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HINDLIMB SKELETAL MUSCLE FUNCTION IN MYOSTATIN DEFICIENT MICE

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Abstract

Introduction—Absence of functional myostatin (MSTN) during fetal development results in adult skeletal muscle hypertrophy and hyperplasia.

Methods—To more fully characterize MSTN loss in hindlimb muscles, the morphology and contractile function of the soleus, plantaris, gastrocnemius, tibialis anterior and quadriceps muscles in male and female null (*Mstn* $^{-/-}$), heterozygous (*Mstn* $^{+/-}$) and wildtype (*Mstn* $^{+/+}$) mice were investigated.

Results—Muscle weights of *Mstn* $^{-/-}$ mice were greater than *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$. Fiber cross-sectional area (CSA) was increased in female *Mstn* $^{-/-}$ soleus and gastrocnemius muscles and in the quadriceps of male *Mstn* $^{-/-}$ mice; peak tetanic force in *Mstn* $^{-/-}$ mice did not parallel the increased muscle weight or CSA. Male *Mstn* $^{-/-}$ muscle exhibited moderate degeneration.

Discussion—Visible pathology in male mice and decreased contractile strength relative to increased muscle weight suggest MSTN loss results in muscle impairment which is dose, sex and muscle dependent.

Keywords

muscle weight; myostatin deficiency; peak tetanic force; muscle weakness; sex differences

INTRODUCTION

Myostatin (MSTN) is a highly conserved negative regulator of skeletal muscle mass and a member of the transforming growth factor - ß (TGF-ß) family [1]. The *Mstn* gene is expressed in adult skeletal muscle, heart and adipose tissue [2]. When functional MSTN is absent during fetal development, the resultant adult muscle mass is significantly increased due to both hypertrophy and hyperplasia of skeletal muscle fibers[3,4]. Though there is extensive evidence of muscle hypertrophy in response to MSTN deficiency, the impact on muscle function[4,5]

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needs further clarification. The reported inconsistencies in skeletal muscle strength and pathology[4,5] may reflect the impact of parental MSTN status on offspring skeletal muscle phenotype[6], sex differences, and variation in mouse genetic background.

The homozygous null myostatin (*Mstn* $^{-/-}$) murine model, first generated and described in 1997 by McPherron et al., was achieved by disrupting the GDF-8 gene by gene targeting [4]. *Mstn* $^{-/-}$ animals are approximately 30% larger than their wildtype (*Mstn* $^{+/+}$) littermates [4]. Heterozygous (*Mstn* $^{+/-}$) animals have been shown to have an intermediate phenotype between control and *Mstn* $^{-/-}$ animals, suggesting a dose-dependent effect of MSTN protein levels on skeletal muscle mass[3,7]. Alterations in muscle mass, fiber contractile properties and fiber type have been reported in the *Mstn* $^{-/-}$ model [5,8,9]. Several studies have been performed on mice with the *Mstn* mutation on different background strains [1,5,10,11], using mice of different ages and sexes [1,4-6,9-11], different breeding strategies and variable amounts of backcrossing [1,5,6,9]. Contractile function has been studied in only a few muscles [5,9,10]. Thus, there is a clear need for a comprehensive evaluation of *in situ* skeletal muscle function in age-matched male and female *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$ mice on a congenic background generated by a consistent breeding strategy.

The following study was performed to provide a comprehensive analysis of several hindlimb skeletal muscles with different fiber type compositions, functions and architectures including the soleus (S), plantaris (P), gastrocnemius (G), tibialis anterior (TA) and quadriceps (Q) muscles. We examined 4 month old male and female *Mstn* $^{+/+}$, *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$ mice, taking into account the documented maternal affect of MSTN[6] while controlling carefully for genetic background. Our studies indicated that male and female *Mstn* $^{-/-}$ skeletal muscles had increased muscle wet weight and reductions in the strength of selected muscles. *Mstn* $^{+/-}$ mice had a similar but milder skeletal muscle phenotype. Frequently, male *Mstn* $^{-/-}$ mice had evidence of moderate muscle pathology, while male *Mstn* $^{+/-}$ mice had evidence of mild pathology and females appeared unaffected.

MATERIALS AND METHODS

Experimental Model

Mature male and female wildtype (*Mstn* $^{+/+}$ m=11, f=11), heterozygous (Mstn $^{+/-}$ m=10, f=15) and homozygous (*Mstn* $^{-/-}$ m=12, f=11) mice maintained on a C57BL/6J (B6) background [12] were evaluated. The B6 background was verified to be > 99% congenic after 11 generations by microsatellite analysis (RADIL, Columbia, MO). B6 *Mstn* $^{+/-}$ animals were then bred to produce the congenic B6 *Mstn* $^{+/+}$, *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$animals[12]. All mice used in the study were the result of *Mstn* $^{+/-}$ x *Mstn* $^{+/-}$ crosses so that maternal MSTN status remained constant[6]. Mice were 4 months of age at time of study to avoid characterizing animals in the rapid growth phase of development. The protocols used for this study comply with the guidelines of the American Physiological Society. All experimental manipulations were performed under an approved University of Missouri Animal Care and Use Protocol.

Contractile properties

The soleus (S), plantaris (P), gastrocnemius (G), tibialis anterior (TA), and quadriceps (Q) muscles were chosen based on their differing fiber type compositions, architecture and contribution to movement. Multiple muscles were studied, as there is evidence that selected muscles may respond differently to MSTN inhibition[10]. The S, P, G, TA and Q muscles are uni- or multi-pennate; span one or more joints and function as anti-gravity, postural or locomotor muscles. The S, P and G muscles serve as plantar flexors, the TA is a dorsal flexor in mice[13] and the Q extends the knee and flexes the hip [14].

To determine *in situ* contractile properties of the S, P, G, and TA muscles in 4 month old *Mstn* $^{+/+}$, *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$ mice, animals were anesthetized with pentobarbital sodium (0.15ml pentobarbital with 0.85ml saline) with 0.15ml as first injection, and anesthesia was maintained with 0.05ml injection given as needed. Each mouse was placed laterally on a water-jacketed heating pad maintained at 37°C. The left S, P, G, and TA muscles were surgically exposed at their distal insertions. The distal tendon of each muscle was attached in turn to a Grass force transducer with 4.0 silk. The sciatic nerve was isolated and placed on a bipolar stimulating electrode. The exposed tendon of each muscle was bathed in saline solution, and the nerve was bathed in 37°C mineral oil.

For contractile testing the left hind-limb and mouse torso were firmly immobilized, and muscles were attached in the order of S→P→G→TA to a force transducer by the distal tendon and adjusted in length so that passive tension was zero grams[15]. A twitch was obtained at that position with parameters of 0.5ms, 0.3Hz, at 6V, and then the micromanipulator was used to progressively lengthen each muscle to the point where peak twitch was attained (L_o). At optimal length, a peak tetanic contraction (P_o) was elicited by pulses delivered at 150Hz, 300ms duration, and an intensity of 6V for each type muscle. Preliminary studies revealed 6V to be supramaximal; the 300ms duration was greater than what was required to achieve P_o. Force curves generated at 15, 50, 75, 100 and 125Hz revealed that all muscles were maximally recruited by the time 100Hz was reached. All data were collected using Power Lab® (ADInstruments). Contractile function testing lasted approximately 15 minutes.

In pilot studies, random testing of muscles was done as well as testing in the order TA→G→P→S, and no differences in tension were observed, regardless of stimulation order. Repeat testing of S and P during preliminary studies indicated that the protocol did not result in decreased force generation.

Tissue Harvest

After contractile properties were obtained, S, P, G, TA, and Q muscles were removed, cleaned, blotted, and weighed. Muscles were placed in 4% paraformaldehyde solution for 24 hours and then transferred to 70% ethanol for future staining with hematoxylin and eosin (H&E) for morphologic evaluation. Right sided muscles, those that were not electrically stimulated, were placed at their *in situ* length, embedded in OCT tissue-freezing medium, frozen slowly in chilled 2-methylbuterol, and then placed in liquid nitrogen and stored at −80°C until analysis for myofibrillar ATPase activity.

Histochemistry and cross-sectional myofiber measurements

Paraformaldehyde and ethanol prepared muscles from *Mstn* $^{+/+}$, *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$ mice were transversely sectioned at the middle of the muscle belly and then sectioned at 5μm and stained with H&E. Digital images were taken from the cross-section at 10x magnification in order to evaluate muscle fiber morphology and to determine fiber cross-sectional area (CSA) measures. Myofiber CSAs were used to measure evidence of atrophy or hypertrophy of discrete muscle fibers, as well as in the calculation of specific P_o [peak tetanic force (P_o) / CSA (μm^2)] for each muscle. Six sections of 50 contiguous myofibers were circled for each muscle to obtain an average of 300 fibers for fiber cross-sectional area measures. Area determinations were done using a calibrated pen by circling each fiber. Image J software (NIH) was used to obtain area data which were subsequently transferred into an Excel spreadsheet (Microsoft Office, 2003).

Myofibrillar ATPase activity

Right-sided S, P, G, TA and Q muscles, which were harvested immediately after contractile studies and stored at −80°C, were thawed to −20°C in a microtome, oriented vertically, and sectioned at 10μm. Sections were stained using traditional acid-stable myofibrillar ATPase stain (4.2 pH acid preincubation) to reveal muscle fiber type[16].

Statistical analysis

Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC). Data from the three groups were analyzed as a 3×2 factorial (3 genotypes, 2 sexes). Mean differences were determined using Fisher's Least Significant Difference (LSD). When heterogeneous variations made it necessary, a log transformation was used to stabilize heterogeneous variation. If this log transformation failed to stabilize the variation, a non-parametric ranked analysis was performed as outlined by Conover et al.[17]. Results are presented as mean ± standard error (SE). Differences were considered to be statistically significant at a p value ≤ 0.05 unless stated otherwise.

RESULTS

Muscle weight

Both male and female whole muscle weights of S, P, G, TA and Q muscles in *Mstn* $^{-/-}$ mice were greater ($p \leq 0.05$) than their *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$ counterparts (Table 1 A,B). Male and female *Mstn* $^{-/-}$ whole muscle weights were 29-47% and 34-50% greater, respectively than their same sex *Mstn* $^{+/+}$ counterparts, respectively. Male *Mstn* $^{+/-}$ P and G muscles were 13% and 17% larger, respectively than male *Mstn* $^{+/+}$ P and G muscles. Female *Mstn* $^{+/-}$ P, G and TA muscles were 11%, 10%, and 14% larger, respectively than female *Mstn* $^{+/+}$ P, G and TA muscles. Relative weights (muscle weight / body weight) of male and female *Mstn* $^{-/-}$ muscles were 17-39% and 22-40% greater ($p < 0.05$), respectively than male and female *Mstn* $^{+/+}$ muscles. Relative weights of male and female S, P, G, TA and Q *Mstn* $^{-/-}$ muscles were also larger ($p < 0.05$) than *Mstn* $^{+/-}$ muscles. In male *Mstn* $^{+/-}$ mice, relative weights of P and G muscles were 13% and 17% greater ($p < 0.05$), respectively than male *Mstn* $^{+/+}$ mice. Female *Mstn* $^{+/-}$ mice had 8% and 7%, respectively greater relative P and G weights ($p = 0.0573$ for P) compared with relative P and G weights of *Mstn* $^{+/+}$ female mice (Table 1 A,B). These findings suggest that MSTN deficiency and absence results in muscle hypertrophy rather than general body growth.

Histomorphometry

Evaluation of histology cross-sections of male and female *Mstn* $^{+/+}$, *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$ S, P, G, TA and Q muscles stained with hematoxylin and eosin (H&E) was performed. Histology cross-sections of male *Mstn* $^{+/+}$ (Fig. 1A), *Mstn* $^{+/-}$ (Fig. 1B) and *Mstn* $^{-/-}$ (Fig. 1C) Q muscle are shown. *Mstn* $^{-/-}$ Q muscles showed varying degrees of segmental degeneration and regeneration (intracytoplasmic vacuoles, enlarged fibers and / or fibers with loss of eosin staining, and centralized nuclei) (Fig. 1C). Male *Mstn* $^{-/-}$ muscles were the most severely affected, particularly the Q muscle. The only lesions noted in male *Mstn* $^{+/-}$ mice were rare intracytoplasmic vacuoles, primarily in the P muscle (not shown). No lesions were detected in female *Mstn* $^{-/-}$ or *Mstn* $^{+/-}$ mice.

Myofiber Cross-sectional Area

In male mice, the S CSA was 20% larger ($p < 0.05$) in *Mstn* $^{-/-}$ compared to *Mstn* $^{+/+}$ mice (Fig. 2A). In male *Mstn* $^{-/-}$ mice, Q CSA was 25% greater than the Q CSA in *Mstn* $^{+/-}$ mice. Q CSA in female mice was not significantly different between genotypes, though female *Mstn* $^{-/-}$ S, P and G had larger ($p < 0.05$) CSAs compared to *Mstn* $^{+/+}$ (31%, 20%,

and 30% larger, respectively) and *Mstn* $^{+/-}$ (21%, 28% and 38% larger, respectively) mice (Fig. 2B). In contrast, CSAs of TA muscles in male and female mice were not different between the genotypes (Fig. 2A,B).

Contractile Force Generating Capacity

Absolute whole muscle contractile generating capacity [peak tetanic force (P_o)] was not significantly different in the S, G or TA between genotypes in either sex (Fig. 3 A,B and 4 A,B). However, the relative muscle contractile generating capacity [peak tetanic force [(P_o) / muscle weight in milligrams (mg)] of S, P, G and TA in *Mstn* $^{-/-}$ male mice was reduced relative to sex-matched *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$ mice ($p < 0.05$, except $p = 0.0579$ for P) (Fig. 3 C,D). G P_o / mg was decreased in both male and female *Mstn* $^{-/-}$ by 39% and 30% compared to male and female *Mstn* $^{+/+}$, respectively, and approximately 26% compared to sex-matched *Mstn* $^{+/-}$ mice (Fig. 3D and 4D). Specific P_o [peak tetanic force [(P_o) / CSA (μm^2)] was impaired only in the S muscle of *Mstn* $^{-/-}$ males, where S P_o / CSA was decreased by 28% compared to the S P_o / CSA in *Mstn* $^{+/+}$ mice (Fig. 3E).

In contrast to male P P_o, female P P_o was 44% greater in *Mstn* $^{-/-}$ mice compared to *Mstn* $^{+/+}$ and 35% greater compared to *Mstn* $^{+/-}$ mice ($p < 0.05$) (Fig. 4A). In female *Mstn* $^{-/-}$ mice, S P_o / mg was 27% less than *Mstn* $^{+/-}$ S P_o / mg ($p = 0.057$). P P_o / mg was 16% less in female *Mstn* $^{-/-}$ compared to female *Mstn* $^{+/-}$ mice (Fig. 4C). Female *Mstn* $^{-/-}$ mice had 49% and 33% impairment of TA P_o / mg compared to *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$ mice, respectively (Fig. 4D).

Myofibrillar ATPase

Fiber-type analysis of the S muscle of male *Mstn* $^{+/+}$, *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$ mice by histochemical staining for ATPase activity (acid preincubation) revealed significant ($p < 0.05$) differences in the absolute number of slow type I fibers in the S muscle in *Mstn* $^{-/-}$ mice as compared to *Mstn* $^{+/+}$ S (Fig. 5 A,B). The P, G, TA and Q muscles were composed almost exclusively of type II fibers, and no differences were observed between the fiber type composition of *Mstn* $^{+/+}$, *Mstn* $^{+/-}$ and *Mstn* $^{-/-}$ muscles (results not shown).

Discussion

Male and female mice appear to be affected differently by MSTN deficiency. Absence of MSTN increases the CSA of the S and Q muscles in male mice but increases S, P and G CSA in female mice. In females, P_o was greater in *Mstn* $^{-/-}$ P compared to female *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$, while there were no significant differences between genotypes in males. Interestingly, there was no difference in amount of P_o / CSA that could be generated in any of the muscles between female genotypes, but in males, *Mstn* $^{-/-}$ S P_o / CSA was less than *Mstn* $^{+/+}$ S P_o / CSA. Perhaps the most striking difference between male and female *Mstn* $^{-/-}$ mice was the presence of moderate segmental degeneration in male *Mstn* $^{-/-}$ muscle which was non-existent in female *Mstn* $^{-/-}$ muscle.

Our data suggests that some of the muscles studied have fiber hypertrophy rather than hyperplasia. These findings are consistent with McPherron et al. who also found that the CSA of the G muscle in MSTN deficient animals was 49% larger compared to *Mstn* $^{+/+}$[4]. Contrary to our findings, McPherron et al. found that the CSA of the tibialis cranialis in *Mstn* $^{-/-}$ mice was 14% larger than in *Mstn* $^{+/+}$ mice.

We anticipated that increased CSA in *Mstn* $^{-/-}$ animals would be accompanied by a commensurate increase in force[9], but this was not true for any of the hindlimb muscles in our study. Unlike exercise training, which stimulates an increase in both myofiber CSA and force generating capacity[18], increases oxidative metabolism[14], and reduces the percentage of

type IIb fibers in favor of type IIa fibers[19-23], MSTN deficiency causes increased myofiber CSA without parallel increases in muscle force. In addition, there are decreases in mitochondrial number and oxidative capacity[5], and there is a reduction in type IIa fibers with a concomitant increase in type IIb fibers[5]. Amthor et al. looked at specific force generation by the EDL muscle and found that normalizing EDL P_o to CSA showed a significant reduction in male *Mstn* $^{-/-}$ specific force generation compared to wildtype mice[5]. In further support, Byron et al. also found that, though MSTN deficiency increased muscle mass and bite force (when measured at 20Hz) of the temporalis muscle, when bite force was normalized to muscle CSA, there were no significant differences between *Mstn* $^{+/+}$ and *Mstn* $^{-/-}$ mice[24]. In contrast to our study, Mendias et al. found that the S P_o / CSA was not different in *Mstn* $^{-/-}$ compared to *Mstn* $^{+/-}$ and *Mstn* $^{+/+}$ mice[9]. Differences between our study and the Mendias study may be due to the method for evaluating contractile force generation (*in situ* versus *in vitro*[9]) and the age of the animals used; as the mice in our study were 4 months old and those in the Mendias et al. study were 10-12 months of age. Alterations in fiber type, number and size in *Mstn* $^{-/-}$ mice as a result of the aging process[25] could explain the subtle differences between contractile function in ours versus the Mendias et al. study.

In contrast to the report by McPherron et al. that *Mstn* $^{-/-}$ skeletal muscle appeared grossly normal without obvious signs of degeneration[4], Amthor et al. observed cytoplasmic inclusions (determined to be tubular aggregates) in the superficial regions of male and female G, TA and EDL muscles in *Mstn* $^{-/-}$ male mice at 7 months of age and in females at 10 months of age. Inclusions were not noted in the S muscle, or in animals at 2 months of age[5]. In our study degenerative changes in muscles are visible at 4 months of age, and the greatest severity and number of lesions was observed in the Q muscle of male *Mstn* $^{-/-}$ mice. Discrepancies between the findings of McPherron et al. and our findings may be due to differences in background strain of mice, as F2 mice used in the McPherron study were generated on a mixed 129 SV/J, C57BL/6 background[4]. Amthor et al. noted that centralized nuclei were rarely reported in *Mstn* $^{-/-}$ mice[5]. We found evidence of many centralized nuclei, especially in the S and Q muscles. These were interpreted as evidence of myofiber regeneration. We saw no lesions in the skeletal muscle of female *Mstn* $^{+/-}$ or Mstn $^{-/-}$ mice. Amthor et al. found that females had far fewer lesions than males, though similar declines in the *in situ* forces were noted. Our results are consistent with Amthor et al., though we found the relative P_o for all muscles examined and specific P_o of the S were slightly more affected in male *Mstn* $^{-/-}$ than female *Mstn* $^{-/-}$ mice. Unfortunately, we were unable to record the contractile force-generating ability of the Q muscle, which had the most severe lesions in male *Mstn* $^{-/-}$ mice. The reported declines in contractile forces of *Mstn* $^{-/-}$ muscle in the Amthor et al. study did not correlate with the tubular aggregates and were attributed to mitochondrial depletion, loss of oxidative characteristics of the muscle, reduced capillary density, and problems in calcium handling[5].

Rehfeldt et al.[26] introduced the murine myostatin mutation Mstn (Cmpt-dl1Abc) (Compact; C) into an inbred mouse line with extreme growth (DUHi) by marker-assisted introgression in order to study the allelic effects on muscle fiber hyperplasia and hypertrophy, capillary density, and muscle fiber metabolism in wildtype, heterozygous (C/+), and homozygous (C/C) mice. They found that in the *Mstn* Cmpt-dl1Abc mouse the average number of capillaries per muscle fiber was 35% lower in homozygous C/C compared to wildtype animals[26]. Since capillaries serve as the means for gas, substrate and metabolite exchange[27], the increased muscle bulk in *Mstn* $^{-/-}$ mice without a concomitant increase in capillary density could lead to a decrease in oxygen and nutrient exchange, thus making *Mstn* $^{-/-}$ animals (males in particular) susceptible to injury.

Another possible factor to consider is the role of muscle fiber type switching. MSTN deficient muscles demonstrate a switch toward type IIb myofibers[5], which have been found to be damaged selectively after lengthening contractions[28]. Type IIb fibers are also preferentially affected in Duchene Muscular Dystrophy (DMD)[29], where the absence of dystrophin leaves muscles extremely susceptible to contraction-induced injury[30]. When the muscle fiber is injured or torn, there is a subsequent influx of Ca^{+2}, and degenerative pathways are activated in the dystrophic muscle[31-33]. In MSTN deficient muscle, though there is no loss of dystrophin, there may be selective contraction-induced injury in type IIb fibers, thus allowing influx of Ca^{+2} and subsequent activation of degenerative pathways. Degenerative lesions are most prominent in muscles that contain high percentages of type IIb fibers and would appear visually as segmental degeneration similar to what is seen in the Q muscle of male *Mstn* $^{-/-}$ mice. The S muscle, which has a higher proportion of type I fibers may have more of a regenerative response. Whereas in dystrophic muscles the degenerative pathways ultimately lead to muscle atrophy with infiltration of fibrous and fatty tissue, this does not appear to be the case in MSTN deficient muscle. Females may also be somewhat protected from this muscle damage and membrane instability by estrogen[34,35], which has been shown to play a significant role in stimulating muscle repair and regenerative processes[34].

By carefully controlling for the maternal influence and genetic background using a consistent breeding strategy and evaluating both male and female adult mice, we found that though *Mstn* $^{-/-}$ skeletal muscles had increased weights and reductions in muscle strength of specific muscles in comparison with *Mstn* $^{+/-}$ and *Mstn* $^{+/+}$ mice. Our study further affirms muscle-specific responses to MSTN inhibition[5,9-11,25], and it demonstrates sex-specific differences in fiber CSA and contractile force generation of MSTN deficient muscle.

Though complete inhibition of MSTN may not be warranted as a therapeutic intervention due to introduction of muscle degeneration, partial inhibition of MSTN may be considered, since there were increases in muscle weight with only mild lesions and no decreases in P_o / CSA in male *Mstn* $^{+/-}$ mice. In support of this, Mosher et al. studied the effects of a 2-bp deletion in the third exon of the MSTN gene in whippet dogs and its affects on racing performance[36]. They found that whippets which were heterozygous for the mutation (*mh*/+) were among the fastest (grade A) racers, demonstrating that heterozygotes carried a performance-enhancing mutation[36]. It would be of interest to explore why *mh*/*mh* whippets do not dominate the racing scene if bigger muscles are supposedly better[37], or if complete loss of MSTN would result in reduced performance[37]. Therefore, there may be a balance between muscle mass and muscular performance as well as therapeutic effectiveness which is optimal in heterozygous but not homozygous null animals[3].

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ABBREVIATIONS

ATP	adenosine triphospahte
C/+	heterozygous for the *Mstn*$^{\text{Cmpt-dl1Abc}}$ allele
C/C	homozygous for the *Mstn*$^{\text{Cmpt-dl1Abc}}$ allele
CSA	cross-sectional area
DUHi	Dummerstorf high-inbred mouse line
EDL	extensor digitorum longus
G	gastrocnemius
GDF-8	growth differentiation factor 8
H&E	hematoxylin & eosin
LSD	least significant difference
MSTN	myostatin
***Mstn*$^{+/+}$**	myostatin wildtype
***Mstn*$^{+/-}$**	myostatin heterozygous
***Mstn*$^{-/-}$**	myostatin homozygous null
Mstn (Cmpt-dl1Abc)	compact murine myostatin allele
P	plantaris
P_o	absolute whole muscle peak tetanic force
P_o/mg	relative muscle tetanic force
P_o/CSA	specific muscle tetanic force
RADIL	research animal diagnostic laboratory
S	soleus
SE	standard error
TA	tibialis anterior
Q	quadriceps
wt	wildtype

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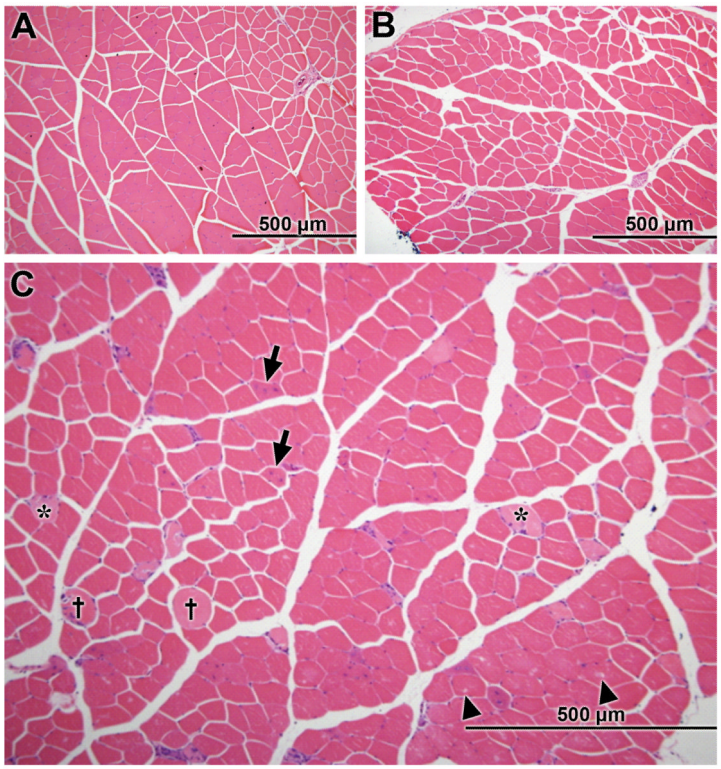

Figure 1.

Hematoxylin and eosin (H&E) stained cross-sections of the quadriceps (Q) muscle from 4 month old male (**A**) wildtype (*Mstn* $^{+/+}$), (**B**) heterozygous (*Mstn* $^{+/-}$) and (**C**) null (*Mstn* $^{-/-}$) mice. *Mstn* $^{-/-}$ Q muscle showed evidence of regeneration, segmental degeneration [swollen fibers (crosses), multiple centralized nuclei (arrows), intracytoplasmic vacuoles (arrow heads), and loss of eosin staining (asterisks)], 10X magnification.

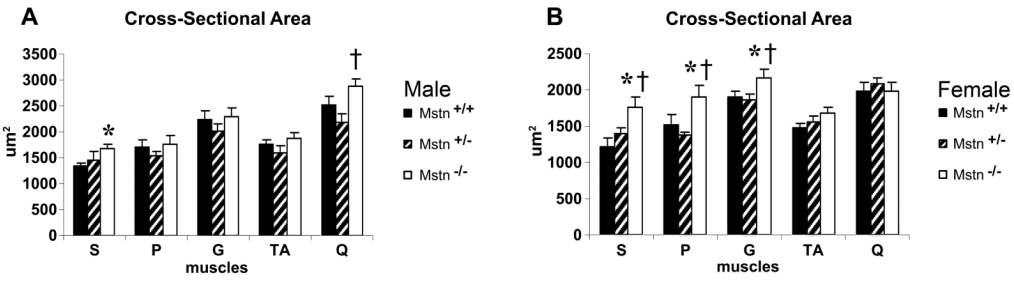

Figure 2.

(**A**) Cross-sectional areas [CSAs (μm^2)] of soleus (S), plantaris (P), gastrocnemius (G), tibialis anterior (TA) and quadriceps (Q) muscles of male *Mstn* $^{+/+}$ (n=9-12; *black solid*), *Mstn* $^{+/-}$ (n=6-8; *diagonal*), *Mstn* $^{-/-}$ (n=7; open) mice (mean ± SE). S CSA was larger in *Mstn* $^{-/-}$ compared to *Mstn* $^{+/+}$ ($p < 0.05$). Male *Mstn* $^{-/-}$ Q CSA was larger than *Mstn* $^{+/-}$ Q CSA ($p < 0.05$). (**B**) CSA of S, P, G, TA and Q muscles of female *Mstn* $^{+/+}$ (n=8; *black solid*), *Mstn* $^{+/-}$ (n=10-14; *diagonal*), and *Mstn* $^{-/-}$ (n=7-11; *open*) mice (mean ± SE). In female *Mstn* $^{-/-}$ mice S, P and G CSAs were larger than *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$ S, P and G CSAs. * $p < 0.05$ compared to same sex *Mstn* $^{+/+}$, † $p < 0.05$ compared to same sex *Mstn* $^{+/-}$.

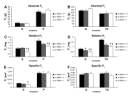

Figure 3.

(**A,B**) In 4 month old male mice, there were no significant differences in the amount of absolute whole muscle contractile generating capacity (P_o) generated by the soleus (S), plantaris (P), gastrocnemius (G), tibialis anterior (TA) muscles of *Mstn* $^{+/+}$ (n=10; *black solid*), *Mstn* $^{+/-}$ (n=9; *diagonal*) and *Mstn* $^{-/-}$ (n=7; *open*) mice (mean ± SE). (**C,D**) Relative muscle contractile generating capacity (P_o/mg) of the S, P, G and TA was impaired in male *Mstn* $^{-/-}$ compared to male *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$ mice ($p = 0.0579$ for P P_o/mg). Male *Mstn* $^{+/-}$ G P_o/mg was significantly reduced compared to *Mstn* $^{+/+}$ mice (mean ± SE). (**E**) Specific tetanic force (P_o/CSA) was significantly less in the S of male *Mstn* $^{-/-}$ mice compared to *Mstn* $^{+/+}$ (mean ± SE). (**F**) There were no specific differences in G P_o/CSA or TA P_o/CSA between genotypes (mean ± SE). * $p < 0.05$ compared to *Mstn* $^{+/+}$ mice, † $p < 0.05$ compared to *Mstn* $^{+/-}$ mice.

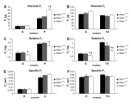

Figure 4.

(**A,B**) In 4 month old female mice, absolute tetanic force (P_o) for the plantaris (P) muscle was increased in *Mstn* $^{-/-}$ (n=11; *open*) compared to *Mstn* $^{+/+}$ (n=10; *black solid*) and *Mstn* $^{+/-}$ (n=13; *diagonal*) (mean ± SE). (**C**) Relative tetanic force (P_o / mg) for the soleus (S) ($p = 0.0577$) and plantaris (P) muscles was impaired in female *Mstn* $^{-/-}$ compared to female *Mstn* $^{+/-}$ mice. (**D**) Female gastrocnemius (G) and tibialis anterior (TA) P_o / mg was significantly reduced in *Mstn* $^{-/-}$ compared to female *Mstn* $^{+/+}$ and *Mstn* $^{+/-}$ G and TA P_o / mg. *Mstn* $^{+/-}$ TA P_o / mg was impaired compared to *Mstn* $^{+/+}$ TA P_o / mg. (**E,F**) There were no significant differences in specific tetanic force (P_o / CSA) generated by any of the female mouse muscles evaluated between genotypes. * $p < 0.05$ compared to *Mstn* $^{+/+}$ mice, † $p < 0.05$ compared to *Mstn* $^{+/-}$ mice.

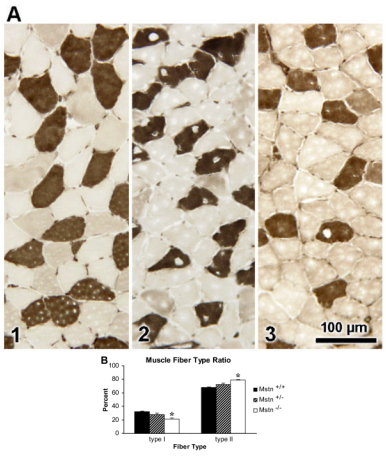

Figure 5.

(A) Myofibrillar ATPase staining (acid preincubation) of the soleus (S) muscle in male *Mstn* $^{+/+}$ (n=2; (**1**) *left*), *Mstn* $^{+/-}$ (n=2; (**2**) middle) and *Mstn* $^{-/-}$ (n=3; (**3**), *right*) revealed that *Mstn* $^{-/-}$ S had significantly ($p < 0.05$) fewer type I fibers and significantly more type II fibers compared to *Mstn* $^{+/+}$ S muscle, 2.5X magnification. **(B)** Distribution of type I and type II fibers of the S muscle expressed as a percent of total muscle fiber number (mean ± SE).

Table 1A

Body and muscle weight of male 4 month old wildtype (*Mstn* $^{+/+}$), heterozygous (*Mstn* $^{+/-}$) and homozygous (*Mstn* $^{-/-}$) mice

PARAMETERS	MOUSE GENOTYPE				
	***Mstn*$^{+/+}$ (n=12)**	***Mstn*$^{+/-}$ (n=10)**	**% change from *Mstn*$^{+/+}$**	***Mstn*$^{-/-}$ (n=11)**	**% change from *Mstn*$^{+/+}$**
Body weight (g)	33.67 ± 0.58	33.75 ± 1.00	+0.2%	38.80 ± 0.73*†	+13%
Muscle wet weight (mg)					
S	11.31 ± 0.7	11.10 ± 0.60	−2%	15.94 ± 1.10*†	+29%
P	21.02 ± 0.40	24.08 ± 0.66*	+13%	39.46 ± 2.70*†	+47%
G	142.79 ± 3.10	171.84 ± 5.60*	+17%	268.30 ± 17.00*†	+47%
TA	57.39 ± 2.7	61.46 ± 3.80	+7%	95.31 ± 5.50*†	+40%
Q	229.00 ± 7.0	255.12 ± 13.2	+10%	386.73 ± 22.50*†	+41%
Relative Wet Muscle Weight [Wet wt (mg)/ Body wt (g)]					
S	0.034 ± 0.002	0.033 ± 0.002	−3%	0.041 ± 0.002*†	+17%
P	0.062 ± 0.001	0.071 ± 0.003*	+13%	0.101 ± 0.006*†	+39%
G	0.425 ± 0.010	0.513 ± 0.030*	+17%	0.689 ± 0.040*†	+38%
TA	0.170 ± 0.007	0.183 ± 0.010	+7%	0.246 ± 0.020*†	+31%
Q	0.683 ± 0.030	0.761 ± 0.050	+10%	0.995 ± 0.060*†	+31%

Values expressed as mean ± SE. S, soleus; P, plantaris; G, gastrocnemius; TA, tibialis anterior; Q, quadriceps; Mstn, myostatin

* $p < 0.05$ compared to *Mstn* $^{+/+}$

† $p < 0.05$ compared to *Mstn* $^{+/-}$

Table 1B

Body and muscle weight of female 4 month old wildtype (*Mstn* $^{+/+}$), heterozygous (*Mstn* $^{+/-}$) and homozygous (*Mstn* $^{-/-}$) mice

PARAMETERS	MOUSE GENOTYPE				
	***Mstn*$^{+/+}$ (n=11)**	***Mstn*$^{+/-}$ (n=15)**	**% change from *Mstn*$^{+/+}$**	***Mstn*$^{-/-}$ (n=11)**	**% change from *Mstn*$^{+/+}$**
Body weight (g)	24.52 ± 0.46	25.32 ± 0.57	+3%	29.32 ± 0.53‡§	+16%
Muscle wet weight (mg)					
S	8.64 ± 0.30	9.05 ± 0.50	+5%	13.14 ± 0.50‡§	+34%
P	14.55 ± 0.20	16.38 ± 0.50‡	+11%	29.24 ± 1.10‡§	+50%
G	115.44 ± 4.50	128.28 ± 2.90‡	+10%	192.13 ± 6.00‡§	+40%
TA	42.14 ± 1.10	48.77 ± 1.80‡	+14%	77.12 ± 3.50‡§	+45%
Q	172.74 ± 5.10	192.14 ± 5.60	+10%	280.73 ± 16.2‡§	+38%
Relative Wet Muscle Weight [Wet wt (mg)/ Body wt (g)]					
S	0.035 ± 0.001	0.036 ± 0.002	+3%	0.045 ± 0.001‡§	+22%
P	0.060 ± 0.001	0.065 ± 0.002‡	+8%	0.100 ± 0.003‡§	+40%
G	0.470 ± 0.010	0.507 ± 0.006‡	+7%	0.655 ± 0.010‡§	+28%
TA	0.163 ± 0.006	0.193 ± 0.007	+16%	0.264 ± 0.010‡§	+38%
Q	0.705 ± 0.020	0.761 ± 0.020	+7%	0.954 ± 0.040‡§	+26%

Values expressed as mean ± SE. S, soleus; P, plantaris; G, gastrocnemius; TA, tibialis anterior; Q, quadriceps; Mstn, myostatin

‡ $p < 0.05$ compared to *Mstn* $^{+/+}$

§ $p < 0.05$ compared to *Mstn* $^{+/-}$