Muscle regeneration in the prolonged absence of myostatin

Kathryn R. Wagner*†‡, Xiaosong Liu§, Xiaoli Chang‡¶, and Ronald E. Allen§

Departments of *Neurology and ¶Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ‡Muscle Biology Group, Department of Animal Sciences, University of Arizona, Tucson, AZ 85721

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Myostatin is an endogenous inhibitor of muscle conserved across diverse species. In the absence of myostatin, there is massive muscle growth in mice, cattle, and humans. Previous studies in the mdx mouse model of muscular dystrophy demonstrate that inhibiting myostatin attenuates several features of dystrophic muscle. These findings have encouraged the development of human therapies to block myostatin. However, little is known of the long-term effects on muscle of myostatin blockade. To evaluate potential sequelae from the prolonged absence of myostatin, senescent myostatin null (mstn−/−) mice were studied. Senescent mstn−/− mice continue to have normal muscle with increased mass and strength relative to controls. Muscles of senescent mstn−/− mice regenerate robustly from both chronic and acute injury. Early markers of regeneration are enhanced in the absence of myostatin, suggesting a mechanism for the attenuation of dystrophic features found in mdx mice lacking myostatin.

Materials and Methods

Animals and Muscle Injury. Myostatin null (mstn−/−) mice and littermate controls were provided by the laboratory of Se-Jin Lee (The Johns Hopkins University School of Medicine) (1). Mdx mice were obtained by Jackson ImmunoResearch and bred to myostatin null mice as described (9). All mice were on a C57BL/6 background. The age range of experimental animals was between 3 weeks and 24 months. Control and disease animals were matched by age and gender. All animal studies were authorized by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

For studies involving muscle injury, mice were anesthetized by metofane inhalation. Tibialis muscle was injected with 0.1 ml of 10 μM cardiotixin (Calbiochem), which was diluted in PBS (14, 15). Muscle was harvested at various times after injection (1, 2, 3, 4, 7, 14, and 30 days). Contralateral, un.injected tibialis muscle was used as a control.

Muscle Histology and Morphometric Analysis. Muscle for histological analysis was flash frozen in isopentane. Cryostat cross sections (10 μm) were stained with hematoxylin and eosin. Muscle fiber diameters were determined by using IPLAB software (Scanalytics, Fairfax, VA) as described (9). We measured 300–500 consecutive fibers in each of three tibialis muscles per genotype per time point. Percentage of central nucleation was determined by counting the total number of central nucleated fibers and total myofibers in cross section of the triceps midbelly.

Evans Blue Dye (EBD). We injected into the peritoneal cavity of 3-week-old animals 1% volume per body mass of 1% EBD, an autofluorescent dye that is excluded by normal sarcolemma but is internalized during membrane permeability and myofiber necrosis (16). Animals were returned to their cages and killed 24 h after injection. EBD-positive fibers and total myofiber number were counted in three different cross sections of each

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Abbreviations: EBD, Evans blue dye; mrf, myogenic regulatory factor.

*To whom correspondence should be addressed: Department of Neurology, The Johns Hopkins University School of Medicine, Meyer 5-119, 600 North Wolfe Street, Baltimore, MD 21287. E-mail: kwagner@jhmi.edu.

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triceps including the midbelly, and the average number of EBD-positive fibers per 1,000 fibers was calculated.

EBD-positive fibers were also quantified in 20- to 24-month-old animals after prolonged exercise. At 1 h after injection with EBD, mice were placed on a motorized treadmill (Eco 3/6, Columbus Instruments, Columbus, Ohio) and run at a 15° downward angle at a rate of 10 m/min for 10 min. Mice were killed 3 h after exercise, and EBD-positive fibers in triceps muscles were quantified as described above.

Functional Muscle Strength. Forearm grip strength was assessed by using an automated grip strength meter (Columbus Instruments). Total peak force (in N) was determined by an electronic strain gauge as described (17). Five measurements within 2 min were taken from each animal. Maximum values were used for statistical analysis (Student’s paired t test).

Myogenic Regulatory Factor (mrf) Expression. Tibialis muscle from senescent, 20- to 24-month-old mstn−/− and WT littermates was injured with cardiotxin, as described above. Muscle was harvested before injury and at 1, 2, 3, 4, and 7 days after injury, and RNA was prepared by using TRizol reagent according to the manufacturer’s instructions (Invitrogen). We separated 10 μg of total RNA on a denaturing agarose gel and transferred to GeneScreen Plus hybridization transfer membrane (PerkinElmer). Membranes were hybridized with myogenin, myoD, and mrf4 cDNA probes provided by Se-Jin Lee. Hybridization was quantified by using a phosphoimager (Fuji). Membranes were then stripped and reprobed with phospho-oligo(dt), and phosphoimager analysis was repeated. The relative abundance of mrf transcripts per lane was expressed in arbitrary units as mrf transcript per polyA transcript × 10−3.

Satellite Cell Isolation and Cell Culture. Satellite cells were isolated from 6- to 7-month-old and 18-month-old WT (C57BL6) and mstn−/− mice as described (18). Satellite cells were plated on polylysine and fibronectin-coated 48-well culture plates at a density equivalent to 0.2 g of muscle tissue per well. The cells were maintained in growth medium consisting of DMEM (HyClone), 10% horse serum (Gemini Biological Products, Calabasas, CA), 1% antibiotic/antimycotic, and 1% gentamicin. For evaluating the effect of myostatin on proliferation and differentiation of WT and mstn−/− satellite cells, cultures were maintained in growth medium from the time of plating to 48 h. The cells were then maintained in growth medium with or without 300 ng/ml recombinant mouse myostatin. Tibios (tric), pectoralis (pect), quadriceps (quad), and gastrocnemius (gast) muscle isolated from C57BL/6 WT (gray, n = 5) and mstn−/−/C57BL/6 (black, n = 3) mice. **, P < 0.01; ***, P < 0.001.

Results

To investigate whether muscle structure and growth remains normal in the prolonged absence of myostatin, senescent, 24-month-old mstn−/− were analyzed and compared with their

![Fig. 1.](https://www.pnas.org/cgi/doi/10.1073/pnas.0408729102) Increased muscle mass of senescent (24-month-old) mice lacking myostatin. Triceps (tric), pectoralis (pect), quadriceps (quad), and gastrocnemius (gast) muscle isolated from C57BL/6 WT (gray, n = 5) and mstn−/−/C57BL/6 (black, n = 3) mice. **, P < 0.01; ***, P < 0.001.

![Fig. 2.](https://www.pnas.org/cgi/doi/10.1073/pnas.0408729102) Muscle histology of senescent (24-month-old) WT and mstn−/− mice. (A) Uninjured tibialis muscle. (B) Tibialis muscle at 1 month after cardiotxin injury. (Scale bar, 50 μm.)
WT littermates. Similar to young animals lacking myostatin as described (1), muscle weights of senescent mstn−/− animals remain elevated by >2-fold compared with their WT counterparts (Fig. 1). Histology of multiple limb muscles is normal with no evidence of myopathy including no significant split fibers, central nucleated fibers, or fibrosis, compared with WT senescent animals (Fig. 2A). Myofibers of senescent mstn−/− animals are not prone to eccentric contraction-induced injury. Prolonged exercise on an declined treadmill produces negligible (mean, ≤2 per 1,000 fibers), scattered prenecrotic fibers, as identified by EBD in both senescent mstn−/− (n = 5) and WT (n = 7) animals.

When acutely injured by cardiotoxin, tibialis muscle from senescent 24-month-old mstn−/− animals retain the ability to regenerate. Histological abnormalities of fully regenerated muscle are limited to variability of fiber size, centralized nuclei, and rare split fibers similar to regenerated WT muscle (Fig. 2B). However, in contrast to WT muscle, mstn−/− muscle more rapidly attains larger diameter myofibers in the first week after injury (Fig. 3). For example, at 7 days after injury, mstn−/− myofibers have diameters 43% larger than WT myofibers (23.5 ± 2.7 μm versus 13.4 ± 3.5 μm, P = 0.04, n = 3 for each group). These diameters correspond to 55% of the respective uninjured myofiber size in the mstn−/− animals and 36% of uninjured myofiber size in the WT animals. WT myofibers do not attain a comparable fiber size (22 ± 1.6 μm) for another week at 15 days after injury. By 30 days after injury, myofibers from both genotypes have reached 85–89% of the myofiber diameter from their respective uninjured counterparts.

Satellite cells are induced to proliferate and differentiate in growth and regeneration. This requires the initiation of a muscle specific gene program involving the expression of basic helix–loop–helix mrf. The expression of these mrf at various time points after injury by cardiotoxin injection was evaluated in mstn−/− and WT counterparts. As shown in Fig. 4, where the relative abundance of transcripts in mstn−/− and control animals is normalized to total polyA+ RNA, the expression of myogenin (4A) is similar in mstn−/− and control animals. However, myoD expression (4B) is more robust in mstn−/− than control animals.
on day 2 after injury and beyond. Similarly, mrf4 expression (4C) is higher in mstn−/− than control animals. Control animals show no mrf4 expression at day 2 after injury (22). In contrast, mstn−/− animals have substantial expression of mrf4 by day 2 after injury and beyond.

Accumulating evidence suggests that, in the absence of myostatin, muscle progenitor cells are disinhibited (8, 23, 24). Similarly, we have observed that satellite cells dissociated from muscle of 6- to 7-month-old mstn−/− animals proliferate and differentiate more rapidly than satellite cells from WT animals in vitro (Fig. 5). This observation implies that the normal function of myostatin is to inhibit satellite cell proliferation and differentiation. Satellite cells isolated from mstn−/− and WT animals were treated with 300 μg of exogenous myostatin in the presence of horse serum. Cultures of mstn−/− satellite cells had significantly decreased rates of proliferation, but WT cultures were not significantly affected at the concentration used in these experiments (Fig. 6A). Likewise, differentiation was also decreased significantly in mstn−/− satellite cell cultures by myostatin but not in WT cultures (Fig. 6B). Satellite cells from mstn−/− muscle are capable of responding to exogenous myostatin, but in WT cultures, the production of endogenous myostatin likely blunts the response to exogenous myostatin under these conditions of relatively low-dose myostatin in serum-containing media.

As muscle ages, satellite cells not only decrease in number but are also less easily activated to proliferate. The rate of activation of quiescent satellite cells can be assessed in culture by evaluating the incorporation of BrdUrd at 40 h after plating as cells move from G0 into the G1, and S phases of the cell cycle (21). In activation assays with satellite cells from 18-month-old animals, the percentage of BrdUrd-positive satellite cells was slightly higher from mstn−/− muscle than from WT muscle (31.4 ± 7.7% versus 27.5 ± 7.6% BrdUrd-positive cells, P = 0.054). Although the differences in satellite cell activation between mstn−/− and WT were not significant, these results indicate that satellite cells isolated from aged mice lacking myostatin have not reached replicative senescence.

We have shown (9) that in the setting of chronic injury, such as the repeated rounds of degeneration and regeneration found in the mdx model of muscular dystrophy, mice lacking myostatin were stronger and more muscular than their mdx counterparts with less fibrosis and fatty infiltration. The amelioration of some of the features of the mdx dystrophic phenotype in the absence of myostatin does not appear to be due to an effect on degeneration. mdx mice undergo their first and major round of fiber degeneration at 3 weeks of age. The number of degenerating fibers was quantified by EBD uptake, EBD being a diazo dye that is impermeable to normal sarcolemma. There was large animal-to-animal variability, and no significant difference in EBD fibers between muscle from 3-week-old mdx (mean, 36 EBD-positive fibers per 1,000 fibers; SD, 35 EBD per 1,000 fibers; n = 24) and mstn−/− per mdx animals (mean, 103 EBD-positive fibers per 1,000 fibers; SD, 105 EBD per 1,000 fibers; n = 12). The number of fibers that had degenerated and subsequently regenerated by 6 weeks of age, as determined by the percentage of central nucleated
fibers, was almost identical between mdx (59 ± 7%, n = 16) and mstn−/−/mdx animals (58 ± 7%, n = 17).

To evaluate further the regenerative potential after chronic injury in the prolonged absence of myostatin, aged mstn−/−/mdx mice were analyzed. Muscle weights of 18-month-old mstn−/−/mdx animals remained elevated compared with mdx controls (Fig. 7). Functional strength, as measured by forearm grip strength, was significantly (P = 1.5 × 10−5) greater in 18-month-old mstn−/−/mdx (mean, 1.51 ± 0.13 N; n = 8) compared with mdx controls (mean, 1.02 ± 0.18 N; n = 9). Diaphragm is the most severely affected muscle in the mdx model and by 18 months frequently consists of only a thin strip of fibrotic tissue (12, 25). In the prolonged absence of myostatin, diaphragm muscle demonstrated some fibrosis and fatty infiltration but consistently contained abundant myofibers (Fig. 8A). Regenerative capacity in the prolonged absence of myostatin was further challenged by acutely injuring aged mdx muscle. Tibialis muscle from 18-month-old mstn−/−/mdx animals injured with cardiotoxin injection was able to regenerate with pathology indistinguishable from regenerated mdx muscle (Fig. 8 B and C).

Discussion

Blockade or loss of myostatin causes increased muscle growth by disinhibition of muscle progenitor cells (reviewed in ref. 26). The effects of long-term myostatin loss are unknown and have been cause for concern, particularly with the current introduction of myostatin inhibitors as potential human therapies. The results presented in this article suggest that there are not long-term deleterious effects to mouse muscle in the prolonged absence of myostatin. Senescent myostatin null mice maintain increased muscle mass and strength relative to controls. No myopathic features or increased susceptibility to contraction-induced injury were observed. Aged mdx mice lacking myostatin have undergone rounds of degeneration and regeneration also continue to maintain increased muscle mass and strength relative to mdx controls.

These results suggested that the regenerative capacity of mouse muscle was not exhausted in the prolonged absence of myostatin. The ability of muscle to regenerate in the prolonged absence of myostatin was challenged by cardiotoxin injury from which senescent myostatin null mice readily regenerated. Also, aged mdx mice lacking myostatin also readily regenerated from cardiotoxin injury.

Contrary to an exhaustion of regenerative capacity, several lines of evidence suggest that in the absence of myostatin, muscle regenerates more robustly. First, as has been shown (9), mdx mice lacking myostatin have decreased fibrosis and fatty infiltration in diaphragm muscles. This was evident, as shown in this article, even in aged 18-month-old mdx animals that have more myofibers and less fibrosis in diaphragm muscle than mdx controls. Second, after injury, muscle lacking myostatin regenerate large diameter myofibers earlier than controls and express myoD and myf4 at least 24 h earlier than controls. Third, in vitro experiments reported in this study are consistent with the enhanced regenerative capacity of myostatin null muscle in demonstrating greater proliferation and earlier differentiation of mstn−/−/satellite cells when compared with WT cells.

Although it has been postulated that lack of myostatin decreases myofiber degeneration in mdx mice, our studies do not support an effect on degeneration (10, 27). Previously, we had not observed a significant difference in creatine kinase, a serum marker of muscle breakdown, in the mdx versus mstn−/−/mdx populations (9). In this study, there was no significant difference between large numbers of mdx and mstn−/−/mdx animals in EBD-positive fibers during the major period of muscle degeneration at 3 weeks of age, and there was a nearly identical number of regenerated fibers at 6 weeks of age. Therefore, a plausible interpretation of studies (9, 10) showing amelioration of the mdx phenotype with blockade or loss of myostatin is that muscle continues to degenerate because of the loss of dystrophin, but that regeneration is enhanced without the endogenous inhibitor myostatin.

The results in this article suggest that mice are not at risk of depleting the pool of muscle progenitor cells and exhausting their regenerative reserve in the prolonged absence of myostatin. This observation can be understood if myostatin normally inhibits satellite cell proliferation as well as differentiation, as shown here and in refs. 8, 23, and 24. In the absence of myostatin, satellite cells are disinhibited to proliferate, replenishing the stem cell pool and subsequently differentiate into new myofibers or fuse with preexisting fibers. In the prolonged absence of myostatin, satellite cells from 18-month-old myostatin null mice are activated to divide at least as well as those from control animals.

These results are encouraging for the human application of myostatin inhibitors, suggesting that myostatin inhibition may provide a sustained benefit of improved muscle regeneration. However, most cells do not replicate indefinitely, potentially because of the loss of telomeric DNA via the end-replication problem. There are substantial differences in the telomerase activity in humans and rodents, and shortening of telomere length has been found in some (28) but not all (29) studies of human dystrophic muscle. Similar experiments of regeneration in the prolonged absence of myostatin need to be conducted in larger mammals or telomerase-deficient mouse models that more closely resemble the replicative aging seen in humans.

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