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Article

Porcine Models of Muscular Dystrophy[1]

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Abstract

Duchenne muscular dystrophy is a progressive, fatal, X-linked disease caused by a failure to accumulate the cytoskeletal protein dystrophin. This disease has been studied using a variety of animal models including fish, mice, rats, and dogs. While these models have contributed substantially to our mechanistic understanding of the disease and disease progression, limitations inherent to each model have slowed the clinical advancement of therapies, which necessitates the development of novel large-animal models. Several porcine dystrophin-deficient models have been identified, although disease severity may be so severe as to limit their potential contributions to the field. We have recently identified and completed the initial characterization of a natural porcine model of dystrophin insufficiency. Muscles from these animals display characteristic focal necrosis concomitant with decreased abundance and localization of dystrophin-glycoprotein complex components. These pigs recapitulate many of the cardinal features of muscular dystrophy, have elevated serum creatine kinase activity, and preliminarily appear to display altered locomotion. They also suffer from sudden death preceded by EKG abnormalities. Pig dystrophinopathy models could allow refinement of dosing strategies in human-sized animals in preparation for clinical trials. From an animal handling perspective, these pigs can generally be treated normally, with the understanding that acute stress can lead to sudden death. In summary, the ability to create genetically modified pig models and the serendipitous discovery of genetic disease in the swine industry has resulted in the emergence of new animal tools to facilitate the critical objective of improving the quality and length of life for boys afflicted with such a devastating disease.

Key words: Becker muscular dystrophy; disease model; DMD; Duchenne muscular dystrophy; dystrophin; mdx; pig

Introduction

The dystrophin gene, found on the X chromosome, is among the largest in the mammalian genome and is more than 2 megabases in length. When fully transcribed and translated, a 427 kDa full-length protein is produced from 79 exons, although several smaller transcripts can also originate from the same gene (Koenig et al. 1987; Muntoni et al. 2003). The dystrophin protein is a functional link between the actin cytoskeleton and

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the dystrophin glycoprotein complex (DGC). Through the DGC, dystrophin transmits force produced during muscle contraction to large extracellular proteins and ultimately to tendons and bones. The dystrophin protein contains four hinge regions and a long rod domain composed of spectrin-like repeats (for review Le Rumeur et al. 2010) and helps to protect the sarcolemma from injury, particularly during eccentric contractions. In the case of dystrophin deficiency, this functional connection is lost, as is the capacity to transmit force via this mechanism along with DGC component abundance and complex assembly. Dystrophin deficiency results in a disease called Duchenne muscular dystrophy (DMD).

DMD is most commonly caused by deletions in the dystrophin gene, although point mutations and duplications also contribute to the DMD population (Aartsma-Rus et al. 2006). In total, approximately two thirds of cases are inherited, with the remainder resulting from de novo mutations (Laing 1993). Boys lacking the dystrophin protein product are afflicted with deteriorating muscles and a devastating prognosis that is typically observed with locomotor deficits in early preschool years, progressing toward death as a result of the failure of muscles supporting cardiac and respiratory function. Becker muscular dystrophy (BMD) is also caused by dystrophin mutations, resulting in either a failure to accumulate sufficient full-length dystrophin and/or the accumulation of a truncated dystrophin gene product. Often this is the result of a deletion that maintains the open reading frame. Disease severity is highly variable in boys with BMD and ranges from nearly asymptomatic to severity that closely parallels that seen in DMD (Beggs et al. 1991; Nicolas et al. 2015).

Whether through increased sarcolemmal Ca^{2+} permeability (Liu et al. 2005; Lynch 2004; Moens et al. 1993; Morris et al. 2010; Petrof et al. 1993; Selsby et al. 2010) or Ca^{2+} channel dysfunction (Franco and Lansman 1990; Lansman and Franco-Obregon 2006; Tutdibi et al. 1999), it is clear that dystrophic muscle suffers a loss of Ca^{2+} homeostasis. In terms of cellular function, loss of Ca^{2+} control leads directly to increased proteolysis. Indeed, both increased activation of calpains and the proteasome system are widely reported in dystrophic skeletal muscle (Selsby et al. 2010; Spencer and Mellgren 2002). Further, mitochondria become a secondary sink for Ca^{2+} storage leading to mitochondrial and metabolic dysregulation (Basset et al. 2006; Vandebrouck et al. 2006). Superoxide production and free radical injury are increased in dystrophic muscle (Selsby 2011; Tidball and Wehling-Henricks 2007), likely due to increased calpain activity and mitochondrial dysfunction. The combination of increased cytosolic Ca^{2+}, free radical production, and mitochondrial dysfunction and apoptotic signaling (Basset et al. 2006; Tews 2006) in dystrophic muscle unfortunately culminates in myonuclear loss and muscle fiber death. This cellular stress contributes to an inflammatory response, further contributing to muscle injury. There is growing evidence that suppressed autophagy may also be contributing to disease severity (De Palma et al. 2014; Hollinger et al. 2013).

While there are numerous secondary pathologies contributing to disease severity, each represents a potential therapeutic target. In addition, the goal of several novel treatment strategies is to convert DMD patients to BMD patients and thereby mitigate disease severity. In one such approach, protein abundance of utrophin, a dystrophin-related protein, is elevated. Numerous reports have established that utrophin and dystrophin are structurally similar (Blake et al. 1996), although not identical (Li et al. 2010), such that utrophin can serve as an adequate substitute for dystrophin in the absence of dystrophin (Tinsley et al. 1998). In addition, utrophin may play a role in satellite cell function, DGC localization, smooth muscle function, and expression of dystrophin splice variants, any one of which could potentially affect muscle function and metabolism (Cohn et al. 2002; Deconinck et al. 1997; Rafael et al. 2000). In another approach called exon skipping, exons containing errors are artificially spliced out, serving to reestablish the correct reading frame, although it also results in the production of a truncated dystrophin protein product (Anthony et al. 2012; Mendell et al. 2013). Lastly, gene therapy techniques attempt delivery of a miniaturized utrophin or dystrophin gene to allow effective packaging in adeno-associated viral particles. Termed micro-utrophins (Odom et al. 2008) and micro-dystrophins (Shin et al. 2013), these protein products have shown efficacy in animal models. Hence, in addition to DMD, studies related to BMD are urgently needed, as the BMD population seems likely to expand. A requisite requirement for the development of therapies for dystrophinopathies is animal models in which to test these important therapeutic approaches.

Conventional Animal Models of Dystrophinopathy

mdx Mouse

The mdx mouse is the research animal most commonly used to study DMD. In the mdx mouse, dystrophic pathology is caused by a point mutation in exon 23 leading to formation of a stop codon and subsequently truncated protein (Bulfield et al. 1984). More recently, mutagenesis techniques have generated at least four novel mutations in the mouse dystrophin gene that are designated $mdx^{2cv\text{-}5cv}$ (Cox et al. 1993; Im et al. 1996). In addition to these models, two mouse dystrophin knockout models have also been developed (Kudoh et al. 2005; Wertz and Fuchtbauer 1998). The mdx mouse is an extremely well characterized model of DMD and has been invaluable in the understanding of disease pathogenesis and improving our mechanistic understanding of the disease. Further, it is widely used in the development of new therapies.

However, for all of the many useful features of mdx mice, there are a few significant drawbacks. For example, the disease phenotype exhibited by mdx mice is much milder than that of human DMD patients. These animals continue to be mobile, experience very little limb muscle fibrosis or adiposity, exhibit no significant contractures, and have only a mildly reduced life span (Banks and Chamberlain 2008; Chamberlain et al. 2007). To date, several reasons explaining these observations have been advanced, though little data supporting or refuting them have been published. It could be that their small size and four-legged means of locomotion spares muscles from continued cycles of degeneration and regeneration (Wells and Wells 2005). Alternatively, mice may have an inherently larger and/or faster means of muscle regeneration than do humans (Wells and Wells 2005). Further, in mdx mice, expression of utrophin is higher than in corresponding muscle from healthy mice and may be better able to compensate for dystrophin deficiency in mice than in humans (Law et al. 1994). As these were recognized as potential confounding variables, an mdx/$utrophin^{-/-}$ mouse line was developed, which has a far more severe phenotype (Deconinck et al. 1997). Indeed, a number of additional mutations have been superimposed on the mdx model in an effort to increase disease severity (e.g., Sacco et al. 2010). While the disease phenotype is typically far more severe in these models, they are no longer accurate genetic models of the human disease as these new

knockouts often increase disease severity independent of the dystrophin mutation.

Additionally, in many instances, there is a poor correlation between effectiveness of therapies in mouse models to that observed in human models (Duan 2011; Wells and Wells 2005). Indeed, scaling up from a mouse to a dog or human presents a number of challenges for conventional drug interventions as well as gene and stem cell transfer-based approaches (discussed below).

Golden Retriever Muscular Dystrophy (GRMD) Model

Identification and characterization of a larger dystrophin-deficient animal model would contribute greatly to the advancement of our understanding of the disease as well as our ability to treat it. As a result, the GRMD model has been used extensively among some research groups. In the GRMD model, the dystrophin gene has a mutation in the 3′ end of intron 6 that results in the aberrant exclusion of exon 7 during splicing, and a frame shift, which introduces a stop codon in exon 8 (Sharp et al. 1992). This model features a phenotype that is similar in severity and selective muscle injury to that seen in human patients (Kornegay et al. 2003). Finally, these dogs are more similar in size to human patients, helping to ease issues related to project scale-up (Duan 2011; Wells and Wells 2005).

Despite these positive aspects of the GRMD model, several deficiencies warrant consideration of new large-animal models of DMD. GRMD dogs have a high degree of variability, despite an identical causative mutation (Cooper et al. 1988; Shimatsu et al. 2003). It is likely that at the root of this issue is variation in the expression of alternatively spliced dystrophin gene products and expression of resultant truncated translational products in the muscles of GRMD dogs, leading to great phenotypic range (Banks and Chamberlain 2008; Schatzberg et al. 1998). This may be indicative of other underlying differences between the canine and human diseases. While identification of mediators of this differential expression is important and has great potential for large therapeutic impacts, the high phenotypic variability makes determining end points difficult and confounds data interpretation (Bretag 2007). For example, despite being dystrophin deficient, Ringo, a member of a Brazilian GRMD colony, behaves normally (running, jumping, opening a door while on hind limbs, and breeding) (Ambrosio et al. 2008). Furthermore, dystrophic dogs treated with corticosteroids exhibited a greater frequency of calcified necrotic fibers and impairment of some measures of muscle function (Liu et al. 2004). This is of great importance because corticosteroids have been routinely used in the care of human DMD patients since the 1970s and have consistently improved muscle function. As corticosteroids are the standard of care, it is important to determine how novel drugs may interact with corticosteroids in an animal model. Because corticosteroids exacerbate the disease in the GRMD model, it is not the ideal large-animal model in which to make these comparisons. Despite the many favorable attributes of the dog model of DMD, several deficiencies remain, necessitating the continued search for alternative large-animal models of DMD.

Other Dystrophinopathy Models

The hypertrophic feline muscular dystrophy (HFMD) cat analog of DMD was first diagnosed in a companion animal. Different spontaneously occurring mutations can lead to HFMD, however these mutations appear to be located at the muscle and Purkinje promoter of the dystrophin gene (Gambino et al. 2014; Winand et al. 1994). The feline model is rarely used as a research animal because tongue and diaphragm hypertrophy lead to feeding difficulties, animal welfare concerns, and death (Carpenter et al. 1989; Gaschen et al. 1992).

Two independent labs have recently employed genome editing tools to induce dystrophin mutations in rats (Larcher et al. 2014; Nakamura et al. 2014). Nakamura and colleagues used clustered, interspaced short palindromic repeats (CRISPR)/Cas to induce deletions in exon 3 and/or exon 16 in inbred Wistar–Imaichi rats. The rats showed muscle pathology consistent with DMD; however, there was large variation in severity between individual animals (Nakamura et al. 2014). A stop codon in exon 23, as found in the mdx mouse, was created in rats by Larcher and colleagues utilizing transcription activator-like effector nucleases (TALEN). The Dmd^{mdx} rats have a phenotype similar to human DMD patients including fatty infiltration and a cardiac defect. In outbred Sprague-Dawley rats carrying this mutation, the phenotype is also consistent between animals. A benefit of rat models is that rats are relatively easy to work with and have inexpensive husbandry needs compared with large animals. However, issues related to scale up, like in the mouse, would seem to limit the usefulness of these models as translational tools.

Zebrafish dystrophinopathy models are commonly used non-mammalian models of DMD. Two strains are available: sapje, with a nonsense mutation in exon 4, and sapje-like, with a deletion of exon 62 leading to a stop codon in exon 63 (Bassett et al. 2003; Guyon et al. 2009). Since zebrafish embryos are translucent, muscle damage can be visualized and quantified using polarized light (Berger et al. 2012). The straightforward quantification facilitates high throughput screening of disease-modifying compounds (Kawahara et al. 2011; Waugh et al. 2014), testing the efficacy of potential treatments (Berger et al. 2011; Li et al. 2014), and studies of disease pathology (Alexander et al. 2013; Bassett et al. 2003). Even though zebrafish have proteins orthologous to those in the mammalian DGC (Chambers et al. 2001), there are limitations to using zebrafish models including differences in taxonomy class, locomotion, and size.

Rationale for Porcine Models of Dystrophinopathy

Despite the fact that DMD remains the most common fatal, X-linked disease, development of therapeutic interventions is moving at an alarmingly slow rate. Certainly, current animal models have been essential in aiding our understanding of DMD and have made contributions as preclinical models. However, their differences in body size, disease presentation, and response to therapeutic interventions compared with humans have hindered progress toward advances in patient care. With a porcine model available, we anticipate that preclinical trials would be far more representative and predictive of human outcomes, because the pig genome is three times more similar to that of the human than is the mouse (Wernersson et al. 2005) and is much closer in anatomical size to humans than is the mouse or dog.

As noted above, a significant hurdle is appropriately scaling experimental medications from doses given to a mouse or dog to those appropriate for a human patient. In many instances, the dog is omitted from the translational pipeline, hence more commonly the hurdle is scaling therapies from mouse to human. In addition to differences in body size, there are also differences in body composition, metabolic rate, and biological chemistry that contribute to varied or underwhelming responses

in humans despite strong preclinical data demonstrating efficacy in mice. Failure to appropriately dose human subjects results in a failure to accumulate sufficient drug-of-interest in the blood and/or target tissues or to effect a desired modification of targeted pathway activity. It may cause significant side effects leading to failure of the clinical trial.

Similar to conventional intervention, scale up of gene therapy approaches also faces significant challenges. Intramuscular gene transfer and intravascular delivery of virus for whole-body transfection have become routine in mouse models (Gregorevic et al. 2006, 2008; Zhang and Duan 2012). Unlike the mdx mouse, after intramuscular viral delivery of microdystrophin to DMD patients, no transgene was detected (Mendell, Campbell, et al. 2010). This may be due, in part, to an immune response to the transgene. The viral vector itself can also provoke an immune response in patients (Mendell, Rodino-Klapac et al. 2010; Mingozzi et al. 2007), which could interfere with transgene expression. Because of the immune response encountered with intramuscular delivery, there is concern that the higher titers required for systemic perfusion could trigger a stronger immune response, which would be counter to the efficacy of the intervention and well-being of the patient. Further, delivering virus directly to the entirety of the musculature is logistically impractical. Large-animal models can facilitate the optimization of approaches for systematic viral delivery while at the same time evaluating the immunological responses in multiple species.

Given these current problematic issues of scale-up, a model of human size would seem to be an invaluable tool. Minipigs (i.e., NIH minipig, Yucatan) can be managed to reach a size of ~150 lbs at one year of age and ~200 pounds at two years of age. Domestic pigs used for agriculture can reach nearly 300 pounds by six months of age and may exceed 500 pounds by one year of age. An advantage of the larger size makes biopsies possible such that longitudinal studies could be performed on the same animal. Furthermore, logistics of scale-up, including dosages, titers, injection sites, dosing regimens, etc., could be better defined prior to use in DMD patients. This has the cumulative benefit of reducing the cost of clinical trials because (1) a very thorough preclinical trial featuring pigs would be much cheaper than most human clinical trials, (2) clinical trials could contain fewer groups as dose determination and scale-up methods have already been determined, and (3) interventions that would fail because of limitations to scale-up or other toxicities discovered in moving to large animals could be halted at the preclinical stage.

In addition to skeletal muscle dysfunction, dystrophinopathy patients suffer a progressive cardiomyopathy. Indeed, respiratory support therapies have been so successful in protecting respiratory function that cardiomyopathy is growing in frequency as the cause of death for DMD patients (McNally 2007). Hence, it is imperative that models of DMD have a cardiovascular system comparable to the human cardiovascular system so that interventions will be in the appropriate physiological context. In that regard, porcine and human hearts are similar in terms of cardiac output, stroke volume, mean arterial pressure, heart rate, and perfusion (Crick et al. 1998; Thein and Hammer 2004). Moreover, the porcine heart is so similar to the human heart that, in preparation for xenotransplantation, transgenic pigs designed to reduce rejection have been developed (Ezzelarab et al. 2009; Lai et al. 2002; Weiss et al. 2009), while pig xenographs are currently being used in humans for valve replacements as well as aortic root replacements (Kon et al. 1999; LeMaire et al. 2009). Finally, a model with a cardiomyopathy provides an opportunity to study the mechanism of this feature of dystrophinopathy as well as a platform to test potential interventions.

Transgenic Porcine Models of Dystrophinopathy

A tremendous amount of progress has been made in the last decade with respect to genetically engineering pigs with specific defects to recapitulate human disease phenotypes in a desirable pig genetic background (Prather et al. 2013; Yang and Ross 2012). The ability to make pigs carrying specific genetic modifications has been accomplished through somatic cell nuclear transfer (Rogers et al. 2008; Ross et al. 2012) and through the implementation of new gene-editing technologies such as TAL effector nucleases (TALENs) (Prather et al. 2013) and the use of the CRISPR/Cas9 system (Whitworth et al. 2014).

We and others have pursued this genome modification approach, resulting in the emergence of several new models of dystrophinopathies in pigs. In 2013, Klymiuk and colleagues reported a transgenic pig model with the exon corresponding to human dystrophin exon 52 replaced with a neomycin selectable marker cassette. The deletion of the exon resulted in a +1 frame shift after the splicing of exon 51 to 53 (Klymiuk et al. 2013). The model produces some of the cardinal features of DMD in humans, such as absence of dystrophin protein, a reduction in the abundance and localization of DGC components, elevated serum creatine kinase activity, and progressive fibrosis of muscle. The model though is not without some significant drawbacks. At a molecular level, the model appears to express significantly greater utrophin than wild-type counterparts, a shortcoming of the mdx mouse. Further, the severity of the disease in this pig model is so great as to significantly limit its use as a research tool as the majority of affected offspring died within the first week of life. Hence, the opportunity to identify biomarkers prior to an observable physical phenotype is greatly reduced, as is the ability to introduce a therapeutic intervention early enough in the course of the disease to curb disease progression and severity over time.

Information regarding another porcine model of muscular dystrophy is available only through the U.S. patent office (Exemplar Genetics Patent US 20140223589 A1). The patent claims that these miniature pigs possess an exon 52 deletion and demonstrate several characteristics consistent with DMD phenotypes observed in boys with the disease, such as elevated serum creatine kinase activity and lack of a functional dystrophin protein. While several key features of this model still require further characterization to determine its full potential, this model is promising. Of potential concern is that the genetics of this model appears to cause the same mutation (exon 52 deletion) as the transgenic dystrophin knockout model above (Klymiuk et al. 2013). Hence, there is the expectation that the severity of the disease would be similar, although until the details of the model are peer reviewed this is speculative.

We have also pursued the development of a miniature pig model of muscular dystrophy. Our modification, accomplished by the introduction of a neomycin selectable marker cassette via homologous recombination, results in the in-frame deletion of an exon in the rod domain of the dystrophin protein (Ross et al., unpublished observations). We have recently begun producing males carrying the modification. Anecdotally, they appear to develop fairly normally for the first two months of life. Both phenotypic and molecular characterizations at multiple time points in the first year are underway to determine the extent to which this model recapitulates fundamental features of dystrophinopathies. Our expectation is that an in-frame dystrophin mutation in the pig would result in a milder dystrophinopathy in comparison with other existing transgenic pig models, such as

the one developed by Klymiuk and colleagues (2013). An additional advantage of a miniature BMD-like model would be their research importance in the event of successful deployment of exon-skipping drugs, which would dramatically increase the number of boys with a BMD-like phenotype.

While genetic modification of the pig genome offers the ability to create and utilize important animal models, the use of transgenic pig models is hampered by federal regulations limiting rapid, widespread use and distribution of the animals (Maxmen 2012). Alternatively, the U.S. swine industry produces over 100 million pigs per year. While the swine industry does not actively pursue the identification of phenotypes for use as biomedical models, some mutations have been identified, such as pigs with malignant hyperthermia (MH) (Fujii et al. 1991), severe combined immunodeficiency (SCID) (Ewen et al. 2014), and our porcine BMD model (see below) (Hollinger et al. 2014; Nonneman et al. 2012). These discoveries of spontaneously occurring disease models are capable of making an impact on the biomedical research community without the complications of regulatory efforts associated with monitoring transgenic animals.

A Spontaneous Porcine Dystrophinopathy Model

Discovery

A porcine stress syndrome symptomatically similar to ryanodine receptor 1 (*RYR1*)-mediated porcine stress syndrome was discovered in the U.S. Meat Animal Research Center's swine research population when two male siblings died of apparent stress after transport at 12 weeks of age (Nonneman et al. 2012). Because the population was free of the *RYR1* mutation, it was initially thought it could be an allelic form of porcine stress syndrome or MH. To evaluate candidate genes associated with the stress syndrome, the coding regions of porcine orthologs of human MH genes *RYR1*, calcium channel, voltage-dependent, L type, alpha 1S subunit (*CACNA1S*), carnitine palmitoyltransferase 2 (*CPT2*), and ryanodine receptor 2 (*RYR2*) were sequenced for SNP discovery and mutation detection in the proband's family. There were no obvious mutations identified when normal and affected siblings were compared, and the syndrome did not cosegregate with SNP alleles in any of the human MH candidate genes. Matings were made with the original sire, dam, and daughters to generate additional offspring to characterize the genetic basis of this syndrome. A pedigree of 250 animals that included 49 affected pigs was genotyped with the Illumina PorcineSNP60 beadchip (Ramos et al. 2009), and a single region on the X chromosome (29–32 Mb) was associated with the stress syndrome. The two most significant markers on the beadchip (ALGA0099513 and ALGA0099514) are located in intron 44 of the dystrophin gene (Nonneman et al. 2012).

Genetics

After discovering that the dystrophin gene was associated with the stress syndrome, the exons and flanking intron boundaries of *DMD* were sequenced from normal and affected pigs, and no polymorphisms were identified near splice sites. Amplification and sequencing of skeletal muscle cDNA from normal and affected animals revealed no evidence of alternative splicing or deletion of exons in the affected animals. Polymorphisms in the promoter regions (P1, P2, and the muscle-specific promoter) did not segregate with the disease. Six nonsynonymous polymorphisms were found in the coding region (Table 1), and only one of these nonsynonymous changes, 85890_783, which causes the amino acid change arginine to tryptophan at amino acid 1958 (R1958W) in exon 41, was significantly associated with the stress syndrome. This SNP was as highly associated with the stress response as the two most significant SNPs in intron 44 on the Illumina PorcineSNP60 Beadchip. The C allele (arginine) was found in all unaffected animals, and the T allele (tryptophan) was hemizygous in affected males and heterozygous in carrier females. This change was predicted by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) to be damaging with a probability score of 0.983 (Nonneman et al. 2012).

To further characterize this variant, additional sequencing of the locus in this population was performed. Because of the size of the dystrophin gene, a DNA capture array (Agilent, Santa Clara, CA) was designed for 2.33 Mb of pig genomic sequence spanning the DMD gene and included 146 kb upstream of the first exon and 20 kb downstream of the last exon. The variation between affected and normal pigs and two normal pools of Duroc/Yorkshire and Landrace boars showed that affected pigs shared a common haplotype that extended for about 750 kb. corresponding to a region ranging from intron 40 to intron 59 of the dystrophin gene (DJ Nonneman, GA Rohrer, unpublished observations). This region includes the arginine to tryptophan substitution in exon 41 and the two most significant Illumina Beadchip markers. This shared haplotype implies that the mutation resides in this interval, and no other mutations were found in this region. Thus the hypothesis that the Arg-Trp mutation is responsible for the phenotype is strongly supported by the genetic evidence.

Phenotype

In affected pigs, dystrophin abundance was decreased in all muscles by ~70% when assessed by Western blot; however, using an immunohistochemical approach, the reduction in dystrophin localization was noted in the diaphragm (70% reduction) and the longissimus (90% reduction) but not the psoas (45% numerical reduction) (Hollinger et al. 2014; Nonneman et al. 2012).

Table 1. Nonsynonymous polymorphisms in pig DMD and their associations with the stress-induced phenotype

Marker	Nucleotide	Polymorphism[a]	Amino Acid	Location	*p*-value
84000_82	1349	CRA	R396G	Exon 11	0.002
84002_411	1629	CAS	H489Q	Exon 12	0.0002
84339_200	2102	ARC	N647S	Exon 16	0.406
84339_333	2235	ACR	T691T	Exon 16	0.559
85890_783	6034	YGG	R1958W	Exon 41	1.06×10^{-23}
ALGA0099513				Intron 44	2.19×10^{-23}
ALGA0099514				Intron 44	2.19×10^{-23}
85904_613	7274	CYG	L2371P	Exon 49	0.005

[a]Polymorphisms are shown by their IUB code within the codon.

Likewise, α-sarcoglycan and dystroglycan abundance and localization followed a similar pattern in these muscles (Fortunato et al. 2014; Hollinger et al. 2014). Litter size and sex distribution of offspring were consistent with that found in a typical production line, suggesting that dystrophin insufficiency does not affect in utero survival. Further, growth of affected pigs and healthy littermates was similar throughout the first 12 months of life. Visually, pigs containing the polymorphism causing dystrophin insufficiency appear similar to their unaffected littermates throughout the first 12 months of life. Consistent with disease-related muscle injury, serum creatine kinase activity is increased in affected animals compared with healthy littermates. At two months of age, a detailed histological study using several muscles was conducted, which revealed focal necrotic lesions in some, but not all, muscles selected for investigation (Hollinger et al. 2014). For example, the diaphragm and longissimus contained necrotic lesions; however, the psoas did not. It is unclear if the muscle-specific response is due to frequency and type of muscle use, where the diaphragm and longissimus are used regularly and the psoas is used sparingly. Alternatively, the fiber-type distribution may also affect muscle injury because the psoas is largely type I, which has been shown to be resistant to dystrophic injury relative to other fiber types (Webster et al. 1988). Indeed, a novel therapeutic approach is to increase the frequency of type I fibers within a muscle group as the complement of proteins associated with the type I phenotype appears protective to dystrophic injury (Hollinger et al. 2013; Ljubicic et al. 2011; Selsby et al. 2012). It is also unclear if the dystrophic psoas contains less injury as a cause or an effect of the higher degree of dystrophin and DGC localization.

Cardiomyopathy is another important consideration for animal models of dystrophinopathies because it is quickly becoming the leading cause of death for dystrophinopathy patients. In this pig model, the myocardium appears free from lesions at two months of age despite dystrophin insufficiency (JT Selsby, JW Ross, DJ Nonneman, K Hollinger, unpublished observations). At 12 months of age, foci of necrosis are apparent. indicating a deteriorating myocardium, which is consistent with the human disease (Nonneman et al. 2012). Regardless of age, these pigs are susceptible to a stress-mediated sudden death. Early experiments implicated the ryanodine receptor because inhalation of isoflurane also caused this sudden death (Nonneman et al. 2012), although it is unclear if the death was due to the isoflurane, per se, or the stress of forced breathing of isoflurane. Regardless, immediately prior to death during isoflurane inhalation, electrocardiogram (EKG) abnormalities were reported (Nonneman et al. 2012). Similarly, we found EKG abnormalities immediately prior to death resulting from acute stress (Figure 1). It is unclear if the ryanodine receptor is affected by dystrophin insufficiency in this model; however, recent evidence (Bellinger et al. 2009; Fauconnier et al. 2010) and a similar susceptibility to sudden death in BMD patients (Nigro et al. 2002) leads to the speculation that there may be post-translational ryanodine receptor modification due to dystrophin insufficiency. Importantly, hyperthermia was not present during stress-mediated death (unpublished observations).

Current Approaches

While histological experiments provide compelling evidence that dystrophin insufficiency leads to disease-related injury in dystrophin-insufficient pigs, we are currently working to determine the extent to which it leads to impaired muscle function. This will provide an objective outcome measure so that disease progression can be clearly tracked and establish a basis on which to evaluate interventions at the preclinical stage. Further, because it is clear in other species that dystrophin insufficiency causes lesions in skeletal muscle and leads to impaired muscle function, establishing this in the porcine model is critical. Currently, we are using changes in gait to objectively measure locomotor function. To accomplish this, pigs are observed as they walk on an ~15-meter linear track that contains an ~5-meter mat with embedded force transducers (Xeno, ProtoKinetics, Havertown, PA; Figure 2). While this is an ongoing study, our preliminary data indicate that, at three months of age, stride length is decreased (Figure 3) in affected pigs, and affected pigs do not roll through a step (strike to toe off) in the same fashion as healthy littermates (Selsby, Ross, Nonneman, Kaiser, unpublished observations). Such changes would be indicative of disease-related modifications to gait and locomotion much like those observed in human patients.

Future Directions

To more fully characterize the model, we intend to measure respiratory function in these pigs because respiratory failure is a serious clinical concern for these patients. That these pigs have such a powerful and repeatable stress-induced death creates some hurdles that we are working to overcome. In an early attempt to measure respiratory function, we used respiratory inductive plethysmography in which bands are placed around the chest and abdomen of the pig. While we were successful in recording a signal, we were not able to calibrate the system due to fears that this process would trigger stress sufficient to kill the animal. We also considered surgical techniques; however, that precludes the possibility of longitudinal studies. We are currently exploring the possibility of using whole-body unrestrained plethysmography and have successfully performed this technique in mice (Selsby et al. 2014).

Of equal importance are changes in cardiac function, and the possibility of using the working heart or Langendorff approaches to measure cardiac function is foreseeable. We may also pursue in vivo imaging using magnetic resonance imaging (MRI) and have recently performed this measure in mice (Ballman et al. 2015; Beyers et al. 2015).

Aside from disease characterization, understanding the molecular mechanism leading to dystrophin insufficiency is important because it will help us to better understand the disease in this model but may also help us better understand similar causes in human patients. The reduction in dystrophin abundance is greater than was anticipated given the identified mutation. Full sequencing of the dystrophin gene did not reveal additional mutations (Nonneman et al. 2012). The mutation did not cause a new microRNA binding site, although the molecular properties of arginine and tryptophan differ. It is reasonable to suggest that this amino acid substitution changed the tertiary structure, making proteolytic attack more likely. Indeed, in silico digests suggest increased susceptibility to calpain (Hollinger et al. 2014). Even this, however, seems inadequate to explain the 70%–90% reduction in dystrophin protein abundance, suggesting that some other mechanism(s) are contributing to decreased dystrophin abundance. These may include decreased stability of the transcript, increased transcript degradation, or decreased protein stability.

Summary

A variety of animal models have contributed to our understanding of dystrophinopathies and to therapy development. Deficiencies in these models necessitate the development of

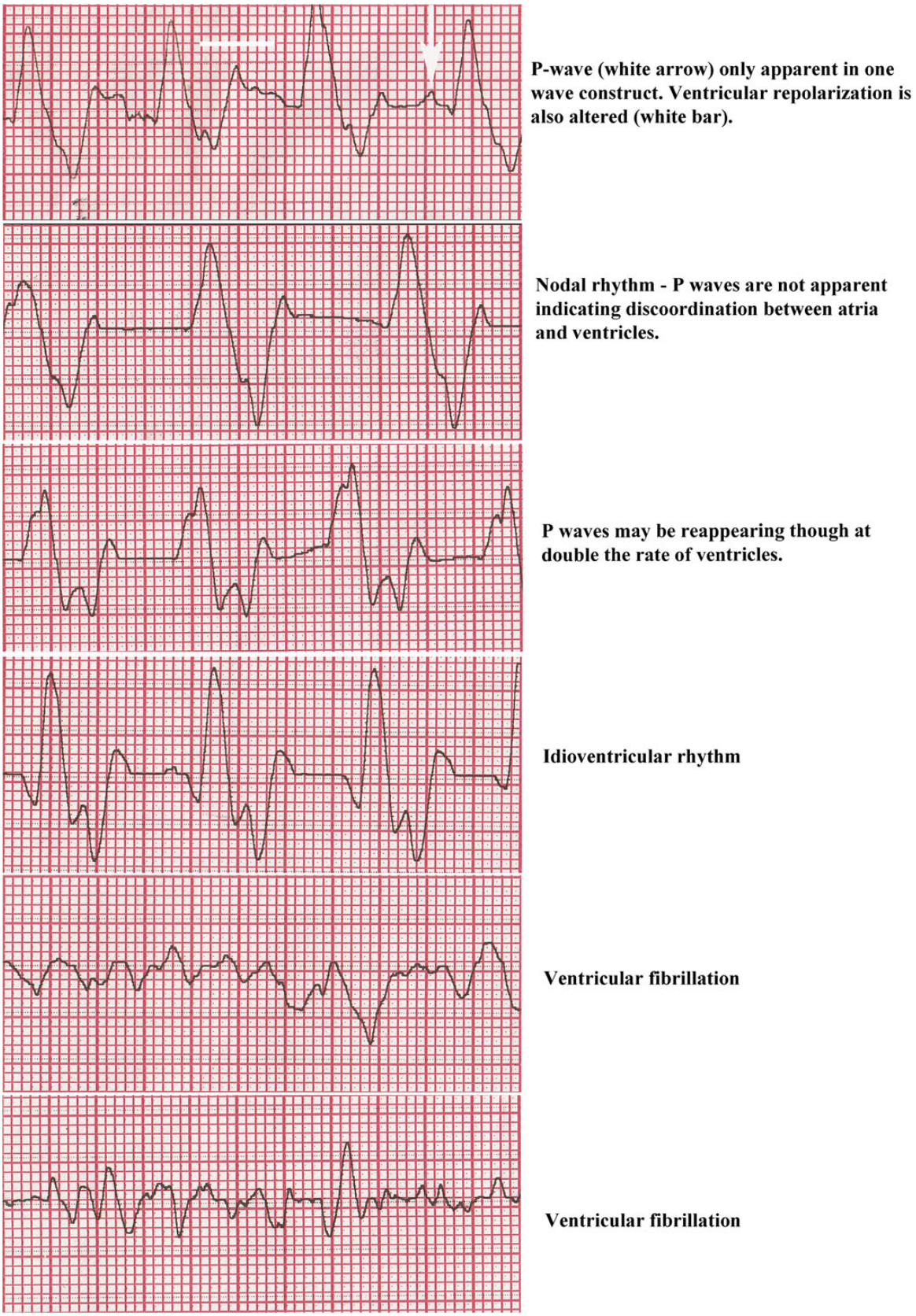

Figure 1. Dystrophic pigs have EKG abnormalities after acute stress. Fifteen minutes after a routine injection, a pig with dystrophin insufficiency collapsed and exhibited rapid, shallow breathing. EKG pads were applied within a few minutes to record electrical activity in the heart. Ultimately, the rhythm degraded to ventricular fibrillation and asystole. The entire duration from the onset of breathing abnormalities to death was less 15 min. Similar recordings, along with an example from a healthy animal have been previously reported (Nonneman et al. 2012). These panels (in order) were taken over the final 8 min.

Figure 2. Gait analysis. The track is ~15 meters with the central 5 meters containing 33,000 embedded force transducers (Zeno, Protokinetics, Havertown, PA). The pigs walked back and forth across the track, and data are collected bidirectionally.

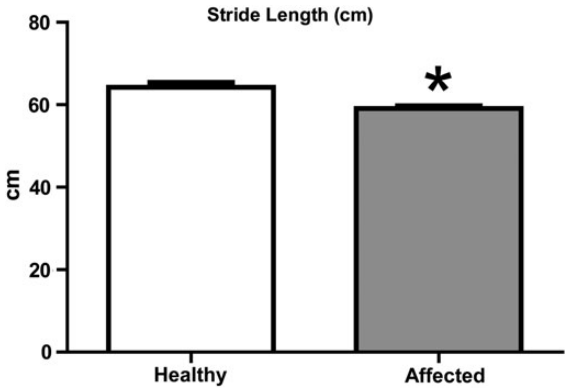

Figure 3. Gait abnormalities in dystrophic pigs. Using the system indicated in Figure 2, data were collected on ~10 walks/pig, which were generally accomplished in a total of 14 walks over ~15 min/pig. At 3 months of age, stride length was decreased in dystrophic ($n = 8$) pigs compared with healthy littermates ($n = 9$). In this preliminary analysis, factors including body weight and limb length were not included in the statistical model but did not differ between groups. * indicates significantly different from healthy; $p < 0.05$.

novel, large-animal models as preclinical tools. The pig represents a human-sized animal that shares many anatomical and physiological features with humans, including the cardiovascular system, which is of importance in the study of dystrophinopathies. Several pig models are being developed by different groups, and we have recently described work characterizing a porcine model with a dystrophin insufficiency and associated muscle injury. We are in the process of characterizing disease progression and are developing techniques to objectively measure physiological outcomes. We are also pursuing the measurement of respiratory and cardiac function. In total, porcine dystrophinopathy models hold tremendous promise as preclinical models in the drug and therapy development pipeline for the treatment of boys afflicted with dystrophinopathies.

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