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Recommendations on the care and handling of the MDC1A mouse model *dy^{W}*

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1. OBJECTIVE

Mouse models for congenital muscular dystrophy, such as the *dy*^{W} strain (*Lama2*^{tm1Eeng}, *Lama2*$^{dy\text{-}W}$), develop muscle weakness, loss of muscle function and impaired mobility that affects their survival. This document describes recommended procedures for the care of these mice, designed to standardize and ensure adequate nutrition, hydration and general environmental conditions necessary for their survival.

2. SCOPE AND APPLICABILITY

Animal models for neuromuscular diseases such as the *dy*^{W} model for laminin α2 (*Lama2*)-deficient (or merosin-deficient) congenital muscular dystrophy (MDC1A) develop severe limitations in coordinated muscle movement that can affect their ability to eat, drink, breath and compete for survival when housed with normal mice. The *dy*^{W} strain, derived by targeted insertion of a LacZ-neo reporter cassette within the *Lama2* locus (Kuang et al., 1998; Kuang et al., 1999), produces low levels of a truncated form of Lama2 that lacks function (Guo et al., 2003). Several additional *Lama2*-deficient mouse models arising from either spontaneous or induced mutations have also been widely studied (http://www.informatics.jax.org/searches/allele_report.cgi?markerID=MGI:99912). The disease severity phenotype correlates with levels of *Lama2* expression (Guo et al., 2003). The guidelines provided here are applicable to all of these mutant strains, as well as other mouse disease models in which mutations compromise animal mobility and behavior.

The *dy*^{W} mice have a severe phenotype in which muscle degeneration begins within 1-2 weeks after birth and is followed by poor muscle regeneration and fibrosis (Kuang et al., 1999). Motor neurons are affected as well because Schwann cells that myelinate the neurons also require *Lama2* for their proliferation and development (Chernousov et al., 2008; Yang et al., 2005; Yu et al., 2007). Myelination defects primarily affect spinal roots and cranial nerve function, while brachial nerves are less affected, possibly due to compensation by other laminin isoforms (i.e. α4, α1) expressed there (Yang et al., 2005; Yu et al., 2007). The combined loss of muscle cells and functional innervation leads to overall muscle weakness and eventual development of hindlimb paralysis. Hindlimb weakness is apparent at approximately 2-3 weeks of age, evident by slight tremors and inward limb retraction when mice are suspended by their tails. The frequency of standing on hindlimbs is reduced (Girgenrath et al., 2004), as is their grip strength and locomotor activity (Meinen et al., 2007; Moll et al., 2001; Qiao et al., 2005) This is

followed later by hindlimb dragging and eventual hindlimb paralysis, accompanied by rigid contractures in mice surviving to 7-8 weeks and beyond ((Kuang et al., 1998), J. Dominov observations). Typically, ~40-65% of these mice die by 4 weeks of age and few, if any survive beyond 4 months of age (Erb et al., 2009; Kuang et al., 1998; Kumar et al., 2011; Meinen et al., 2007; Moll et al., 2001; Qiao et al., 2005).

Progressive *dy*^{W} pathology impairs animal mobility, therefore extra attention and care are required to ensure that mice have adequate access to food and water, and are housed under conditions that minimize stress. It is important to establish criteria for care and oversight to avoid experimental variation caused by inadequacies in these basic elements of animal care.

3. CAUTIONS

As with all animal model studies, care should be taken to minimize experimental variation due to inconsistencies in animal handling and environmental conditions. In the case of animals with impaired mobility or behavior such as *dy*^{W} mice, extra precautions as described below can help to minimize variation.

4. MATERIALS

4.1 Husbandry supplies

In addition to standard mouse caging, bedding, water supply and food pellets (e.g. ISO Pro 3000, LabDiet, St. Louis, MO), additional housing materials could include:

- High fat breeder diet food pellets (e.g. PicoLab 5058, LabDiet, St. Louis, MO)
- Gel food/ hydration supply such as HydroGel (ClearH2O, Portland, ME) or Napa Nectar (Systems Engineering, Napa, CA)
- Nestlets (Anacare, Bellmore, NY,)
- Rodent huts (Bio-Serv, Frenchtown, NJ) or Shepherd Shacks (Shepherd Specialty Papers, Watertown, TN)

4.2 Other supplies

- Clidox (Pharmacal Research Labs, Waterbury, CT)
- Isoflurane (Aerrane, Baxter, Deerfield, IL)
- Anesthetizing jar, glass, 1-1.5 liter with lid and 50 ml plastic centrifuge tube

- Tattoo paste (Ketchum, Inc., Brockville, ON Canada)
- Sterile 26g needles
- Sterile cotton balls
- Scissors or scalpel
- Microcentrifuge tubes (1.5 ml)
- 70% ethanol
- Isopropanol wipes
- Kwik-Stop styptic powder

4.3 Genotyping reagents

- 25mM NaOH / 0.2 mM EDTA
- 40 mM Tris HCl (pH 5.5)
- HotMaster Taq polymerase Kit (5 Prime, Inc., Gaithersburg, MD)
- Agarose gel apparatus and materials for DNA (PCR product) analysis

5. METHODS

5.1 Animal handling and care

All animal handling, care and experimentation must be approved and monitored by institutional bodies responsible for animal care and use. Procedures must follow institutional policies and guidelines, such as those established in the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and described in the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. In compliance with these guidelines and good standards of animal care, mice should be housed in a well-maintained, pathogen-free environment, with controlled temperature (~21°C), air flow, humidity (~50%), light cycles (12 hr light, 12 hr dark), and under conditions that minimize noise, vibrations, odors (e.g. perfumes or chemicals), and traffic flow. All experimental procedures should be performed in rooms separate from animal housing rooms. Routines should be in place for daily animal monitoring by caretaker staff to identify any health issues, and for bedding, food and water changes, as appropriate. Procedures should strive to minimize disturbance of cages, particularly those with litters. Those in contact with animals should be diligent in following procedures that maintain a pathogen-free environment. This includes use of gloves and other personal

protective equipment as required, frequent use of sanitizing agents (e.g. Clidox) to disinfect gloves and equipment, and compliance with animal quarantine rules. Veterinary staff must be available for consultation and animal care as needed.

5.1.1. Breeding

Mice that are breeding can be fed a breeder diet with higher fat content (e.g. PicoLab 5058) to improve outcome. *Lama2*-deficient dy^W mice must be bred as heterozygotes ($dy^W/+$ males x $dy^W/+$ females) due to the early lethality of homozygous *Lama2*-deficient animals. At birth, progeny exhibit typical Mendelian ratios, (1:2:1, (+/+): (+/-): (-/-)) (Wardrop and Dominov, 2011), with a typical litter size in the range of 6-10 pups. By 1 week of age, mutant dy^W/dy^W mice are smaller than siblings (Wardrop and Dominov, 2011), and by weaning age (~3 weeks) the difference in size among normal and mutant siblings is more evident, and mutants often appear more frail and some animal death occurs. By 4 weeks of age up to two-thirds of dy^W/dy^W mice die and hindlimb weakness is more evident in those surviving to this point and beyond. For this reason, mice should be monitored daily after weaning for signs of impaired mobility, weakness, and distress.

5.1.2. Food Supply and Environment

Lama2-deficient mice, from an early age, are smaller and weaker than siblings expressing *Lama2*, therefore extra precautions should be taken to ensure an adequate nutrition supply and to minimize stress. Mutant mice in larger litters might have difficulty competing with siblings for access to maternal milk. Introduction of a foster mother to provide additional nutrition supply could be helpful if such competition becomes apparent. Here, the foster female should be at a similar stage post-partum as the mother of the litter to provide optimal milk availability (i.e. not a foster mother from a litter close to weaning). Two small litters born in one cage as a result of harem mating could accomplish a similar result, providing that animal numbers do not exceed desirable cage limits.

At weaning (3 weeks of age), weakness of mutant mice or onset of impaired hindlimb mobility could limit their access to solid food pellets and water suspended above them in the cage. From the time of weaning, a gel food/ hydration source such as HydroGel, Napa Nectar, or softened food pellets soaked in water, along with normal food pellets should be provided on the floor of the cage thereby allowing easy access. This should be done consistently to minimize nutrition variation from litter to litter that might arise from variability in hindlimb weakness. For individually ventilated microisolator cage systems with high airflow, the gel should be replaced daily as it becomes dehydrated quickly.

Additional materials can be routinely added to mouse cages to enrich the environment and minimize animal stress. Examples include the addition of shreddable nesting material such as Nestlets along with plastic huts or Shepherd Shacks to provide a secure nesting environment. Stress associated with reduced body temperature could occur in young pups that are isolated from their litters for prolonged periods of time, so this time should be minimized. If necessary, an external heat source (heating pad or lamp) could be used to maintain warmth, using caution to avoid heat stress by maintaining the ambient temperature between 26-34°C.

5.1.3. Genotyping

DNA from small tail biopsies can be used in PCR reactions to determine the genotypes of pups. Use sterile techniques and materials for tissue collection. Pups as young as 1 day-old can be genotyped, but there is an increased risk of the mother abandoning the litter if they are disturbed within the first 2-3 days after birth. Brief exposure of the mother to the inhalant anesthetic (isoflurane) before proceeding with the pups can be helpful by masking that smell which will be associated with the pups. Mice are briefly (<1 min) exposed to isoflurane in a small closed, sanitized glass jar until they don't react to touch. For this, a sterile cotton ball wet with a few drops isoflurane is placed in an open 50 ml sanitized plastic tube (to prevent the mouse from directly contacting the anesthetic), and the chamber atmosphere is allowed to equilibrate for several minutes before use. Mouse weight is recorded, the distal tip of the tail is wiped with 70% ethanol, then a ~1-2 mm tip if the tail is removed with scissors or scalpel (sterilized in a heated glass bead sterilizer or 70% isopropanol or ethanol) and collected in a 1.5 ml microcentrifuge tube for DNA preparation. To identify mice, small permanent spots can be tattooed on the skin using a small gauge (e.g. 26g) needle dipped in tattoo ink, marking ears or the skin at edges of paws (avoiding paw pads, which are particularly sensitive areas). Alternatively, numbered ear tags or ear clip/hole punch methods can be used to identify mice. Toe clipping (an amputation) is generally discouraged. Little residual tail bleeding typically occurs with young pups but could occur with older pups. Ensure that bleeding stops by applying pressure to the tip of the tail or by using styptic powder (e.g. Kwik-Stop).

Numerous methods can be used to prepare DNA samples of varying levels of purity from tail biopsies. Commercial kits (e.g. Puregene Genomic DNA Purification Kit, Qiagen) result in purified DNA suitable for a variety of downstream applications. Alternatively, crude tail lysates that are adequate for routing genotyping can be efficiently prepared in streamlined 1-tube procedures such as that using the DirectPCR Lysis Reagent (Tail) (Viagen Biotech) with 0.2 mg/ml proteinase K following the manufacturer's protocol. We routinely use a streamlined tail DNA preparation protocol from Jackson Laboratories that works well, even for genotyping that involves more difficult PCR reactions (e.g. high GC content amplicons)

(http://jaxmice.jax.org/support/genotyping/dna-isolation-protocols.html). Tail biopsies are suspended in 75 µl of 25mM NaOH /0.2 mM EDTA then heated at 98°C for 1 hour. At 3 times during this incubation, (e.g. at 15, 30, 45 min.) and at the end samples are vortexed and centrifuged for a few seconds to break up tissue, then the temperature is reduced to 15-20°C until ready to proceed to the next step. 40 mM Tris HCl (pH 5.5) is added, 75 µl/ tube, along with 600 µl water to dilute the sample 5-fold. The tubes are vortexed and centrifuged at 1,500 x g for 3 minutes. Aliquots of the resulting tail lysate supernatants (0.5 µl) are then directly used in 20 µl PCR reactions. A single multiplex PCR reaction with combined primer pairs allows detection of both wild-type and mutant *dy*^{W} alleles when the PCR products are run on standard agarose gels. Primers that distinguish wild-type and *dy*^{W} mutants (Kuang et al., 1998), with one primer slightly modified (Dominov et al., 2005), are listed below. PCR reactions using hot-start amplitaq polymerase reagents (e.g. HotMaster Taq DNA Polymerase Kit, 5 Prime) are as follows:

Tail DNA	0.5 µl
Nuclease-free ddH_2O	15.4 µl
10X HotMaster Reaction Buffer with 25 mM Mg^{2+}	2.0 µl
dNTP mixture (2.5 mM each: dATP, dCTP, dGTP, dTTP)	1.6 µl
Primer mix 1 (15 µM each: wt forward + wt reverse)	0.2 µl
Primer mix 2 (15 µM each: wt forward + *dy*^{W} reverse)	0.2 µl
HotMaster amplitaq DNA polymerase	0.1 µl
Total volume	20.0 µl

Primers (listed 5'- 3'):

Wild-type allele	wt forward	actgccctttctcacccaccctt	247 bp product
	wt reverse	gttgatgcgcttgggac	
Mutant allele	wt forward	actgccctttctcacccaccctt	454 bp product
	dy^{W} reverse	gtcgacgacgacagtatcggcctcag	

PCR conditions:

94°C 2 min

94°C 20 sec
62°C 20 sec
65°C 1 min
} 30 cycles

65°C 10 min

4°C hold

5.2. Experimental issues and data recording

5.2.1. Animal Health and Experimental Endpoints

Individual experimental designs will dictate the age at which mice will be analyzed for physiological or behavior characteristics and the experimental endpoints when mice will be euthanized for tissue analysis. Experiments must be designed to accommodate anticipated loss of animals due to disease and with consideration of the poor health arising in older animals (beyond 7 – 8 weeks of age typically) that will dictate euthanasia. Mice that exhibit significant signs of distress or pain must be euthanized. These signs include agitation, abnormal breathing or activity, hostility, guarding, restlessness, lethargy, lack of movement or severely impaired movement such as that associated with the hindlimb contractures developing in these *Lama2*-deficient mice. Experiments involving older mice should have specific goals and endpoints that warrant maintaining animals with impaired mobility. Investigators should seek advice from veterinary staff as needed.

5.2.2 Routine Experimental Parameters

Body weight

Changes in body weight can be routinely monitored during the course of experiments using *dy*^{W} mutant mice and provide a measure of disease severity. Tracking body weight (g) at weekly intervals will demonstrate the disease effects on animal growth. Differences in body weight between *Lama2*-deficient mice and siblings expressing *Lama2* are evident from 7 days

after birth when mean body weight of dy^W/dy^W mice is ~82% that of normal siblings (Wardrop and Dominov, 2011). Body weight differences (gender matched) become greater with age, such that dy^W/dy^W mouse weight is ~58-60% that of wild-type at 3 weeks and ~38-50% at 6 weeks of age (Dominov et al., 2005). Others studies have noted similar differences in body weight (Girgenrath et al., 2009; Girgenrath et al., 2004; Guo et al., 2005; Kuang et al., 1998; Kumar et al., 2011; Li et al., 2005; Moll et al., 2001; Qiao et al., 2005).

Muscle weight

The weight of isolated muscles is also reduced significantly in dy^W mutant mice and serves as a useful parameter to monitor disease-related pathology. For instance gastrocnemius/ soleus, tibialis anterior and quadricep muscles of dy^W/dy^W mice have ~ 18-25% the weight of wild-type mice (Girgenrath et al., 2004). Muscles to be measured should be dissected carefully away from bones with the aid of magnification (e.g. using a dissecting microscope), ensuring that the entire muscle is collected by cutting at tendons or as close to the insertion point as possible. Fat, overlying muscle and any other extraneous tissue should be removed and if necessary, the muscle blotted briefly with a tissue to remove excess moisture prior to weighing, working quickly to avoid tissue dehydration. Muscle weights can be compared directly, or represented as a percentage of total body weight (i.e. g (muscle)/ g (total body)) (Carmignac et al., 2011) to compensate for the overall reduced size of mutant mice with age.

Muscles for Analysis

A number of muscles are useful for monitoring disease progression and severity in the *Lama2*-deficient mouse models. Evaluation of these muscles might include histological, morphometric, immunohistological, biochemical or molecular analyses of gene expression as described in other protocols in this series. Hindlimb muscles, which become severely affected in *Lama2*-deficient mutant mice as a result of muscle degeneration along with hindlimb paralysis, include the tibialis anterior, gastrocnemius, soleus and quadriceps (rectus femoris) (Connolly et al., 2001; Gawlik et al., 2004; Gawlik and Durbeej, 2010; Girgenrath et al., 2004; Kuang et al., 1999; Li et al., 2005; Meinen et al., 2007; Xu et al., 2007). Forelimb muscles (e.g. triceps brachii, biceps brachii), which are not secondarily affected by the severe paralysis due to compromised neuronal activity, along with the diaphragm are also useful for analysis of muscle disease progression (Connolly et al., 2001; Dominov et al., 2005; Erb et al., 2009; Gawlik et al., 2004; Meinen et al., 2007; Moll et al., 2001; Qiao et al., 2005). Individual experimental goals and designs will dictate which muscles should be used, but analysis of multiple muscles from each

mutant animal along with their unaffected cohorts can determine whether any observed experimental effects are common to all muscles or restricted to particular muscle types. Such information could provide new insight into disease progression.

6. EVALUATION AND INTERPRETATION OF RESULTS

The *dy*^{W} mouse strain is a useful experimental model for MDC1A but these mice require extra care due to the degenerative nature of this disease. As with all animal models, it is important to ensure that standard guidelines for maintaining these mice are established and diligently followed to ensure that interpretation of experimental results is not encumbered by improper or inconsistent quality of care. In order to evaluate results compiled from multiple litters and mice with declining health maintained over prolonged periods of time it is important to ensure:

- a pathogen-free environment
- adequate availability of nutrition/hydration

  (particularly post-weaning when mouse mobility becomes compromised)
- a minimal stress environment

  (provide enrichments, avoid overcrowding or competition)
- frequent mouse monitoring for health problems that require intervention or euthanasia

7. REFERENCES

Carmignac, V., Quere, R., and Durbeej, M. (2011). Proteasome inhibition improves the muscle of laminin {alpha}2 chain-deficient mice. Hum Mol Genet *20*, 541-552.

Chernousov, M.A., Yu, W.M., Chen, Z.L., Carey, D.J., and Strickland, S. (2008). Regulation of Schwann cell function by the extracellular matrix. Glia *56*, 1498-1507.

Connolly, A.M., Keeling, R.M., Mehta, S., Pestronk, A., and Sanes, J.R. (2001). Three mouse models of muscular dystrophy: the natural history of strength and fatigue in dystrophin-, dystrophin/utrophin-, and laminin alpha2-deficient mice. Neuromuscul Disord *11*, 703-712.

Dominov, J.A., Kravetz, A.J., Ardelt, M., Kostek, C.A., Beermann, M.L., and Miller, J.B. (2005). Muscle-specific BCL2 expression ameliorates muscle disease in laminin {alpha}2-deficient, but not in dystrophin-deficient, mice. Hum Mol Genet *14*, 1029-1040.

Erb, M., Meinen, S., Barzaghi, P., Sumanovski, L.T., Courdier-Fruh, I., Ruegg, M.A., and Meier, T. (2009). Omigapil ameliorates the pathology of muscle dystrophy caused by laminin-alpha2 deficiency. J Pharmacol Exp Ther *331*, 787-795.

Gawlik, K., Miyagoe-Suzuki, Y., Ekblom, P., Takeda, S., and Durbeej, M. (2004). Laminin alpha1 chain reduces muscular dystrophy in laminin alpha2 chain deficient mice. Hum Mol Genet *13*, 1775-1784.

Gawlik, K.I., and Durbeej, M. (2010). Transgenic overexpression of laminin alpha1 chain in laminin alpha2 chain-deficient mice rescues the disease throughout the lifespan. Muscle Nerve *42*, 30-37.

Girgenrath, M., Beermann, M.L., Vishnudas, V.K., Homma, S., and Miller, J.B. (2009). Pathology is alleviated by doxycycline in a laminin-alpha2-null model of congenital muscular dystrophy. Ann Neurol *65*, 47-56.

Girgenrath, M., Dominov, J.A., Kostek, C.A., and Boone Miller, J. (2004). Inhibition of apoptosis improves outcome in a model of congenital muscular dystrophy. J Clin Invest *114*, 1635-1639.

Guo, L.T., Shelton, G.D., Wewer, U.M., and Engvall, E. (2005). ADAM12 overexpression does not improve outcome in mice with laminin alpha2-deficient muscular dystrophy. Neuromuscul Disord *15*, 786-789.

Guo, L.T., Zhang, X.U., Kuang, W., Xu, H., Liu, L.A., Vilquin, J.T., Miyagoe-Suzuki, Y., Takeda, S., Ruegg, M.A., Wewer, U.M*., et al.* (2003). Laminin alpha2 deficiency and muscular dystrophy; genotype-phenotype correlation in mutant mice. Neuromuscul Disord *13*, 207-215.

Kuang, W., Xu, H., Vachon, P.H., Liu, L., Loechel, F., Wewer, U.M., and Engvall, E. (1998). Merosin-deficient congenital muscular dystrophy. Partial genetic correction in two mouse models. J Clin Invest *102*, 844-852.

Kuang, W., Xu, H., Vilquin, J.T., and Engvall, E. (1999). Activation of the lama2 gene in muscle regeneration: abortive regeneration in laminin alpha2-deficiency. Lab Invest *79*, 1601-1613.

Kumar, A., Yamauchi, J., Girgenrath, T., and Girgenrath, M. (2011). Muscle Specific Expression of Insulin-Like Growth Factor 1 Improves Outcome in Lama2Dy-w mice, a model for Congenital Muscular Dystrophy Type 1A. Hum Mol Genet *in press*.

Li, Z.F., Shelton, G.D., and Engvall, E. (2005). Elimination of myostatin does not combat muscular dystrophy in dy mice but increases postnatal lethality. Am J Pathol *166*, 491-497.

Meinen, S., Barzaghi, P., Lin, S., Lochmuller, H., and Ruegg, M.A. (2007). Linker molecules between laminins and dystroglycan ameliorate laminin-alpha2-deficient muscular dystrophy at all disease stages. J Cell Biol *176*, 979-993.

Moll, J., Barzaghi, P., Lin, S., Bezakova, G., Lochmuller, H., Engvall, E., Muller, U., and Ruegg, M.A. (2001). An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy. Nature *413*, 302-307.

Qiao, C., Li, J., Zhu, T., Draviam, R., Watkins, S., Ye, X., Chen, C., Li, J., and Xiao, X. (2005). Amelioration of laminin-alpha2-deficient congenital muscular dystrophy by somatic gene transfer of miniagrin. Proc Natl Acad Sci U S A *102*, 11999-12004.

Wardrop, K.E., and Dominov, J.A. (2011). Proinflammatory Signals and the Loss of Lymphatic Vessel Hyaluronan Receptor-1 (LYVE-1) in the Early Pathogenesis of Laminin {alpha}2-Deficient Skeletal Muscle. J Histochem Cytochem *59*, 167-179.

Xu, R., Chandrasekharan, K., Yoon, J.H., Camboni, M., and Martin, P.T. (2007). Overexpression of the cytotoxic T cell (CT) carbohydrate inhibits muscular dystrophy in the dyW mouse model of congenital muscular dystrophy 1A. Am J Pathol *171*, 181-199.

Yang, D., Bierman, J., Tarumi, Y.S., Zhong, Y.P., Rangwala, R., Proctor, T.M., Miyagoe-Suzuki, Y., Takeda, S., Miner, J.H., Sherman, L.S.*, et al.* (2005). Coordinate control of axon defasciculation and myelination by laminin-2 and -8. J Cell Biol *168*, 655-666.

Yu, W.M., Yu, H., and Chen, Z.L. (2007). Laminins in peripheral nerve development and muscular dystrophy. Mol Neurobiol *35*, 288-297.