. 2012; Liu et al. 2004) occur, at least partially, from reduced inflammation. In support of this, mdx mice treated with prednisone had reduced inflammatory cell numbers and adhesion while showing reduced sarcolemma damage and myofiber degeneration-regeneration (Wehling-Henricks et al. 2004). Dystrophic dogs given prednisone alone or with cyclosporine generally show an improved phenotype but have increased numbers of calcified myofibers, potentially due to a reduction of macrophage activity (Barthélémy et al. 2012; Liu et al. 2004). Interestingly, DMD patients treated with another antiinflammatory agent, azathioprine, did not have a comparable beneficial effect despite suppression of the same inflammatory cell populations, suggesting mechanisms other than immunosuppression are involved (Griggs et al. 1993; Kissel et al. 1993). Similarly, numbers of CD4 and CD8-positive cells in GRMD dogs treated with the prednisone-cyclosporine regimen were comparable with those in untreated GRMD controls (Barthélémy et al. 2012). Although prednisone has a well-established catabolic effect on protein, treated DMD patients have a net increase in muscle mass, thought to occur through decreased muscle proteolysis versus increased protein synthesis (Rifai et al. 1995). But, prednisolone also promotes myogenesis, with associated utrophin upregulation, in mdx myoblast cultures (Passaquin et al. 1993). Other compounds, like VBP15 (Heier et al. 2013) and a proprietrary drug (Compound A) (Huynh et al. 2013), which have been extensively studied in the Hoffman and Nagaraju laboratories, benefit the mdx mouse through inhibition of the NF-κB pathway (Figure 6) (Table 3), without side effects of glucocorticoid receptor stimulation. Importantly, OPN levels were decreased in mdx mice treated with Compound A but increased in those receiving prednisone (Huynh et al. 2013).

Muscle Fibrosis

Fibrosis is readily seen histologically in DMD (Pearce and Walton 1962) and the mdx (Stedman et al. 1991) and GRMD (Figure 4D) (Kornegay et al. 1988; Valentine et al. 1989) models. Increased endomysial connective tissue occurs early in DMD and correlates with clinical disease progression (Desguerre et al. 2009). Mediators of fibrosis, therefore, are logical therapeutic targets (Klingler et al. 2012). Attention has centered on TGFβ, which is involved in fibrosis in DMD (Bernasconi et al. 1995), the mdx mouse (Gosselin et al. 2004), and GRMD dog (Passerini et al. 2002). As discussed above under *Muscle Inflammation*, NF-κB induction of OPN also may contribute to fibrosis in myocardial infarction (Zhang et al. 2010), providing an additional potential target for therapy. Angiotensin activates or increases expression of TGFβ (Morales et al. 2012), NF-κB (Muller et al. 2000), and OPN (Remus et al. 2013). Accordingly, ACE inhibitors can provide benefit at several levels and are recommended for DMD patients at the onset of cardiac clinical dysfunction (Spurney 2011d). Studies in mdx mice have suggested that ACE inhibitors may also benefit skeletal muscle and that

treatment during the preclinical stage of DMD could be indicated (Cozzoli et al. 2011). Early treatment of mdx mice with the ACE-inhibitor, lisinopril, together with the aldosterone inhibitor, spironolactone, reduced fibrosis and improved function of both cardiac and skeletal muscle (Rafael-Fortney et al. 2011). In other studies, treatment of mdx mice with the angiotensin II receptor antagonist, losartan, reduced fibrosis in the heart and skeletal muscles (Bish et al. 2011a; Cohn et al. 2007; Spurney et al. 2011b). Although the cardiac phenotype was improved, functional benefit in skeletal muscle varied. Treatment of GRMD dogs with the vasodilator peptide, bradykinin, which is degraded by ACE and increased by ACE inhibitors, improved a number of cardiac functional parameters (Su et al. 2012). TGFβ-inhibitors, acting through different mechanisms, have shown variable effects in the mdx mouse, with some demonstrating functional and/or pathologic benefit (Cohn et al. 2007; Nelson et al. 2011; Taniguti et al. 2011) and others showing only minimal effects (Gosselin et al. 2006, 2007). Thus, ACE inhibitors and angiotensin receptor blockers have a clear indication in reducing cardiac fibrosis and potential analogous benefit in skeletal muscle.

Muscle Atrophy

All of the lesions/mechanisms discussed above, extending from necrosis to fibrosis, contribute to muscle wasting and the ultimate atrophy (sarcopenia) that characterizes DMD (Shin et al. 2013). Analogous changes occur in the mdx mouse and GRMD models. However, the dramatic muscle wasting seen in DMD does not occur until late in mdx mice, when muscles are approximately one-half normal size (Lefaucheur et al. 1995). In contrast, atrophy occurs earlier in GRMD (Kornegay et al. 2003; Valentine et al. 1990). Neither mdx mice nor GRMD dogs show the same degree of fatty deposition seen in DMD. Reasons for these species differences are not clear. Anderson et al. (1993) found higher expression of basic fibroblast growth factor in the mdx mouse versus DMD patients and GRMD dogs. Given that basic fibroblast growth factor promotes proliferation of muscle precursor cells (Abdel-Salam et al. 2009), they speculated this could explain the greater regenerative response in mdx mice.

Muscle regeneration is dependent on differentiation of resident satellite cells to myoblasts, which fuse to form myotubes and, ultimately, new myofibers (Briggs and Morgan 2013). Cellular proliferation is facilitated by telomeres, regions near the ends of chromosomes that deter degradation of genes during chromosome replication (Bojesen 2013). The enzyme telomerase is necessary for addition of DNA sequence repeats to the telomere (Holysz et al. 2013). Muscle atrophy in DMD is associated with satellite cell senescence (Heslop et al. 2000; Jejurikar and Kuzon 2003; Webster and Blau 1990) and telomere shortening (Decary et al. 2000). Species variation in normal telomere length and telomerase activity could account for the more severe phenotype of DMD and GRMD versus the mdx mouse. Cells of humans and dogs have relatively short telomeres, in the 5- to 15-kb range, and low telomerase activity (Argyle and Nasir 2003; Cross et al. 1989). Mice have considerably longer telomeres, in the 20- to 70-kb range and as long as 140 kb (Kipling and Cooke 1990), and proportionally higher telomerase activity (Prowse and Greider 1995). Lending support for this mechanism of phenotypic variation is the fact that knocking out the mdx mouse telomerase gene results in more severe signs (Sacco et al. 2010).

Muscle atrophy in DMD could result from either increased protein degradation or reduced production (Griggs and Rennie 1983). An early study completed before dystrophin was identified, and drawing somewhat on protein data from nongenetic animal models available at the time, concluded that DMD resulted from reduced production versus degradation (Rennie et al. 1982) (also see effects of prednisone under *Muscle Inflammation* above). Of course, with *DMD* gene mutations, there is inherent reduced production of stable dystrophin protein. Genetic therapies are directed at restoring dystrophin and, in turn, additional members of the dystrophin-glycoprotein complex. Other approaches are less selective and involve the use of anabolic agents and hormones. Much attention has focused on the anabolic hormone, insulin-like growth factor, which promotes muscle hypertrophy by activating the phosphatidylinositol 3-kinase/Akt pathway and, in turn, mTOR and further downstream targets. Insulin-like growth factor was among a group of compounds that improved the mdx phenotype in one preclinical screen using whole-body strength as an outcome parameter (Granchelli et al. 2000) and increased force generation in miniature bioartificial mdx muscles in an ex vivo system (Vandenburgh et al. 2009). On the other hand, mdx mice made transgenic for insulin-like growth factor developed muscle hypertrophy only during periods of muscle regeneration subsequent to bouts of necrosis (Shavlakadze et al. 2010). An additional study showed benefit in mdx mice treated early but not those with more chronic involvement or in dko mice (Gehrig et al. 2012). As discussed above under *Muscle Inflammation*, prednisone has a net anabolic effect on muscle. A novel androgen receptor modulator, GLPG0492, improved both functional and pathologic biomarkers in mdx mice (Cozzoli et al. 2013).

Inhibition of the UPS and calpain systems, which contribute to muscle degradation in DMD, has been proposed as a treatment based largely on evidence that activities of involved enzymes are elevated in the mdx mouse and that blockade can improve their phenotype (Badalamente and Stracher 2000; Bonuccelli et al. 2007; Spencer et al. 1995; Spencer and Mellgren 2002). In support of this hypothesis, systems involved in protein degradation are misregulated in the muscular dystrophies (De Palma et al. 2012; Sandri et al. 2013). Moreover, a defect in autophagy in mdx mice, associated with abnormal Akt and mTOR signaling, was reversed by a low-protein diet, with reduced histopathologic changes and improved function (De Palma et al. 2012). Additionally, proteasome inhibition restored members of the dystrophin-glycoprotein complex in DMD muscle explants and reduced the severity of histopathologic lesions in mdx mice (Gazzerro et al. 2010). But, other mdx mouse studies failed to show efficacy for either calpain inhibition (Selsby

et al. 2010) or overexpression of an antiapoptosis protein (Dominov et al. 2005). Similarly, we were unable to demonstrate benefit of a novel calpain inhibitor in GRMD dogs (Childers et al. 2012). In a separate collaboration with the Willis laboratory at the University of North Carolina-Chapel Hill, mRNA expression of UPS and calpain system components and calpain 1 and 2 and proteasome activities were measured in several GRMD muscles and the heart at 6 months of age (Wadosky et al. 2011). A majority of muscles had decreased levels of proteasome activity, and only one-half had increased calpain activity. More alarmingly, numerous UPS components were decreased in the heart. Taken together, these results do not support pharmacologic inhibition of the UPS and calpain systems in DMD and suggest that there could be unintended deleterious consequences.

Another strategy for reversing muscle atrophy involves inhibition of the myostatin gene (growth/differentiation factor 8), which serves as a key negative regulator of muscle growth (Lee 2004; McPherron et al. 1997). Mdx mice in which myostatin is knocked out (Wagner et al. 2002) or postnatally inhibited (Wagner et al. 2005) have a less severe phenotype. In collaboration with the Xiao Xiao laboratory at the University of North Carolina-Chapel Hill, we showed that regional limb AAV8-mediated overexpression of the inhibitory myostatin propeptide enhanced muscle growth in normal dogs (Qiao et al. 2009). A similar systemic approach generated analogous results in GRMD dogs evaluated by the Sweeney laboratory at the University of Pennsylvania (Bish et al. 2011b). In an effort to validate the mdx mouse-myostatin knockout studies, we crossbred dystrophin-deficient GRMD dogs with whippets carrying a spontaneous two-nucleotide myostatin gene deletion (Mosher et al. 2007). Surprisingly, rather than showing improvement, the dystrophic myostatin-heterozygous *GRippet* dogs were more severely affected than their dystrophic myostatin-wild type littermates (JN Kornegay, DJ Bogan, JR Bogan, JL Dow, J Wang, Z Fan, LC Warsing, RW Grange, M Ahn, Z Zhu, MA Styner, KR Wagner, unpublished data). Disproportionate enlargement and atrophy/hypoplasia of certain muscles appeared to lead to postural instability and joint contractures in the *GRippets*.

An antibody intended to reduce myostatin's activity was assessed in adult muscular dystrophy patients, including some with BMD (Wagner et al. 2008). While no safely issues were seen, the trial was not sufficiently powered to show clinical efficacy.

Pharmacogenomic Drug Targeting

The present low rate of translation of pharmacologic therapies between animal models and their human counterparts highlights a strong need for better, more effective ways to extract meaningful information from cross-species studies. Part of the solution, undoubtedly, lies in increasing experimental rigor and transparency in animal experiments, as outlined above. A critical paradigm shift in designing and interpreting studies requires expanding the perception of animal models to "many-to-many" rather than "one-to-one" when investigating their correlations with the human diseases they model. It's also important to note that species differences often reflect individual differences within a species. Another piece of the puzzle likely will be based on moving from the pathologic lesion/pathogenetic mechanism approach that has traditionally been used to identify drug targets to one that is based more in specific gene/protein targets that have been clearly associated with disease phenotype (Hoffman et al. 2003). Towards this end, gene expression profiling studies have been published for DMD, the mdx mouse, and, more recently, GRMD dogs. Correlations across species can be interpreted when comparing profiles built using analogous tissues and conditions. In this way, gene expression profiles from different species can illustrate the similarities and differences in species-specific responses to pharmacologic agents of interest. These comparisons give insight into the relevancy of each animal model and its applicability for investigating specific aspects of human disease pathogenesis and treatment (Yu et al. 2012). Ultimately, these studies should provide a better understanding of disease pathogenesis and identify molecular therapeutic targets, with cross-species relevance, to modify or arrest disease progression (Barrie et al. 2012).

mRNA Arrays

Remarkable phenotypic variation occurs among dystrophin-deficient species that share the same genetic lesion. Similar differences occur among individuals and muscles within a species, raising questions about primary versus secondary effects of dystrophin deficiency (Hoffman and Gorospe 1991; Porter et al. 2003). Microarray gene expression profiling and follow-up protein studies in DMD and dystrophin-deficient animals have provided insight into how genes other than dystrophin modify the disease phenotype. Porter et al. (2003) showed that differential gene expression in the mdx mouse is closely associated with the degree of individual muscle involvement and longitudinal disease progression. Differential gene expression was far more pronounced in more severely affected limb muscles compared with the relatively spared extraocular muscles. Temporal gene expression measurements in limb muscle were associated with distinct stages of disease (e.g. development) and different gene ontologies. Gene expression differences between normal and affected mice early in the disease were associated with inflammation, proteolysis, and the extracellular matrix/fibrosis pathways. Furthermore, these differences tracked with the progression of histopathologic changes. Specific reference was made to a potential role for OPN in disease pathogenesis. A later paper compared gene expression between an mdx limb muscle and the diaphragm, which shows more progressive disease (Porter et al. 2004). Overall, gene expression patterns were similar, suggesting that general disease mechanisms are shared between both tissues. With this said, evidence of differential gene expression pointed to disease processes that are potentially tissue specific. As an example, the diaphragm showed more persistent

expression of genes tied to muscle regeneration, in keeping with progressive disease. Thus, again, the microarray expression data tracked well with disease phenotype.

Tseng et al. (2002) compared mRNA expression in the mdx mouse at 16 weeks of age, when the disease is relatively quiescent, with previously published array results from 6- to 9-year-old DMD boys with ongoing disease (Chen et al. 2000). They found several genes that could account for the less-severe mdx phenotype. Most notably, mRNAs for actin-related proteins and mast cell chymase were upregulated in mdx mice versus DMD patients. Actin-related proteins might stabilize the dystrophic myofiber membrane, whereas mast cell chymase activates matrix metalloproteinases involved in collagen degradation. In addition, the expression levels of certain genes involved in mitochondrial function and energy metabolism were downregulated in DMD, potentially contributing to a so-called "metabolic crisis" in affected patients, but unchanged in mdx mice, relative to control samples. Finally, mRNA for myostatin was decreased 4-fold in mdx mice (myostatin was also downregulated in DMD, but data were not discussed).

In an extension of their earlier study, Chen et al. (2005) assessed gene expression in three DMD age groups: fetal, presymptomatic infant (8–10 months), and symptomatic 5- to 12-year olds. Genes involved in inflammation and muscle regeneration were upregulated in the fetal group, and this increased gene expression persisted in the older patients. TLR and NF-κB pathway genes were upregulated even in presymptomatic infants, whereas expression levels of TGFβ and major histocompatibility complex (MHC) genes were higher in the 5- to 12-year-old group. On the other hand, a cluster of genes associated with metabolism was downregulated, in keeping with the proposed "metabolic crisis" of DMD. Pathways potentially involved in muscle wasting/atrophy were also queried. Although the AKT1/atrogin ubiquitin ligase pathway has been implicated in a number of muscle-wasting disorders, it was not activated in any DMD age group. Similarly, while blocking myostatin to enhance muscle regeneration has been logically proposed as a therapy for DMD, myostatin levels were already downregulated in symptomatic patients, and the myostatin inhibitor, follistatin, was increased in all three age groups.

An additional DMD array study found differential expression, mostly upregulation, of genes involved in inflammation, muscle regeneration, and cell signaling (Haslett et al. 2002). These authors commented that gene expression patterns largely paralleled histopathological lesion progression, providing credence for the traditional approach used to identify drug targets. In particular, OPN was markedly upregulated and there were less pronounced increases in biglycan and TGFβ. Another paper from this group emphasized that genes involved in cell signaling, particularly survival cascades, were differentially expressed in DMD (Haslett et al. 2003), thus bringing attention to an important, but somewhat overlooked, role of the dystrophin glycoprotein complex.

A recent meta-analysis of DMD mRNA array data largely confirmed conclusions reached through earlier published studies (Baron et al. 2011). In particular, differentially expressed genes fell largely into two clusters, namely overexpressed genes coding for proteins involved in extracellular cell-matrix adhesion and inflammatory-immune response and underexpressed genes coding for mitochondrion proteins. The meta-analysis validated published observations that differentially expressed genes largely reflect infiltrating inflammatory cells and proliferating connective tissue and that the patterns of gene expression correspond to major pathological features of DMD.

Protein studies in GRMD dogs have identified abnormalities in energy metabolism (Guevel et al. 2011) and signaling (Feron et al. 2009) consistent with those defined by gene expression microarrays in DMD. Extending those studies, we have assessed mRNA microarrays in tandem with real time quantitative reverse transcription PCR (qRT-PCR) and protein expression (proteomic profiling, Western blotting, and immunohistochemistry) to define the molecular basis for phenotypic variation and, in particular, CS hypertrophy in GRMD dogs (Nghiem et al. 2013). Notably, we found that CS size was inversely correlated with myostatin expression in 6-month-old GRMD dogs. Utrophin mRNA was upregulated in the CS, but levels did not correlate with size. Using a quantitative trait analysis, we queried mRNAs that strongly correlated with CS hypertrophy at 6 months. A total of 250 transcripts were entered into Ingenuity Pathway Analysis (Redwood City, CA) software to determine molecular networks associated with CS hypertrophy. The top-ranked pathway included genes coding for two dystrophin-associated mRNAs, namely dystrophin-associated glycoprotein 1 (DAG1) and LARGE enzyme responsible for O-linked glycosylation of DAG1. DAG1 qRT-PCR expression values directly correlated with CS circumference at 6 months, validating the microarray data and suggesting that DAG1 may support CS hypertrophy. Spectrin was identified as an additional potential dystrophin surrogate via proteomic profiling. The growth factor myotrophin was also increased in the CS, implying that it might play a role in muscle hypertrophy. Taken together, these data indicated that multiple factors, including myostatin downregulation, upregulation of potential dystrophin surrogates DAG1 and spectrin, and increased expression of the growth factor myotrophin are involved in CS hypertrophy.

In another collaboration with colleagues at Vanderbilt University, we have studied mRNA arrays from skeletal and cardiac muscle at three different ages (6 and 12 months and 4–8 years) of separate groups of GRMD dogs. Preliminary data demonstrate a substantial downregulation of many skeletal muscle genes associated with metabolism in the younger dogs but not the other two groups. Thus, GRMD dogs seem to undergo a "metabolic crisis" akin to that of DMD patients and then recover to some extent (CL Galindo, CL Brinkmeyer-Langford, LW Markham, JH Soslow, JN Kornegay, DB Sawyer, unpublished data).

GWAS

GWAS are used to identify genetic markers (typically single nucleotide polymorphisms [SNPs]) that may be associated with complex traits (Edwards et al. 2013; Hou and Zhao, 2013). GWAS offer a powerful tool to identify common genetic

variants that mark regions of the genome to correlate with phenotypic variation in DMD. Genes within these regions may play a role in the manifestation of the phenotype, making them potential targets for drug therapy. The GRMD model is well suited to complement DMD studies (Brinkmeyer-Langford and Kornegay 2013). Completion of a draft sequence of the canine genome has provided data for the design of SNP chips to support canine GWAS to define the genetic bases for phenotypic features in dogs, including, as an example, the wide variation in canine skull shapes (Schoenebeck and Ostrander 2013). We have utilized GWAS to identify SNPs associated with individual variation for phenotypic markers in age-matched normal and GRMD dogs. For sake of these studies, the focus has been on the association between TTJ flexion and extension torque values and genetic profiles of the involved cranial tibialis and medial head of the gastrocnemius muscles (Figure 7) (Brinkmeyer-Langford and Kornegay 2013). Several candidate genes marked by SNPs associated with phenotypic diversity are currently being validated.

Gene expression profiling using microarrays (or RNA sequencing) works in tandem with GWAS to elucidate the genetic background underlying phenotypic variation in DMD and its animal models. The types of information generated by these two distinct, yet complementary, methods should be used concurrently to provide a multifocal, integrated pathway toward identifying drug targets. GWAS data show the extent to which a particular region of the genome (and the genes therein) are statistically associated with some phenotype of interest, thus providing context but not insight into the mechanism affected; gene expression data cannot directly give context to the differentially expressed genes but can show pathways that are variably affected and, thereby, provide insight into possible mechanistic effects. Together, both GWAS and gene expression microarray paint a holistic picture of the genetic factors influencing phenotypic variation.

The template offered by Plenge et al. (2013) in their recent review of drug target identification should be carefully considered when using GWAS and other genetic studies to identify potential drug targets. The review cited a number of human genes for which alleles (often a SNP) associated with disease phenotype. These were identified using various biochemical, genetic linkage, and GWAS methods. Plenge et al. (2013) stressed that certain criteria must ideally be fulfilled in identifying gene-drug pairs in drug discovery (Figure 8). Although not cited in the review, the protein OPN offers a contemporary example relevant to DMD. As discussed above under *Muscle Inflammation*, an OPN promoter polymorphism is associated with a more severe phenotype in DMD (Bello et al. 2012; Pegoraro et al. 2011) and enhanced muscle mass in normal human females (Hoffman et al. 2013). OPN was identified as a candidate gene by correlating DMD functional and gene expression data. Confidence in OPN's relevancy was provided by (1) an established high rate of spontaneous human polymorphisms (Giacopelli et al. 2004) and their association with disease (Chiocchetti et al. 2004), (2) the influence that the same polymorphism had on normal human muscle mass, and (3) the association of increased OPN expression with disease in the mdx mouse. Therapeutic strategies to target OPN, both nonspecifically as with NF-κB inhibition and, potentially, with humanized antibodies, could have benefit in DMD.

Epigenetics

The numerous SNPs identified through GWAS account for only a small proportion of phenotypic variation in complex diseases (Butcher and Beck 2008). Nongenetic factors encompassed by the field of epigenetics play an additional major role in disease pathogenesis. Epigenetics has traditionally been defined as the study of "heritable changes in gene expression that are not due to changes in DNA sequence" (Eccleston et al.

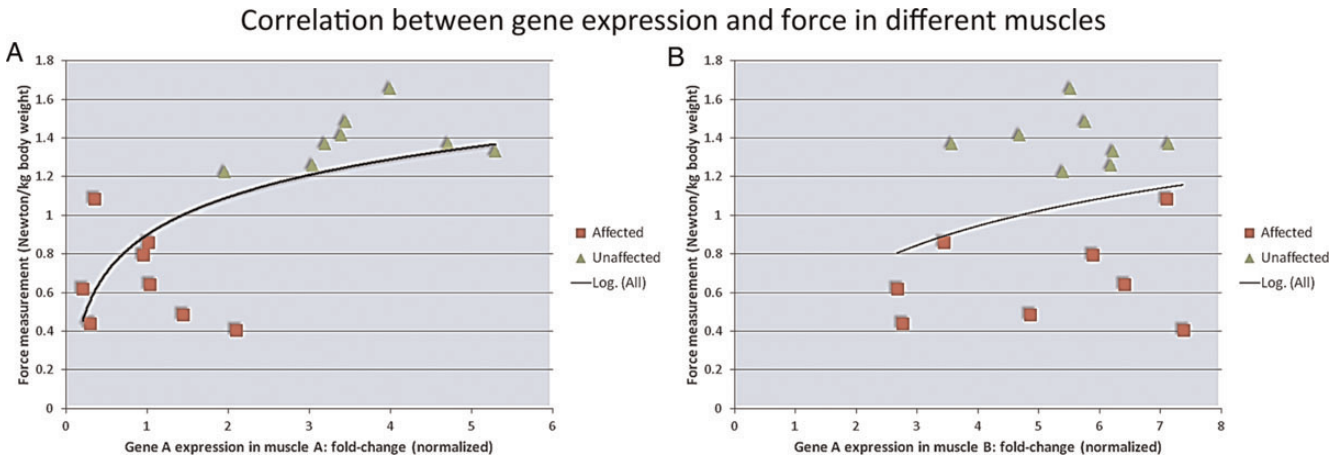

Figure 7 Data from GWAS and gene expression microarrays help to identify trends that provide important clues for drug development. Panels A and B illustrate that the same gene ("Gene A") may have distinct effects on a particular biomarker (here, force) in different muscles ("Muscles A and B"). (A) Expression of Gene A positively correlates with force values in GRMD and normal (unaffected) dogs. (B) Expression of Gene A only minimally correlates with force values (if at all), though higher values are still clearly found in unaffected dogs. Therefore, Gene A expression levels are distinct in the two different muscles from which RNA was extracted. This suggests that Gene A could be considered as a therapeutic target in Muscle A but not Muscle B.

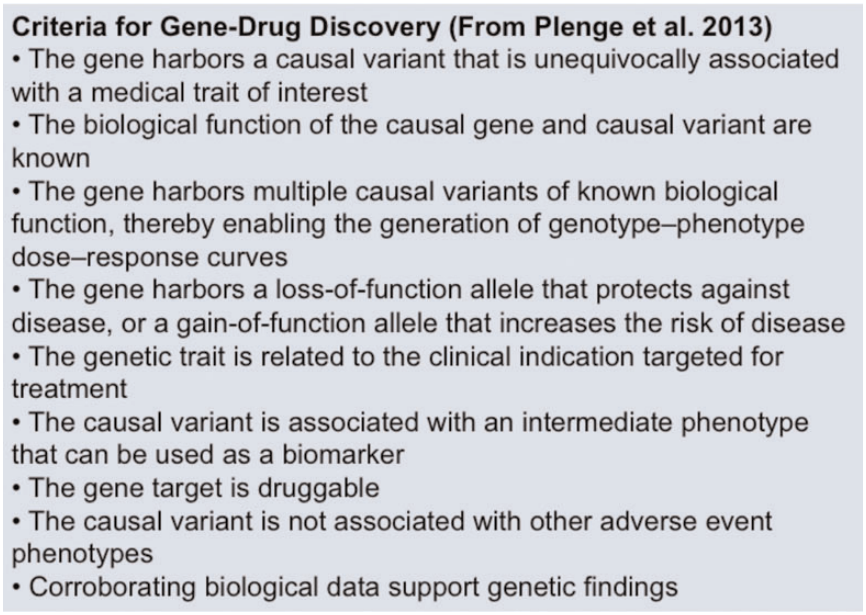

Figure 8 Criteria proposed by Plenge et al. (2013) to apply genetic findings to target validation in human disease. These criteria generally fit with the role that OPN plays in DMD disease pathogenesis. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Drug Discovery.* Validating therapeutic targets through human genetics, 12:581–594, © 2013.

2007). Bird offered a more contemporary definition of "the structural adaptations of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird 2007). The epigenome consists of chemical agents that modify, or mark, the genome, but are separate from the DNA itself. Effects of epigenetics are principally achieved by DNA and histone modifications that enhance or suppress gene expression (Barros and Offenbacher 2009). Epigenome-wide association studies are done to determine whether differences in histone and/or DNA modifications are associated with a trait (Michels et al. 2013). Any agent that interferes with the normal programming of the epigenome can interfere with activities of the affected gene(s) and their downstream targets. In some cases, drugs targeting the epigenome can be used to restore normal function, for example, by inhibiting DNA methyltransferase and histone deacetylase to enable the transcription of affected genes (Baer-Dubowska et al. 2011). Relevant to this review, epigenetic cues likely are important in driving differentiation of stem cells during muscle regeneration, potentially dictating whether pleuripotency is maintained or cell death and fibrosis occur (Giordani and Puri 2013). Puri and his colleagues have shown that histone deacetylase inhibitors promote muscle regeneration and reduce fibrosis in mdx mice (Consalvi et al. 2013) and have suggested that this effect is mediated through inhibition of fibroadipogenic progenitors while promoting differentiation of satellite cells (Mozzetta et al. 2013).

Conclusions

Pharmacologic approaches have been used to increase *DMD* gene expression and enhance production of dystrophin surrogates. Drugs that target genes downstream from the primary biochemical defect are also critically needed to lessen DMD disease morbidity and mortality. Unlike many genetic diseases for which animal models are lacking, the mdx mouse and GRMD dog provide homologous models in which disease pathogenesis and therapeutic efficacy can be tested. However, just as with other disease states, data derived from these models do not uniformly translate to DMD patients. This failure of translation likely occurs due to both physiologic differences between animals and humans and the lack of sufficient rigor in preclinical studies. With regard to preclinical studies, more care should be taken in experimental design to include random assignment of animals to treatment groups, blinding of data assessment, greater attention to power analysis of outcome parameters used to establish benefit, and increased stringency in data handling. Consideration should also be given to applying criteria of the "Animal Rule" to therapy development, especially if treatments carry substantial risk. Pharmacologic approaches have typically targeted characteristic pathologic changes, such as necrosis, inflammation, and fibrosis. But, although the mdx and GRMD models are genetically homologous to DMD, they are not analogous. Differences in histologic changes and disease progression undoubtedly reflect disparate pathogenetic mechanisms among species. New assays provide a means to better identify genes/drug targets more closely tied to disease pathogenesis, especially if genetic changes correlate with objective phenotypic outcome parameters. Genes, such as OPN, in which polymorphisms have been shown to affect the DMD phenotype are particularly attractive targets for therapy.

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involved in animal care. We hope that knowledge gained through these studies will improve not just the lives of humans but also animals with analogous diseases.

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