

## Animal Models for Genetic Neuromuscular Diseases

Mariz Vainzof · Danielle Ayub-Guerrieri ·  
Paula C. G. Onofre · Poliana C. M. Martins ·  
Vanessa F. Lopes · Dinorah Zilberztajn ·  
Lucas S. Maia · Karen Sell · Lydia U. Yamamoto

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**Abstract** The neuromuscular disorders are a heterogeneous group of genetic diseases, caused by mutations in genes coding sarcolemmal, sarcomeric, and cytosolic muscle proteins. Deficiencies or loss of function of these proteins leads to variable degree of progressive loss of motor ability. Several animal models, manifesting phenotypes observed in neuromuscular diseases, have been identified in nature or generated in laboratory. These models generally present physiological alterations observed in human patients and can be used as important tools for genetic, clinic, and histopathological studies. The mdx mouse is the most widely used animal model for Duchenne muscular dystrophy (DMD). Although it is a good genetic and biochemical model, presenting total deficiency of the protein dystrophin in the muscle, this mouse is not useful for clinical trials because of its very mild phenotype. The canine golden retriever MD model represents a more clinically similar model of DMD due to its larger size and significant muscle weakness. Autosomal recessive limb-girdle MD forms models include the SJL/J mice, which develop a spontaneous myopathy resulting from a mutation in the Dysferlin gene, being a model for LGMD2B. For the human sarcoglycanopathies (SG), the BIO14.6 hamster is the spontaneous animal model

for  $\delta$ -SG deficiency, whereas some canine models with deficiency of SG proteins have also been identified. More recently, using the homologous recombination technique in embryonic stem cell, several mouse models have been developed with null mutations in each one of the four SG genes. All sarcoglycan-null animals display a progressive muscular dystrophy of variable severity and share the property of a significant secondary reduction in the expression of the other members of the sarcoglycan subcomplex and other components of the Dystrophin-glycoprotein complex. Mouse models for congenital MD include the dy/dy (dystrophia-muscularis) mouse and the allelic mutant  $dy^{2J}/dy^{2J}$  mouse, both presenting significant reduction of  $\alpha 2$ -laminin in the muscle and a severe phenotype. The myodystrophy mouse (Large<sup>myd</sup>) harbors a mutation in the glycosyltransferase Large, which leads to altered glycosylation of  $\alpha$ -DG, and also a severe phenotype. Other informative models for muscle proteins include the knockout mouse for myostatin, which demonstrated that this protein is a negative regulator of muscle growth. Additionally, the stress syndrome in pigs, caused by mutations in the porcine RYR1 gene, helped to localize the gene causing malignant hypertermia and Central Core myopathy in humans. The study of animal models for genetic diseases, in spite of the existence of differences in some phenotypes, can provide important clues to the understanding of the pathogenesis of these disorders and are also very valuable for testing strategies for therapeutic approaches.

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M. Vainzof · D. Ayub-Guerrieri · P. C. G. Onofre ·  
P. C. M. Martins · V. F. Lopes · D. Zilberztajn · L. S. Maia ·  
K. Sell · L. U. Yamamoto  
Human Genome Research Center,  
Dept of Genetics and Evolutionary Biology,  
University of Sao Paulo,  
Sao Paulo, Brasil

M. Vainzof (✉)  
Human Genome Research Center, IBUSP, R. do Matão,  
106 Cidade Universitária,  
Sao Paulo 05508-900, Brazil  
e-mail: mvainzof@usp.br

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### Introduction

The neuromuscular disorders are a heterogeneous group of genetic diseases, causing a progressive loss of the motor

ability. More than 30 genetically defined forms are recognized, and in the last decade, mutations in several genes have been reported, resulting in the deficiency or loss of function of different important muscle proteins. Biochemical and immunohistological analysis have localized these proteins in several compartments of the muscle fiber. The proteins dystrophin, sarcoglycans, and dysferlin are sarcolemmal or peri-sarcolemmal proteins,  $\alpha$ 2-laminin and collagen VI are extracellular matrix proteins, telethonin and actin are sarcomeric proteins, calpain 3 and FKRP are cytosolic enzymes, and emerin and lamin A/C are nuclear proteins.

Defects in components of the dystrophin-glycoprotein complex (DGC) are known to be an important cause of different forms of muscular dystrophies (Ervasti and Campbell 1993; Yoshida and Ozawa 1990). The DGC is an oligomeric complex that connects the subsarcolemmal cytoskeleton to the extracellular matrix. The DGC consists of dystroglycan ( $\alpha$ - and  $\beta$ -DG), sarcoglycans ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -, and  $\epsilon$ -SG) and syntrophin/dystrobrevin subcomplexes. The intracellular link of the DGC is the protein dystrophin, which plays an important structural role in muscle fibers. Mutations in the dystrophin gene cause the most common form of X-linked Duchenne muscular dystrophy (DMD; Hoffman et al. 1987). Dystrophin binds its amino-terminal and rod domain to actin and with its carboxy terminal to the integral membrane protein  $\beta$ -DG. The sarcoglycan sub-complex is also linked to  $\beta$ -DG and includes  $\alpha$ -SG,  $\beta$ -SG,  $\gamma$ -SG, and  $\delta$ -SG, which are tightly associated and inserted into the membrane. Mutations in the genes coding the four SG proteins cause severe forms of limb-girdle muscular dystrophies type LGMD2D, 2E, 2C, and 2F, respectively. The peripheral membrane glycoprotein  $\alpha$ -DG, a receptor for the heterotrimeric basement membrane protein laminin-2, binds to  $\beta$ -DG and so completes the connection from the inside to the outside of the cell (Straub and Campbell 1997). Mutations in

the LAMA2 gene, encoding the  $\alpha$ 2 chain of laminin-2, cause  $\alpha$ 2-laminin deficiency and a severe form of congenital muscular dystrophy (CMD1A) linked to chromosome 6q (Tomé et al. 1994). In addition, some forms of muscular dystrophy have recently been associated with genes encoding putative or known glycosyltransferases. Muscle protein analysis in these patients show a hypoglycosylation of  $\alpha$ -dystroglycan and a consequent reduction of numerous ligands components of the extracellular matrix, such as laminin 2 (Muntoni et al. 2004). Other milder forms of muscular dystrophy are caused by mutations in genes coding the enzyme calpain 3 (LGMD2A), the sarcolemmal protein dysferlin (LGMD2B), and the sarcomeric protein telethonin (LGMD2G; revision in Vainzof and Zatz 2007).

#### Animal Models for Neuromuscular Diseases

Several animal models manifesting phenotypes observed in specific genetic diseases have been identified in nature. In addition, a number of genetically engineered murine models for muscular dystrophy have been generated. These animals generally present physiological alterations frequently observed in human patients and can be used as important tools for genetic, clinic, and histopathological studies, providing important clues to the understanding of the pathogenesis of these disorders (Table 1; Fig. 1). Animal models are also very valuable for testing strategies for therapeutic approaches.

#### Dystrophinopathies

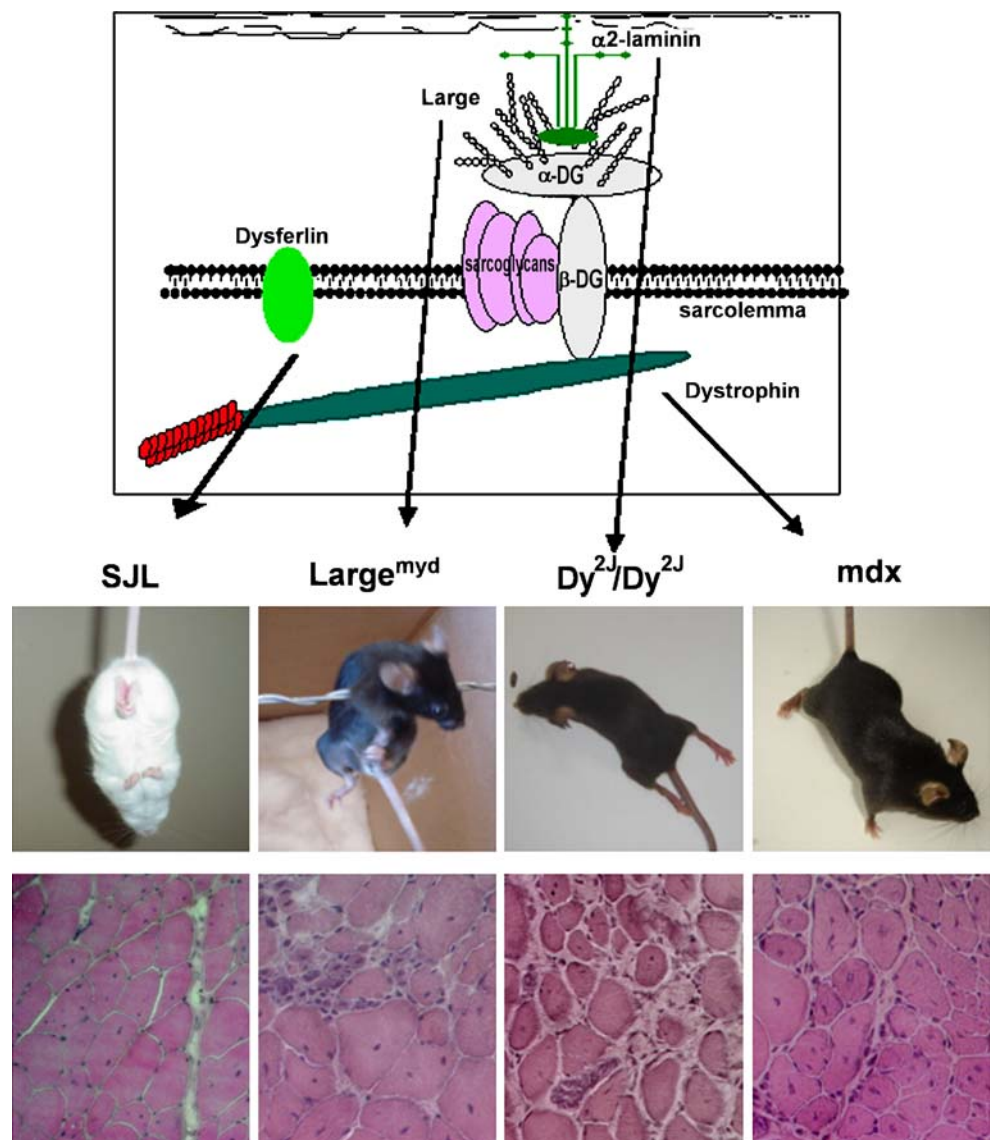
The mdx mouse is the most widely used animal model for DMD. It was first described in 1984 because of the observation of elevated plasma levels of muscle creatine kinase and pyruvate kinase enzymes and histological lesions characteristic of muscular dystrophy. The mutants show mild

**Table 1** Mice models for muscular dystrophy

Genotype (protein absent)	Lifespan	Skeletal dystrophy	Cardiomyopathy	Human disease
Mdx (dystrophin)	>1 year	Mild/moderate	Mild	DMD
MyoD/mdx (MyoD, dystrophin)	1 year	Severe	Severe	
Utrn <sup>-/-</sup> /mdx (utrophin, dystrophin)	4–20 weeks	Severe	Severe	
Sgca <sup>-/-</sup> ( $\alpha$ -sarcoglycan)	>1 year	Moderate	None	LGMD2D
Sgcb <sup>-/-</sup> ( $\beta$ -sarcoglycan)	>1 year	Severe	Severe	LGMD2E
Sgcg <sup>-/-</sup> ( $\gamma$ -sarcoglycan)	20 weeks	Severe	Severe	LGMD2C
Sgcd <sup>-/-</sup> ( $\delta$ -sarcoglycan)	>1 year	Severe	Severe	LGMD2F
DG <sup>-/-</sup> (dystroglycan)	Embryonic lethal	NA	NA	
Myd (large)	Reduced	Moderate	None	CMD1D
Dy/dy ( $\alpha$ 2-laminin)	6 months	Severe		CMD1A
Dy <sup>2J</sup> /dy <sup>2J</sup> ( $\alpha$ 2-laminin)	Reduced	Moderate/severe		CMD1A
SJL (dysferlin)	>1 year	Mild		LGMD2B

Adapted from Durbeej and Campbell, 2002

**Fig. 1** Examples of four mice models for neuromuscular diseases showing the respective histopathological alterations in the muscle



clinical symptoms and are viable and fertile. Linkage analysis localized the gene in the murine X chromosome (Bulfield et al. 1984). Molecular analysis by Sicinski et al. (1989) identified the mdx mutation as a single base substitution in exon 23 resulting in a premature stop codon in the mouse dystrophin gene, which leads to the absence of detectable dystrophin in the muscle, except in rare revertant myofibers (Hoffman et al. 1987, 1990). The absence of dystrophin also affects the expression of the other DGC components at the sarcolemma (Ohlendieck and Cambell 1991). However, the mdx shows a mild non-progressive phenotype, associated to a comparatively moderate muscle pathology, and muscle degeneration is followed by subsequent significant regeneration (Dangain and Vrbova 1984). Therefore, the mdx is a good genetic and biochemical model for DMD, but it is not useful for clinical evaluation in therapeutic trials. For this reason, histological analysis of the diaphragm, one of the most severely affected muscles of

the mdx, is more often used as a marker of weakness progression, as it reproduces the degenerative changes of muscular dystrophy. Other allelic variants of the mdx-dystrophin deficient mouse were discovered or produced in laboratory, including a knockout mouse for dystrophin (Araki et al. 1997). However, all models show similar phenotype and little advantage over the original mdx model (revisions in Allamand and Campbell 2000; Durbeej and Campbell 2002; Collins and Morgan 2003).

Some double mutants using the mdx background have been created and show more severe phenotypes. Among them, the mdx mice lacking the muscle-specific transcription factor MyoD is possibly more severely affected due to a deficiency in its regenerative capacity. Another double mutant is the mdx lacking utrophin. Utrophin is a developmentally regulated protein, an autosomal homologue to dystrophin. The *utr<sup>-/-</sup>/mdx* mice are severely affected and show many signs of the human dystrophy, with reduced

lifespan and severe muscle weakness with joint contractures, growth retardation, and cardiomyopathy, suggesting the important role of utrophin in muscle development (Durbeej and Campbell 2002).

Numerous sporadic cases of canine muscular dystrophy (MD) have been recognized in the last two decades, but the original description of the dystrophic Golden Retriever dog (GRMD), as a model for the human DMD, was done by Cooper et al. (1988) in an X-linked breed. This canine model represents a more clinically similar model because of its larger size and significant muscle weakness. However, there is also a significant phenotypic variability between litters. The GRMD mutation in the dystrophin gene consists of a splicing site substitution in intron 6 leading to the skipping of exon 7 and to a premature stop codon in exon 8, resulting in the absence of the protein dystrophin in the muscle (Valentine et al. 1992). Canine dystrophinopathies have also been reported in many other purebred and mixed breed dogs, and in addition to the Golden Retriever, genetic mutations have also been characterized in Rottweiler and the German Shorthaired Pointer.

The clinical signs in the GRMD are progressive, with a gradual weakness and loss of muscle mass, development of contractures, and skeletal deformities. Tongue hypertrophy is associated to pharyngeal and esophageal dysfunction and a decreased respiratory function is common (Shelton and Engvall 2005). The histopathological changes in the muscle are similar to the ones in humans and include muscle fiber degeneration and regeneration, fiber splitting, numerous fibers with centrally located myonuclei, and intense connective tissue replacement. On immunohistochemistry using anti-dystrophin antibodies, dystrophin is greatly deficient, with the observation of scattered reverted fibers (personal observation). An interesting Becker-like dystrophy with a truncated form of dystrophin was recently identified in a family of Japanese Spitz dogs (Shelton and Engvall 2005). Some sporadic cases of feline MD with dystrophin deficiency were also reported, with a typical clinical presentation, showing shoulder and neck muscle hypertrophy and dramatic tongue enlargement (Shelton and Engvall 2005).

#### Sarcoglycanopathies

The BIO14.6 hamster is the spontaneous animal model for sarcoglycanopathies, presenting a mutation in the  $\delta$ -SG gene. Clinically, this animal shows a significant cardiomyopathy, with the heart muscle more affected than skeletal muscle (Straub et al. 1998). More recently, using the homologous recombination technique in embryonic stem cell, several mouse models have been developed with null mutation in each one of the SG genes. All sarcoglycan-null animals display a progressive muscular dystrophy of variable severity and share the property of a significant

secondary reduction in the expression of the other members of the sarcoglycan subcomplex and other components of the DGC (Allamand and Campbell 2000).

Mice deficient in  $\alpha$ -SG (SGCa null mice), a protein exclusively expressed in striated muscle, develop a progressive muscular dystrophy and a concomitant deficiency in  $\beta$ -,  $\gamma$ -, and  $\delta$ -SG along with sarcospan in skeletal muscle. These mice, however, do not develop cardiomyopathy. On the other hand, mice deficient in  $\beta$ - and  $\delta$ -SG (SGCb and SGCd null mice, respectively) that are expressed in both striated and smooth muscle develop a more severe progressive muscular dystrophy. Differently than observed in the SGCa null mice, they present large focal areas of necrosis/fibrosis in skeletal muscle. Both SGCb and SGCd null mice also develop cardiomyopathy, and morphologically, this is recognized by extensive areas of necrosis/fibrosis in their hearts (Durbeej and Campbell 2002). Mice deficient for  $\gamma$ -SG (SGCg null model) also exhibit a severe muscular dystrophy and cardiomyopathy, but differently from the other models, the expression of dystroglycans is not altered, and the mice show normal resistance to mechanical strain, and no evidence of contraction-induced injury after exercise. This suggests a different physiopathological mechanism in the SGCg model (Durbeej and Campbell 2002).

Some canine models with deficiency of SG proteins have been identified in a young Boston Terrier, Cocker Spaniel, and Chihuahua, but their specific mutations have not been identified. The phenotype includes failure to thrive and exercise intolerance. Serum CK is highly elevated, and muscle histopathology shows a dystrophic pattern and a variable degree of loss of SG proteins staining (Shelton and Engvall 2005).

#### Laminin $\alpha$ 2 Deficiency

Two mouse models for laminin- $\alpha$ 2 deficiency were identified in the Jackson Laboratories (<http://www.jax.org/>), including the *dy/dy* (dystrophia-muscularis) mouse and the allelic mutant *dy<sup>2J</sup>/dy<sup>2J</sup>* mouse. Both mice are models for merosin-deficient Congenital MD (CMD1A). Neither of these mouse models exhibits a complete deficiency of laminin  $\alpha$ 2 chain. Yet, they both display a muscular dystrophy, although the muscular dystrophy in the *dy<sup>2J</sup>/dy<sup>2J</sup>* presents itself in a milder form compared to the *dy/dy* mouse.

Clinically, these mice are considerably smaller and weaker than littermates and have premature deaths. Mice homozygous for the *dy/dy* mutations are characterized by progressive weakness and paralysis beginning at about 3 1/2 weeks of age. The hindlimbs are affected first; later, the axial and forelimb musculature. Death usually occurs before 6 months of age, and the mice are usually sterile. Skeletal muscle shows degenerative changes with proliferation of sarcolem-

mal nuclei, increase in amount of interstitial tissue, and size variation among individual muscle fibers. They also present myelination defects of the peripheral nerves, which can be attributed to an intrinsic defect in the dystrophic process. Molecular analysis of Lama2 expression of dystrophic *dy/dy* mice revealed a deficiency of this mRNA in skeletal muscle, cardiac muscle, and peripheral nerve, but the specific mutation is still unknown (Jackson Laboratories).

The mutant  $dy^{2J}/dy^{2J}$  arose in the WK/Re strain and consists in a G to A mutation in a splicing site consensus sequence, which causes abnormal splicing and a deletion in domain VI of the  $\alpha 2$ -polypeptide (Xu et al. 1994; Vilquin et al. 2000). Effects of the Lama2  $dy^{2J}$  mutation are milder than those previously described for Lama2 *dy/dy*, but the two mutations may present similar clinical and histological alterations on the same genetic background. The homozygous  $dy^{2J}/dy^{2J}$  mouse develops muscle weakness at about 3 weeks of age, which progressively worsens. Clinical analysis shows flexed hind legs to the trunk when lifted by the tail at 2–3 weeks of age. The mice are larger than the *dy/dy* model, more active, and may breed two or three times. Their life expectancy is almost normal (Vilquin et al. 2000). Histopathological analysis reveals muscle degeneration, muscle fiber necrosis and fibrosis, and regenerative activity. Immunostaining of  $\alpha 2$  chain using specific antibodies is significantly reduced at the basal lamina surrounding each muscle fiber (Sunada et al. 1995).

### Glycosylation Defects

The two subunits of the dystroglycan, the extracellular  $\alpha$ -DG and the transmembrane  $\beta$ -DG, derived from post-translational cleavage of one precursor polypeptide encoded by the DAG1 gene, at 13 p21 (Holt et al. 2000). Dystroglycans are considered to be the most broadly expressed DGC components and are very important in the early stages of development because, when the dystroglycan gene was disrupted in the mouse, null embryos fail to progress beyond the early egg cylinder stage of development. Structural and functional perturbations of Reichert's membrane were detected in these animals (Williamson et al. 1997). A chimerical mice, lacking dystroglycan only in skeletal muscle were thus generated, and interestingly, these mice develop progressive muscle pathology similar to muscular dystrophies in humans (Cote et al. 1999).

In recent years, it became increasingly clear that post-translational modification of muscle cell proteins, in particular  $\alpha$ -DG, is important for normal muscle function. The myodystrophy mouse, or LARGE model (Large<sup>myd</sup>) harbors a mutation, a deletion that removes exons 4–6 of the mouse gene (Browning et al. 2005), in the glycosyltransferase Large, which leads to altered glycosylation of  $\alpha$ -DG (Grewal et al. 2001). Mutations in the human Large gene

causes a severe congenital muscular dystrophy type 1D (CMD1D). Affected mice have abnormal shuffling gaits, a diffuse and progressive myopathy, and a shortened lifespan. Homozygous mutant mice can be recognized at 12–15 days by their small size and abnormal posturing of their hind limbs (Browning et al. 2005). Growth/size phenotype can be observed with decreased body weight, postnatal growth retardation, abnormal bone structure, and kyphosis, but with no heart involvement. Clinical signals includes abnormal gait, hind limbs held close to the body producing a short, shuffling gait, and hind limbs that never extended and dragged. Severe contraction of hind limbs sometimes by 3–4 months of age can also be observed. Muscle histopathology demonstrates abnormal muscle morphology with degeneration, focal areas of acute necrosis, variable fiber size, loss of striation, and central migration of nuclei.

Highlighting the importance of the mechanism of glycosylation, the group of Kevin Campbell has recently showed that overexpression of LARGE ameliorates the dystrophic phenotype of Large<sup>myd</sup> mice and induces the synthesis of glycan-enriched  $\alpha$ -DG with high affinity for extracellular ligands, in distinct types of congenital MD. The results are very promising and suggest that the modulation of Large expression or activity can be a viable therapeutic strategy for glycosyltransferase-deficient congenital muscular dystrophies (Barresi et al. 2004).

### Dysferlinopathy

SJL/J mice develop a spontaneous myopathy resulting from a mutation in the Dysferlin gene. A 171-bp in-frame deletion in the encoded mRNA is predicted to remove 57 amino acids from the corresponding protein (Bittner et al. 1999). This region corresponds to most of the fourth C2 domain of the protein, and the deletion likely results in instability of the protein. The molecular basis for the mutation is due to a splicing mutation in the gene, resulting from a deletion of a small tandem repeat. This Dysferlin-mutated allele has been shown to result in decreased levels of dysferlin protein in SJL/J mice and makes this strain a good model for limb girdle muscular dystrophy 2B.

This spontaneous myopathy is characterized by a slowly progressive loss of muscle mass and strength corresponding to an increase in muscle pathology including muscle fibers with central nuclei, size variation, splitting, inflammatory infiltrate, necrosis, and eventual replacement of muscle fiber with fat tissue. Whereas muscle weakness can be detected as early as 3 weeks of age, the greatest pathology occurs after 6 months of age. SJL/J mice have also been shown to have an increased rate of muscle regeneration after injury when compared to BALB/c mice (Jackson Laboratories).

This model has been extensively studied in immunology because of the facility to induce on it a chronic inflamma-

tory myopathy. However, inflammation is a feature of the early stages of most muscular dystrophies, and older SJL mice have the usual signs of MD with extensive fibrosis in the muscle (Shelton and Engveall 2005).

### Other Muscle Related Animal Models

#### Myostatin

Myostatin is a member of transforming growth factor beta family and is a negative regulator of muscle growth. Myostatin knockout mice display an increased body weight of about 30% during development and up to 250% in some specific muscles. The additional muscle mass is due to both hyperplasia (increase in the number of muscle fibers) and hypertrophy (increase in the size of individual fibers). Additionally, a normalization of body weight in adult animals is observed in spite of the excessive muscle size. This has been attributed to a decreased fat accumulation in myostatin knockout mice, suggesting that the lack of myostatin also prevents excessive fat deposition (revision in Patel and Amthor 2005). Because of the important role of myostatin in muscle development and regeneration, therapies blocking myostatin have been tested for muscular dystrophies in animal models (Bogdanovich et al. 2002).

However, it is not clear whether the excessive muscle mass caused by loss of myostatin can result in improved muscle performance. In some cattle strain with “muscle doubling” and mutations in the myostatin gene, such as the Belgian Blues and Piedmontese breeds, forced exercise resulted in precocious exhaustion, acute rhabdomyolysis, and increase in serum CK levels. Additionally, a decreased reproductive fitness was also observed in these animals. On the other hand, a recent publication has shown a huge benefit in dogs, heterozygote for a mutation in the myostatin gene. A new mutation in MSTN gene (a two-base-pair deletion in the third exon of MSTN leading to a premature stop codon at amino acid 313) was found in the Whippet dog breed that results in a double-muscled phenotype Whippet. Interestingly, heterozygotes individuals are, on average, more muscular than wild-type individuals and are significantly faster than individuals carrying the wild-type genotype in competitive racing events. These results highlight the utility of performance-enhancing polymorphisms and associated for the first time mutations in MSTN with increased athletic performance (Mosher et al. 2007).

#### Malignant Hyperthermia

Malignant Hyperthermia (MH) is a potentially lethal pharmacogenetic predisposition associated with a susceptibility to volatile anesthetics and depolarizing muscle relaxants

that lead to a fulminant anesthetic crisis with hyperthermia, skeletal muscle rigidity, respiratory and metabolic acidosis, and muscle rhabdomyolysis. MH crises are caused by an abnormal regulation of the calcium release mechanism, which reflects the consequences of disturbed skeletal muscle calcium homeostasis. MH susceptibility is caused predominantly by dominant mutations in the RYR1-ryanodine receptor gene at chromosome 19 q13.1. When exposed to triggering agents, MH-susceptible individuals show uncontrolled  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum via the ryanodine receptor, the main calcium release channel of the sarcoplasmic reticulum. Excess myoplasmic  $\text{Ca}^{2+}$  leads to hypermetabolism, muscle rigidity, massive rhabdomyolysis, local and systemic acidosis, and eventual hyperthermia. MH has also been identified in several domestic species such as the pig, the dog, and the horse. In all species studied to date, MH has been associated with mutations in proteins that influence excitation–contraction coupling. Porcine, canine, and equine MH are each thought to be caused by a missense mutation in the RyR1 gene, which can be homozygous in porcine or heterozygous, as in canine and equine (revision in Yang et al. 2006). The porcine model is especially important, as it was identified in 1968. This was called porcine stress syndrome because of stress-triggered events, mainly during transportation. Afterward, it was discovered that certain pigs were sensitive to Halotane, an anesthetic that caused MH in humans, and linkage analysis showed an association in pigs between a mutation in the RYR1 protein coding gene and the development of MH. In five breeds of pigs, the mutation Arg615Cys in the RYR1 gene was identified (Fujii et al. 1991). The discovery of this gene led to the identification of an homologous gene in humans (revision in Brandom 2006). The use of the porcine model has been historically useful, especially in the development of dantrolene as a therapy for preventing the MH episode. However, the porcine MH is an autosomal recessive trait and limited to a single mutation (Arg615Cys), which accounts for less than 2% of human MH cases. Thus, some mice models have been created by genetic manipulation techniques, carrying more common mutations. The most studied one carries the Arg615Cys mutation in hot spot 1 of the RYR1 gene in heterozygote state. This model develops fulminant MH episodes after exposure to low levels of halotane or an ambient temperature of 42°C or more and has been considered a new good model for studying tests and therapies for malignant hyperthermia (Yang et al. 2006).

### Conclusion

Analysis of mouse models for neuromuscular diseases has unraveled novel pathogenetic mechanisms for the development of muscular dystrophy. The studies done in the mdx

mice were fundamental to the elucidation of the role of inflammation in regeneration and fibrosis during the dystrophic process. More importantly, the use of mice models have been of immense benefit for evaluating possible stem cells and drug therapies for muscular dystrophy and cardiomyopathy (Gussoni et al. 1999; Durbeej and Campbell 2002).

In summary, the study of animal models for genetic diseases, in spite of the existence of differences in the phenotype, genetics, or primary molecular defect will continue to be an important tool for the elucidation of the mechanisms of diseases and for experimental treatments.

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