The aging myostatin null phenotype: reduced adiposity, cardiac hypertrophy, enhanced cardiac stress response, and sexual dimorphism

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Abstract

The natural aging process results in the physiological decline of multiple tissues and organ systems. Changes commonly occur with middle age and include decreased skeletal muscle mass, bone mineral density, cardiac output, and insulin sensitivity, and increased adiposity, all of which can contribute to the onset of sarcopenia, osteoporosis, heart failure, or type 2 diabetes. Recent studies suggest that myostatin may influence many of these systems. We therefore sought to determine whether they are affected by aging, especially in ‘middle-aged’ *Mstn*^{−/−} mice (12–20 months old (m.o.)). Although body weights were similar in wild-type (WT) and *Mstn*^{−/−} mice, lean fat-free mass and skeletal muscles composed of predominantly type I, II, and mixed fibers were significantly heavier in *Mstn*^{−/−} mice. These differences were accompanied by lower total adiposity, especially in female mice, white and brown fat pad weights,

and adipocyte size. Hearts were heavier in *Mstn*^{−/−} mice across a large age range (3–24 m.o.) and exhibited signs of dilated cardiomyopathy at rest, which include lower strain measurements compared with WT myocardium. However, *Mstn*^{−/−} mice responded better to isoproterenol stress tests with greater increases in fractional shortening and ejection fraction—differences that were again more apparent in females and which are consistent with physiological cardiac hypertrophy. Spleens and kidneys were also smaller, although histologically normal, in *Mstn*^{−/−} mice. These data together suggest that attenuating myostatin could potentially prevent or possibly treat pathological conditions that develop with age. Additional studies are nevertheless needed to definitively assess potential risks to cardiac function.

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Introduction

The aging process is the physiological accumulation of changes over time and is unfortunately associated with increased disease susceptibility. Sarcopenia, the age-related and progressive loss of skeletal muscle mass and function, afflicts a large percentage of the elderly (Cruz-Jentoft *et al.* 2010) and can be a significant contributing factor to cardiovascular and bone mineralization diseases (Lang *et al.* 2010). Increasing skeletal muscle mass through exercise can also have beneficial effects on obesity-related disorders, on preventing frailty, and on mitigating cardiovascular disease (Winett *et al.* 2009). Thus, a better understanding of sarcopenia or mechanisms to enhance skeletal muscle mass could help to develop novel treatments for these related disorders.

Potential therapeutics for sarcopenia and other muscle-wasting diseases include those that target myostatin, a myokine best known for negatively regulating skeletal muscle mass and for the extreme musculature generated in myostatin

null animals often referred to as ‘double muscling’ (Rodgers & Garikipati 2008). Several recent studies have also indicated that myostatin is not only expressed in skeletal muscle but also in cardiac muscle (Sharma *et al.* 1999, Shyu *et al.* 2005, Artaza *et al.* 2007, Rodgers *et al.* 2009), where it inhibits several growth processes including basal and insulin-like growth factor (IGF)-stimulated proliferation of cardiomyocytes, protein synthesis, and cellular hypertrophy (Morissette *et al.* 2006, McKoy *et al.* 2007, Rodgers *et al.* 2009, Bish *et al.* 2010). Myostatin also circulates and has recently been suggested to regulate hepatic production of IGF1 and several IGF-binding proteins (Williams *et al.* 2011). These factors similarly regulate skeletal and cardiac muscle growth, and, thus, myostatin’s actions on these tissues are likely mediated locally, via the autocrine production of myostatin, and systemically, which includes the endocrine regulation of IGF axis components.

In both human and animal models, cardiac expression of myostatin protein and/or mRNA is elevated under

different pathophysiological states, including ischemic and nonischemic (dilated cardiomyopathy) heart failure (Sharma *et al.* 1999, Shyu *et al.* 2006, George *et al.* 2010). Conversely, we have recently reported that myostatin negatively regulates physiological cardiac hypertrophy (Rodgers *et al.* 2009, Valdivia 2009) as myostatin null (*Mstn*^{-/-}) mice had larger hearts, due to eccentric hypertrophy, and an enhanced stress response, due in part to enhanced Ca²⁺ handling. Analysis of primary ventricular myocytes indicated that [Ca²⁺]_i transients and total cellular loads were greater in *Mstn*^{-/-} mice and this corresponded to enhanced cellular contractility. Hearts and cells from *Mstn*^{-/-} mice lacked the fetal gene expression profile that occurs with pathological hypertrophy and, from a biophysical perspective, were functionally normal or superior to wild-type (WT) tissues. Thus, myostatin inhibits cardiac muscle excitation–contraction coupling and appears to similarly act as a chalone in both cardiac and skeletal muscles. The myokine has also been linked to other disease states including obesity and type 2 diabetes, as genetic crosses of *Mstn*^{-/-} mice with genetically obese mice produce offspring that are neither obese nor insulin resistant (McPherron & Lee 2002). *Mstn*^{-/-} mice do not become obese when fed a high-fat diet (Wilkes *et al.* 2009) as the increased musculature consumes circulating carbohydrates and prevents the *de novo* synthesis of triglycerides (Guo *et al.* 2009). Preventing the activation of endogenous activin receptors, which also bind myostatin and possibly growth differentiation factor 11 (GDF11), by injecting a soluble form of the extracellular domain, prevents the cancer-induced development of skeletal and cardiac muscle cachexia while simultaneously reducing fat stores (Zhou *et al.* 2010). Other studies suggest that myostatin blockade can enhance skeletal muscle regeneration, exercise performance, and whole-body metabolism in senescent mice (Siriett *et al.* 2007, Yablonka-Reuveni 2007, Lebrasseur *et al.* 2009). Preventing activin/myostatin receptor activation, therefore, has the potential to treat many common pathological states of aging.

Our objective was to determine the long-term effects of myostatin deletion on various organ systems in aging and ‘middle-aged’ mice as many age-related disorders (e.g. sarcopenia, obesity, and some forms of heart failure) develop progressively from middle age through senescence. This is particularly important, as no study to date has evaluated this age group. Our results indicate for the first time that cardiac hypertrophy and enhanced β-adrenergic responsiveness occur in mice of different ages and that brown adipose tissue (BAT) is reduced in the middle-aged and senescent *Mstn*^{-/-} mice. Several sexually dimorphic differences were also noted, for the first time, especially during cardiac stress tests and in adipose stores. Our studies therefore suggest that the age-related decline of different organ systems is less severe in *Mstn*^{-/-} than WT mice. Thus, attenuating the actions of myostatin could potentially ameliorate many pathological conditions that develop with age and may be more effective in women than men.

Materials and Methods

Ethics statement

C57BL/6 WT and myostatin null (*Mstn*^{-/-}) mice were housed and bred in environmentally controlled rooms with 12 h daily light. They were fed *ad libitum* and were used in strict accordance with protocols preapproved by the Institutional Animal Care and Use Committee of Washington State University.

Animals and tissue collection

Aging male and female mice of both genotypes (WT, *Mstn*^{+/+} vs *Mstn*^{-/-}) were used (see figure legends for *n* values) and were born on different days. The average age of mice used to assess differences in everything except heart weight over time was 14 months old (m.o.) for females and 13 m.o. for males. Measurements include bone density and body composition analyses using a dual-energy X-ray absorptiometry (DXA) on mice anesthetized with 2.5% isoflurane in oxygen. Mice were also asphyxiated with CO₂ and skinned before removing and weighing several tissues, some of which were used for histology. This includes hearts, individual skeletal muscles (gastrocnemius, tibias anterior, extensor digitorum longus, and soleus), livers, spleens, and kidneys. Hearts were first cut to drain the blood, trimmed to remove noncardiac tissue, rinsed in PBS, and blotted dry before weighing. The other tissues were similarly processed.

Histology

Three white adipose tissue (WAT) fat pads, subscapular, inguinal, and gonadal, and the intrascapular brown fat (BAT) pad were analyzed. These tissues were fixed in 4% paraformaldehyde (PFA) overnight, embedded in paraffin, sectioned at 8 μm (WAT) or 4 μm (BAT), and stained with hematoxylin and eosin (Thermo Fisher Scientific). For WAT, six nonconsecutive sections were stained for each fat pad per animal and one random 200× image was taken using a Leica DFC295 from each section. For BAT, five nonconsecutive sections were stained per animal and one random 400× image was taken for each section. Average cell size was then measured from each image using Adobe Photoshop CS4. Mitochondria were labeled by placing isolated WAT and BAT in DMEM (Thermo Fisher Scientific) containing MitoTracker Orange (400 nM; Invitrogen) and left to incubate for 30 min. The tissues were washed twice with media and fixed in 4% PFA for 5 h. After fixation, the BAT was placed in PBS containing 15% sucrose overnight and then embedded in 15% sucrose containing 7.5% gelatin and frozen. BAT was sectioned at 4 μm using a cryostat and imaged using a Leica DFC295 as previously mentioned. WAT was placed between two coverslips and imaged using a confocal microscope (Zeiss LSM 510 META).

The right kidney and spleens were also assessed histologically

Both were fixed in 4% PFA overnight, although kidneys were first cut in half longitudinally to expose the cortex, medulla, and pelvis. Both tissues were then embedded in paraffin, sectioned at 3.5 μm (spleen) or 4.5 μm (kidney), and stained with hematoxylin and eosin. Sections were evaluated by standard light microscopy using a Leica DM3000 microscope. The renal cortex was also imaged at 100 $\times$ and spleens at 50 $\times$ using a Leica DFC420. Cortical thickness and tubule and glomeruli size were measured in a subset of images and the number of kidney nephrons per field was determined by counting all glomeruli. In addition, total surface area of lymphoid nodules was quantified by first outlining nodules in spleen images, using Paint.net, and comparing these regions to the total surface area.

Echocardiography

For standard echocardiography, we used the Mylab 70 XVision echocardiography system (Biosound Esaote, Inc., Indianapolis, IN, USA) with a 18 MHz linear epicardial transducer while a 10 MHz-phased array transducer was used for strain acquisition. Mice were anesthetized in a closed system chamber with 2.5% isoflurane in oxygen and were maintained for the duration of the procedure with a nasal cone delivering 1% isoflurane. Echocardiography was performed on anesthetized mice in order to assess basal and maximal cardiac performance using isoproterenol (ISO) stress tests, which cannot be performed on nonanesthetized mice as handling these mice induces a stress response. Standard imaging planes, M-mode, Doppler, and functional calculations were obtained according to American Society of Echocardiography guidelines.

The left ventricle (LV) parasternal long axis four-chamber view was used to derive ejection fraction (%EF) as well as ventricular dimensions and volumes. The left parasternal short axis view was used to obtain M-mode ventricular wall measures, fractional shortening (%FS), and for radial strain analysis. Eight equispaced tracking points were placed circumferentially along the endocardial surface at the papillary muscle level (short axis). The four tracking points of the left ventricular free wall revealed the greatest signal consistency and were used for analysis. Finally, the subcostal long axis view, from the left apex, was used for Doppler imaging of mitral inflow and aortic ejection profiles. Stress tests were performed after first obtaining a baseline echocardiogram followed by i.p. injections of ISO (10 mg/kg, Isuprel-R; Abbott Laboratories). Echocardiograph measures were then recollected 3 min after injection.

Statistical analysis

Differences between means were determined by a one- or two-way ANOVA coupled to Bonferroni *post hoc* test for multiple mean comparisons or by a Student's *t*-test when

appropriate ($P \leq 0.05$). A regression analysis was also used to determine the differences in heart weights of differently aged mice by calculating slopes, to determine the effect of time, and y-intercepts, and to distinguish overall differences in the two populations/genotypes. Separately analyzing data by age groups allowed us to determine whether a particular age group influenced the differences detected when all animals were included in the analysis.

Results

Body morphology

Aging is often associated with a decrease in skeletal muscle mass and bone mineral density as well as an increase in fat mass. We therefore performed DXA scans to assess body and bone composition and weighed individual muscles and organs from WT and *Mstn*^{-/-} mice. We have previously reported that body and heart weights do not diverge until after ~100 days (Rodgers *et al.* 2009). It was therefore surprising to see that body weights were similar in aged WT and *Mstn*^{-/-} mice (Fig. 1A). This was due to an age-related gradual increase in body weight among WT mice that did not occur in *Mstn*^{-/-} mice (data not shown). Lean fat-free mass was greater in both sexes of *Mstn*^{-/-} mice and total and percent body fat content was less (Fig. 1A), particularly in females. This sexual dimorphism in adiposity among *Mstn*^{-/-} mice is highly novel and to our knowledge has not been previously reported. Bone mineralization was also different as total content in both sexes was less in *Mstn*^{-/-} mice, although when normalized to bone area and expressed as bone mineral density, differences were only seen in male mice (Fig. 1B). Body compositions therefore differed, despite similar weights, as *Mstn*^{-/-} mice were lean, had greater fat-free mass, and possibly less bone mineralization while WT mice had more fat.

Differences in lean body mass are usually reflected in muscle mass

We therefore weighed four hindlimb muscles to determine whether the maintenance of muscle mass differed between muscles with different fiber types particularly as aging is not only associated with the loss of muscle mass in general but also a shift in type II to type I fibers (Lang *et al.* 2010). Although changes in fiber typing was not assessed, the weights of muscles composed of predominantly type I (gastrocnemius and soleus), type II (tibialis anterior), and mixed fiber types were 50–150% greater in aged *Mstn*^{-/-} mice (Fig. 1C). The enhanced musculature that occurs in both sexes, therefore, occurs in muscles with different fiber types, although it is not known whether the age-related change in fiber type is altered in *Mstn*^{-/-} mice. By contrast, liver, kidney, and spleen weights were all less in male and female *Mstn*^{-/-} mice (Fig. 1D). Histological analysis of the kidneys failed to identify differences in cortical thickness; the size of tubules

Figure 1 Morphological differences between WT and *Mstn*^{-/-} mice. Data are expressed as percentage of WT in order to include all parameters in one figure. Body composition (A) and bone mineralization (B) were determined using DXA (BW, body weight; lean and fat refer to mass; BMC, bone mineral content; BMD, bone mineral density). (C) Hindlimb muscles include gastrocnemius (Gastroc, mixed fiber type), tibialis anterior (TA, type II); extensor digitorum longus (EDL, type I), and soleus (type I). (D) Organs were rinsed in PBS and blotted dry before weighing ($n=6-14$). Significant differences between WT and *Mstn*^{-/-} mice, within a particular sex, are indicated by asterisks ($P \leq 0.05$). (A, B and C, $n=5$ or 6 ; D, $n=6-14$; average age = 14 m.o. for female and 13 m.o. for male).

and glomeruli; and the number of glomeruli or the relative area of medullary, pelvic, and perirenal adipose tissue (data not shown). The amount of white pulp in the spleens was also similar in both genotypes. This suggests that the smaller organ size in *Mstn*^{-/-} mice is either due to hypoplasia or general atrophy but not to changes in any particular cell type.

Adiposity

Aged *Mstn*^{-/-} mice have a visibly leaner phenotype compared with WT mice (Fig. 2A) as the amount of s.c. fat accumulation is significantly reduced. In fact, the weights of several fat pads, including three WAT and the intrascapular BAT, were reduced in *Mstn*^{-/-} mice, albeit with notable sex differences (Fig. 2B). Compared with WT females, the weights of BAT, subscapular, inguinal, and gonadal fat pads were all less in *Mstn*^{-/-} female mice. In male *Mstn*^{-/-} mice, however, only the inguinal fat pad was smaller, although the difference in subscapular weights between WT and *Mstn*^{-/-} males was nearly significant ($P=0.08$). We also analyzed the weights of these various fat pads in senescent mice (20 m.o.)

and found that all were significantly smaller ($P < 0.01$) in *Mstn*^{-/-} male and female mice (data not shown). Assessing cell morphology of inguinal fat pads and of BAT from male and female mice (Fig. 2C) indicated that not only cell size was smaller in *Mstn*^{-/-} WAT and BAT but also the latter stained differently in female *Mstn*^{-/-} mice, reddish instead of purple, which is often due to greater eosin staining of mitochondria. Labeling mitochondria using Mitotracker, in both BAT and WAT, indicated that mitochondrial number was similar in fat from both genotypes (Fig. 2D). It is therefore likely that the differential eosin staining results from higher cellular density in *Mstn*^{-/-} fat pads due to the lower relative fat content.

Compared with WT mice, there was a greater distribution of small adipocytes in *Mstn*^{-/-} WAT of both sexes (Fig. 3A and B). More cells were counted in sections of *Mstn*^{-/-} fat pads as the average cell diameter in subscapular and inguinal fat pads, again in both sexes, and also in gonadal fat pads of female *Mstn*^{-/-} mice, was smaller (Fig. 3C). Similar differences in the adipocyte size distribution and average cell diameter were also seen in BAT, but only with female mice (Fig. 3D, E and F). This is consistent with differences in tissue

Figure 2 Deletion of myostatin reduces fat accumulation in aged mice. (A) Representative phenotype of skinned WT and *Mstn*^{-/-} mice fed *ad libitum*. Ten (WT) and 12 (*Mstn*^{-/-}) m.o. male mice are shown and respective fat pads are outlined. (B) Fat pad weights are expressed as percentage of WT to enable grouping of all data on one graph ($n=5$ or 6 ; average age = 14 m.o. for female and 13 m.o. for male; $*P \leq 0.05$). (C) WAT (inguinal fat pads) stained with hematoxylin and eosin (i and ii) or Mitotracker Orange (iii and iv) and imaged at $200\times$. (D) BAT stained and imaged as in (C), but imaged at $400\times$.

Figure 3 Differences in adipose tissue mass and cell size among WT and *Mstn*^{-/-} mice. (A and B) Distribution and number of differently sized cells from WAT among male (A) or female (B) mice. Cell diameters were measured from sections of subscapular, inguinal, and gonadal fat pads removed from 5 to 6 mice/genotype and averages (C; mean ± S.E.M., **P* ≤ 0.05) were determined by pooling all data. Cell size and number were determined from an equal number of sections/fat pad/mouse. Thus, sections with smaller sized cells contained more cells. (D, E and F) The distribution and average cell diameters of BAT were determined as in A, B and C. (Average age = 14 m.o. for female and 13 m.o. for male).

weights (Fig. 2B) and suggests that reductions in cell size and not cell number are responsible for the lean phenotype of *Mstn*^{-/-} mice.

Cardiac hypertrophy

Body weights are often used for normalizing heart weights, although this is inappropriate with older mice (Yin *et al.* 1982). It can also be inappropriate when testing factors that influence both cardiac and skeletal muscle as both numerator and denominator are affected. In fact, absolute heart weights and those normalized to tail lengths, which did not differ

between genotypes, were larger in *Mstn*^{-/-} mice of all ages (Fig. 4A, B, C, D, E and F). A regression analysis indicated no effect of time (i.e. identical slopes), although the WT and *Mstn*^{-/-} populations were distinct as indicated by highly significant differences in Y-intercept values. Differences were independent of age and were highly significant in younger adults (100–300 days old (d.o.)) and in aging mice (~300–700 d.o.) after normalizing to tail length. Tibia lengths were also used for normalization in a small subset of animals and produced similar differences (data not shown). A regression analysis was additionally performed on HW/TL ratios for all animals in order to produce a shared Y-intercept of 0.02.

Figure 4 Age-dependent changes in heart weight among WT and *Mstn*^{-/-} mice. (A, B and C) Absolute heart weights in mice ranging in age from (A) 90–600, (B) 90–300, or (C) 300–600 d.o. In the same mice, (D, E and F) heart weights were also normalized to tail length or (G, H and I) body weights. A regression analysis was performed and differences in slopes and y-intercepts are indicated (*Mstn*^{-/-}, dashed line).

The number of hearts above this value was then calculated for age-matched WT and *Mstn*^{-/-} mice. In fact, there were 50% more *Mstn*^{-/-} hearts above this value than WT hearts (WT, $44 \pm 8\%$; *Mstn*^{-/-}, $66 \pm 7\%$; $P=0.026$). By contrast, no differences were detected when heart weights were normalized to body weights (Fig. 4G, H and I). Cardiac hypertrophy, although mild, therefore occurs in *Mstn*^{-/-} mice of all ages.

Cardiac performance

Many recent studies indicate that myostatin regulates cardiac muscle growth in a manner similar to that of skeletal muscle (Cook *et al.* 2002, Gaussin & Depre 2005, Shyu *et al.* 2005, Morissette *et al.* 2006, McKoy *et al.* 2007). In addition, we recently identified cardiac hypertrophy in young *Mstn*^{-/-} mice that more closely resembles physiological, not pathological, hypertrophy especially as excitation–contraction coupling and responses to ISO stress tests were enhanced in *Mstn*^{-/-} cardiomyocytes and mice respectively (Rodgers *et al.* 2009). We therefore sought to determine whether aged *Mstn*^{-/-} mice possess a similar phenotype.

The resting systolic left ventricle internal diameters and volumes were higher in *Mstn*^{-/-} mice, although wall and septum measurements, stroke volume, cardiac output, left ventricle isovolumic relaxation time, aortic ejection time, and

aortic acceleration/ejection time ratio were similar to those of WT mice (Fig. 5A, Table 1). This was true for both sexes and suggests that a mild form of eccentric cardiac hypertrophy occurs in aged *Mstn*^{-/-} mice. Stroke volume, cardiac output, and most hemodynamic parameters were again similar in both genotypes. However, FS and EF were less in *Mstn*^{-/-} mice of both sexes as were radial velocity and strain measurements, which are indices of myocardial deformation (Saghir *et al.* 2007). The atrial contraction velocity (MV A wave) was also greater in *Mstn*^{-/-} mice, although variability in female measurements prevented significance. Nevertheless, reductions in EF, FS, radial velocity, and strain combined with elevated atrial contraction velocity and internal diameters and volumes are usually indicative of systolic and diastolic dysfunction and dilated cardiomyopathy. By contrast, ISO stress tests, which are assessments of maximal cardiac functional reserve, revealed enhanced responsiveness in aged *Mstn*^{-/-} mice that was more pronounced in females (Fig. 5B and C, Table 1). Indeed, the ISO-induced change in FS and EF was larger in *Mstn*^{-/-} mice of both sexes than in WT mice. This was accompanied by better contractility in female *Mstn*^{-/-} mice as the reductions in internal diameters and volumes were both enhanced, as was the ISO-induced heart rate ($P=0.07$). In males, preservation of diastolic filling contributed to enhanced EF as heart rate and systolic volumes were similar to those in WT mice. The overall differences

between WT and *Mstn*^{-/-} mice are similar to those previously described in young adult *Mstn*^{-/-} mice (Rodgers *et al.* 2009) and suggest that although resting cardiac performance appears pathological, response to ISO stress tests indicates functional improvement over WT mice.

Discussion

Recent studies have reported enhanced skeletal muscle mass and reduced WAT mass in senescent (24–30 m.o.) *Mstn*^{-/-} mice (Wagner *et al.* 2005, Siriott *et al.* 2006, Morissette *et al.* 2009). We have corroborated these results using aging or ‘middle-aged’ *Mstn*^{-/-} mice and, for the first time, demonstrated reductions in BAT mass and cell size. These

studies are the first to report significant sexual dimorphism in *Mstn*^{-/-} mice, particularly in adiposity and in cardiac function. They are also the first to quantify strain differences in the myocardium, regardless of age, and again corroborate the increased ISO responsiveness previously characterized in young adult mice. Furthermore, our results resolve a current controversy in the field and explain why cardiac hypertrophy is often mischaracterized in *Mstn*^{-/-} animals (see below). These studies complement those with senescent mice and together suggest that targeting myostatin could potentially help to treat many age-related disorders (Siriott *et al.* 2007, Yablonka-Reuveni 2007, Lebrasseur *et al.* 2009). This naturally assumes that the phenotypes described can be duplicated by attenuating myostatin in adults and that several precautions are carefully considered.

The progressive loss of skeletal muscle mass and increased adiposity that normally occurs with aging is in contrast to the lean *Mstn*^{-/-} phenotype that is maintained with age in both male and female mice (Fig. 1A). Previous studies have nevertheless demonstrated age-associated declines in fat-free lean body mass and skeletal muscle mass even in *Mstn*^{-/-} mice (Morissette *et al.* 2009). Indeed, the reported decline was similar in both WT and *Mstn*^{-/-} mice. Our data indicate that muscle weights, regardless of fiber-type classification, are still significantly greater than those of WT mice (Fig. 1C). This suggests that a *Mstn*^{-/-} environment does not prevent age-associated losses in skeletal muscle *per se*, but it can enhance musculature to the degree that may avoid some related complications. The equally enhanced weights of muscles with different fiber types further suggests that a *Mstn*^{-/-} environment could potentially avoid the loss of type II fibers

Figure 5 Resting and stress-induced cardiac performance. (A) Echocardiography was performed on the LV parasternal long axis, left parasternal short axis, subcostal long axis, and endocardial short axis views. Data are presented as percentage differences from WT values, which are represented by the horizontal dashed line (LVIDd, left ventricle internal diameter (end diastole, mm); LVIDs, LVID systole; IVSd, intraventricular septum (dimension end diastole, mm); LVWd, LV wall dimension (systole, mm); LV mass, left ventricular mass (g); Diast vol, LV end diastolic volume (ml); Syst vol, LV end systolic volume (ml); FS, percentage of fractional shortening; EF, percentage of ejection fraction; St. vol, stroke volume; C.O., cardiac output; LV IVRT, LV isovolumic relaxation time (ms); HR, heart rate (beats/min); Ao velo, max aortic ejection velocity (cm/s); VTI, velocity time integral (cm); acel, ejection acceleration time (ms); ET, ejection time (ms); ac/ET, ratio of accel to ET; MV E, max LV early filling velocity (cm/s); MV A, max LV late filling (atrial contraction) velocity (cm/s); MV E/A, ratio of E to A velocities; MV DT, deceleration time of early LV filling (ms); Rad vel-S, radial velocity during ventricular systole (cm/s); Rad vel-EA, radial velocity during diastole (early and late filling combined, cm/s); ST-S, LV strain during ventricular systole (%); ST-EA, LV strain during diastole (early and late filling combined, %); SR-S, LV strain rate during ventricular systole (1/s); SR-EA, LV strain rate during diastole (early and late filling combined, 1/s); NA, not applicable due to insufficient *n*). (B and C) Male (B) or female (C) mice were injected i.p. with 10 mg/kg of isoproterenol (ISO) and then assessed by echocardiography. Asterisks denote significant differences (*n* = 5 or 6; average age = 14 m.o. for female and 13 m.o. for male; *P* ≤ 0.05).

Table 1 Cardiac performance in WT and *Mstn*^{-/-} mice

Parameter	WT	WT + ISO	<i>Mstn</i> ^{-/-}	<i>Mstn</i> ^{-/-} + ISO
LVIDd (M)	3.68 ± 0.24 ^a	2.78 ± 0.38 (4) ^b	3.80 ± 0.11 ^a	2.96 ± 0.13 ^b
LVIDd (F)	3.32 ± 0.27 ^a	2.90 ± 0.12 ^{a,b}	3.74 ± 0.18 ^a	2.76 ± 0.10 ^b
LVIDs (M)	2.14 ± 0.24 ^a	0.95 ± 0.13 (4) ^c	2.76 ± 0.09 ^b	1.33 ± 0.17 ^c
LVIDs (F)	1.83 ± 0.20 ^a	1.31 ± 0.15 ^{a,c}	2.66 ± 0.27 ^b	1.30 ± 0.04 ^c
LVWd (M)	1.03 ± 0.12 ^a	1.12 ± 0.08 (4) ^a	1.00 ± 0.05 ^a	1.07 ± 0.07 ^a
LVWd (F)	1.05 ± 0.08 ^a	1.23 ± 0.04 ^a	0.88 ± 0.05 ^{a,b}	0.87 ± 0.03 ^b
Diast vol (M)	0.059 ± 0.009 ^a	0.032 ± 0.01 (4) ^b	0.062 ± 0.004 ^a	0.034 ± 0.003 ^b
Diast vol (F)	0.047 ± 0.008 ^a	0.033 ± 0.003 ^{a,b}	0.061 ± 0.007 ^a	0.029 ± 0.003 ^b
Syst vol (M)	0.016 ± 0.004 ^a	0.002 ± 0.001 (4) ^c	0.029 ± 0.002 ^b	0.005 ± 0.002 ^c
Syst vol (F)	0.011 ± 0.003 ^a	0.005 ± 0.001 ^{a,c}	0.028 ± 0.007 ^b	0.004 ± 0.0003 ^c
FS (M)	41.4 ± 4.3 ^a	66.8 ± 2.3 (4) ^c	27.2 ± 2.0 ^b	56.8 ± 3.1 ^c
FS (F)	45.2 ± 3.3 ^a	56.3 ± 3.2 ^{c,d}	28.6 ± 4.2 ^b	52.2 ± 0.6 ^d
EF (M)	77.6 ± 4.4 ^a	95.8 ± 0.9 (4) ^c	59.8 ± 3.1 ^b	90.2 ± 2.3 ^c
EF (F)	80.8 ± 4.0 ^a	90.2 ± 2.0 ^{a,c}	60.6 ± 6.5 ^b	88.6 ± 0.4 ^c
Ao velo (M)	61.1 ± 2.7 ^a	78.7 ± 7.2 ^{a,b}	63.2 ± 2.7 ^a	90.0 ± 6.5 ^b
Ao velo (F)	52.1 ± 4.6 ^a	67.0 ± 7.5 ^a	57.2 ± 3.3 ^a	61.7 ± 4.2 ^a
Ao VTI (M)	2.38 ± 0.21 ^a	3.34 ± 0.46 ^{a,b}	2.44 ± 0.14 ^a	3.82 ± 0.35 ^b
Ao VTI (F)	2.23 ± 0.17 ^a	2.88 ± 0.26 ^a	2.50 ± 0.23 ^a	2.64 ± 0.16 ^a
Ao acel (M)	17.6 ± 1.0 ^a	20.6 ± 4.1 ^a	15.8 ± 1.7 ^a	14.0 ± 2.1 ^a
Ao acel (F)	16.0 ± 1.6 ^a	21.5 ± 2.3 ^a	14.2 ± 1.4 ^{a,b}	12.8 ± 2.4 ^b
MV E (M)	50.1 ± 0.9 (4) ^a	NA	51.4 ± 1.6 (4) ^a	63.5 ± 16.5 ^A
MV E (F)	46.6 ± 1.8 ^A	NA	47.7 ± 6.9 ^A	52.2 ± 19.8 ^A
MV A (M)	26.8 ± 3.3 (4) ^a	NA	36.5 ± 2.4 (4) ^b	41.5 ± 9.5 ^A
MV A (F)	25.7 ± 5.0 ^A	NA	33.8 ± 12.3 ^A	40.3 ± 3.3 ^A
MV E/A (M)	1.98 ± 0.23 (4) ^a	NA	1.43 ± 0.09 (4) ^b	1.55 ± 0.05 ^A
MV E/A (F)	1.87 ± 0.26 ^A	NA	1.76 ± 0.50 ^A	1.30 ± 0.60 ^A
MV DT (M)	30.6 ± 2.8 ^a	39.0 ± 2.2 ^b	26.4 ± 1.9 ^a	34.0 ± 2.0 ^{a,b}
MV DT (F)	31.4 ± 0.8 (4) ^a	37.0 ± 1.63 ^a	32.2 ± 0.5 ^a	32.4 ± 4.6 ^a
HR (M)	420 ± 8.4 ^a	482 ± 2.0 ^b	450 ± 14.8 ^a	506 ± 7.5 ^b
HR (F)	406 ± 23.0 ^a	432 ± 21.8 ^a	424 ± 9.8 ^a	482 ± 16.4 (4) ^a
Rad vel-S (M)	1.1 ± 0.15 (4) ^a	NA	0.74 ± 0.10 ^b	NA
Rad vel-S (F)	0.74 ± 0.07 ^a	NA	0.49 ± 0.04 (4) ^b	NA
Rad vel-EA (M)	-0.96 ± 0.11 (4) ^a	NA	-0.63 ± 0.09 ^b	NA
Rad vel-EA (F)	-0.73 ± 0.13 ^a	NA	-0.41 ± 0.005 (4) ^b	NA
ST-S (M)	-20.5 ± 2.5 (4) ^a	NA	-16.9 ± 2.3 ^a	NA
ST-S (F)	-20.3 ± 1.9 ^a	NA	-14.8 ± 2.3 (4) ^a	NA
ST-EA (M)	14.3 ± 4.1 (4) ^a	NA	7.40 ± 1.08 ^b	NA
ST-EA (F)	10.6 ± 2.52	NA	3.65 ± 0.41 ^A	NA
SR-S (M)	-7.43 ± 1.02 (4) ^a	NA	-5.22 ± 0.82 ^a	NA
SR-S (F)	-5.06 ± 0.59 ^a	NA	-3.85 ± 0.39 (4) ^a	NA
SR-EA (M)	6.6 ± 1.12 (4) ^a	NA	4.44 ± 0.73 ^a	NA
SR-EA (F)	5.14 ± 0.66 ^a	NA	3.13 ± 0.25 (4) ^b	NA

Refer to Fig. 5 for definition of abbreviations. NA, not available. Different letters denote statistical differences, the same letters represent no difference and male (M) and females (F) were assessed separately. (average age = 14 m.o. for female and 13 m.o. for male).

^ANot statistically assessed due to insufficient replicates; *n* = 5, 6 unless otherwise noted in parentheses.

and enrichment of type I fibers that normally occurs with aging. This is in fact supported by recent studies that quantified age-related changes in fiber type and size among WT and *Mstn*^{-/-} mice (Siriett *et al.* 2006, Matsakas *et al.* 2009). Muscles of senescent *Mstn*^{-/-} mice also regenerate quicker from chronic or acute injury (Wagner *et al.* 2005) and short-term attenuation of myostatin restores muscle regenerative properties (Siriett *et al.* 2007). Thus, myostatin-attenuating therapies could be potentially used to prevent skeletal muscle loss and/or to restore damaged or atrophied muscle in the elderly.

Although the enhanced musculature in young *Mstn*^{-/-} mice results in heavier body weights compared with WT

mice, this difference is lost in aging mice due to greater adiposity in wild types (Fig. 1A). These differences were more prominent in 'middle age' and senescent females and were reflected in fat pad weights and in adipocyte number and size (Figs 2 and 4). Previous studies reported similar differences in 7–9 w.o. and 9–10 m.o. male WAT (McPherron & Lee 2002, Dilger *et al.* 2010) and our data indicate that BAT is also affected. In addition, we identified sexually dimorphic differences in WAT and BAT that were age dependent in that some differences were detected only in females at middle age, but in both sexes at senescence. Such sex- and depot-dependent differences may simply be due to the lower % body fat in female rather than in male *Mstn*^{-/-} mice (Fig. 1A).

However, it is also possible that myostatin influences adipogenesis directly. In fact, myostatin is expressed at low levels in adipose tissue and has been hypothesized to stimulate or inhibit adipogenesis (Rodgers & Garikipati 2008). However, feed intake is normal in *Mstn*^{-/-} mice despite a higher total energy expenditure (McPherron & Lee 2002, Choi *et al.* 2011). Guo *et al.* (2009) determined that myostatin attenuation in skeletal muscle, but not adipose tissue, decreases fat mass and improves glucose homeostasis via the diversion of carbohydrates away from adipose stores and into muscle. This suggests that the reduced adiposity in *Mstn*^{-/-} mice, regardless of age, is likely due to muscle depletion of metabolic reserves rather than to direct effects on adipogenesis or to fat turnover.

Bone mineral density was ~6% smaller in male but not in female *Mstn*^{-/-} mice (Fig. 1B), although other studies report increased density and regeneration with activin/myostatin receptor antagonism (Kellum *et al.* 2009, Zhou *et al.* 2010) or higher density in young and senescent *Mstn*^{-/-} mice (Hamrick *et al.* 2002, 2003, Hamrick 2003, Morissette *et al.* 2009). We have previously reported that age-dependent changes in aggregate bone growth (tail and tibia length) and bone growth rate (tibia epiphyseal plate width) are similar in WT and *Mstn*^{-/-} mice (Williams *et al.* 2011). It is unknown, therefore, why the small difference was noted in the current study. Nevertheless, differences were also detected in other nonmuscle tissues as liver, kidney, and spleen weights were smaller in *Mstn*^{-/-} mice of both sexes (Fig. 1D). We have recently reported that liver expression of IGF1 as well as the total and estimated free levels in circulation are higher in young adult (7 m.o.) *Mstn*^{-/-} mice than in age-matched WT mice (Williams *et al.* 2011). As with aging *Mstn*^{-/-} mice, liver weights are also smaller in these mice as well as in juvenile (1–3 m.o.) *Mstn*^{-/-} mice (Lin *et al.* 2002), which is consistent with increased negative feedback on pituitary GH, the primary regulator of liver size (Ohlsson *et al.* 2009). Myostatin's role in the spleen is unknown, although it is minimally expressed in the spleens of zebrafish and mice and is significantly upregulated when the former are stressed (Helterline *et al.* 2007). No cellular phenotype was detected in any of these tissues, indicating that the differences were likely due to tissue hypoplasia or atrophy. Regardless, these results together suggest that the development of enhanced muscling in young and old *Mstn*^{-/-} mice may impact other nonmuscle tissues.

Several early studies documented myostatin expression in the hearts of different animal models including nonmammalian vertebrates (Rodgers & Garikipati 2008). More recent studies have further demonstrated myostatin inhibition of different cardiac muscle growth processes *in vitro* and *in vivo* (Shyu *et al.* 2005, Morissette *et al.* 2006, Artaza *et al.* 2007, McKoy *et al.* 2007, Rodgers *et al.* 2009, Bish *et al.* 2010, Zhou *et al.* 2010). It is surprising, therefore, that some studies have failed to identify cardiac hypertrophy in *Mstn*^{-/-} mice (Morissette *et al.* 2006, Cohn *et al.* 2007, Heineke *et al.* 2010) especially as overexpressing myostatin stimulates cardiac

atrophy (Artaza *et al.* 2007, Bish *et al.* 2010). We have previously reported eccentric cardiac hypertrophy in 7 m.o. *Mstn*^{-/-} mice (Rodgers *et al.* 2009), although heart weights were normalized to tail lengths, rather than to body weights, as we believe the latter misrepresents the phenotype. Myostatin inhibits the growth of cardiac and skeletal muscle and, hence, both numerator and denominator when normalizing heart weight to body weight. Similar problems occur when experimenting with older mice as treatments can often disproportionately affect body morphology as well as heart weight, necessitating the use of tibia or tail length for normalization (Yin *et al.* 1982). This likely explains why studies that normalized to body weights failed to identify cardiac hypertrophy.

Physiological concentric hypertrophy arises from isometric exercise that also significantly increases skeletal muscle mass. By contrast, physiological eccentric hypertrophy results from aerobic exercise, which does not increase skeletal muscle mass (McMullen & Jennings 2007, Catalucci *et al.* 2008). Thus, the eccentric hypertrophy that develops in *Mstn*^{-/-} mice or with myostatin attenuation does not appear to be a compensatory response to the increased load brought upon by enhanced skeletal muscle growth as this would have produced concentric rather than eccentric hypertrophy. Several apparent systolic and diastolic dysfunctions were also detected in resting hearts as FS, ES, radial velocity, and myocardial strain measurements were lower in aging *Mstn*^{-/-} mice. Many elite human and canine athletes (e.g. Tour de France cyclists, triathletes, racing greyhound and whippets, and sled dogs) possess eccentric physiological hypertrophy that is remarkably similar to that of *Mstn*^{-/-} mice. This often includes reduced EF and FS in resting hearts (Rippe *et al.* 1982, Pape *et al.* 1984, Schaible *et al.* 1986, Colan 1992, Snyder *et al.* 1995, Lonsdale *et al.* 1998, Stepien *et al.* 1998, Whyte *et al.* 2000, Bavegems *et al.* 2005, 2007). Reductions in these parameters are not necessarily pathological as cardiac output is normal in all these subjects including *Mstn*^{-/-} mice. In fact, EF and FS values rise to expected levels when elite athletes stop training (Pavlik *et al.* 1986, Snyder *et al.* 1995) as the reductions are due to reduced relative preload in the presence of enhanced after load at rest (Abergel *et al.* 2004), which was interestingly demonstrated in *Mstn*^{-/-} mice by the ISO stress tests. Indeed, the ISO-induced change in FS and EF was significantly greater in both young (Rodgers *et al.* 2009) and aging *Mstn*^{-/-} mice (Fig. 5B and C). It is unknown why the enhanced responsiveness to ISO was more prominent in female than male *Mstn*^{-/-} hearts nor why differences in internal volumes were similarly greater in females. These data are consistent, however, with sexual dimorphism in other *Mstn*^{-/-} tissues and suggest that sexual development, or even gonadal steroids, predispose tissues differentially to the effects of myostatin or even its attenuation.

Morissette *et al.* (2009) recently reported that left ventricle chamber volumes were smaller and FS was greater in senescent (27–37 m.o.) *Mstn*^{-/-} than WT mice. This

conflicts with their previous study where these parameters were similar in 8 w.o. WT and *Mstn*^{-/-} mice. It also conflicts with our previous study (Rodgers *et al.* 2009) using 7 m.o. adults and with the data presented herein, both of which indicate exactly the opposite. These studies together suggest that a cardiac phenotype slowly develops in a *Mstn*^{-/-} environment and may not be readily recognized in young animals, which is in fact reflected by age-associated changes in heart weight (Rodgers *et al.* 2009). Functionally, however, the phenotype first presents in young adults and includes notable contractile dysfunctions at rest but enhanced functional reserve. The phenotype appears to change with age, as many aspects of normal cardiac senescence, reduced contractility for example (Morissette *et al.* 2009), are less pronounced in senescent *Mstn*^{-/-} mice. More mechanistic studies are nevertheless required to determine whether enhanced contractility and ISO responsiveness are due to similarly enhanced β AR signaling or elevated levels and activity of Ca²⁺ handling proteins.

Future studies will also determine the clinical relevance of myostatin attenuation in different tissues as well as the evolutionary significance of *Mstn*^{-/-} phenotypes, or possibly more importantly, the lack thereof. In fact, many clinically relevant tissues and organ systems that are affected by aging are also impacted by myostatin. These naturally include skeletal muscle as well as cardiac muscle, adipose tissue, and possibly bone. Changes in adiposity have the potential to impact other physiological systems, thermogenesis, and reproduction in particular. This in turn may explain why *Mstn*^{-/-} phenotypes have not been described in nature as the relative gains in fitness brought upon by 'double muscling' would hypothetically be balanced by comparable costs. Clinically, however, attenuating myostatin has great potential and could possibly be used to treat obesity and type 2 diabetes especially as glucose homeostasis is improved in a *Mstn*^{-/-} environment (McPherron & Lee 2002, Guo *et al.* 2009, Morissette *et al.* 2009). The *Mstn*^{-/-} cardiac phenotype resembles physiological, not pathological, hypertrophy in many ways that include enhanced ISO responsiveness and Ca²⁺ handling, reduced age-associated fibrosis (Morissette *et al.* 2009), and normal fetal gene expression profiles (Rodgers *et al.* 2009). The contractile dysfunction at rest, however, raises some concern just as it does in many elite athletes. Future studies are therefore needed to determine whether the myostatin-blocking technologies currently being developed to treat skeletal muscle disorders (Khurana & Davies 2003, Bogdanovich *et al.* 2005, Zhou *et al.* 2010) may also be appropriate for treating cardiac disorders and in addition, whether myostatin is directly or indirectly responsible for the differences described herein.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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