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Myostatin Does not Regulate Cardiac Hypertrophy or Fibrosis

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Abstract

Myostatin is a negative regulator of muscle growth. Loss of myostatin has been shown to cause increase in skeletal muscle size and improve skeletal muscle function and fibrosis in the dystrophin-deficient *mdx* mouse, an animal model for Duchenne muscular dystrophy. We evaluated whether lack of myostatin has an impact on cardiac muscle growth and fibrosis *in vivo*. Using genetically modified mice we assessed whether myostatin absence induces similar beneficial effects on cardiac function and fibrosis. Cardiac mass and ejection fraction were measured in wild type, *myostatin-null*, *mdx* and double mutant *mdx/myostatinnull* mice by high resolution echocardiography. Heart mass, myocyte area and extent of cardiac fibrosis were determined post mortem. *Myostatin-null* mice do not demonstrate ventricular hypertrophy when compared to wild type mice as shown by echocardiography (ventricular mass 0.69 ± 0.01 g vs. 0.69 ± 0.018 g, $P = 0.75$, respectively) and morphometric analyses including heart/body weight ratio (5.39 ± 0.45 mg/g vs. 5.62 ± 0.58 mg/g, $P = 0.59$ respectively) and cardiomyocyte area $113.67 \pm 1.5 \mu\text{m}^2$, $116.85 \pm 1.9 \mu\text{m}^2$; $P = 0.2$). Moreover, absence of myostatin does not attenuate cardiac fibrosis in the dystrophin deficient *mdx* mouse model for DMD (12.2% vs. 12% respectively, $P = 0.88$). The physiological role of myostatin in cardiac muscle appears significantly different than that in skeletal muscle as it does not induce cardiac hypertrophy and does not modulate cardiac fibrosis in *mdx* mice.

Introduction

Duchenne muscular dystrophy (DMD) is a common X-linked disorder caused by mutations in the dystrophin gene and complete absence of dystrophin protein from the sarcolemma, leading (1) to severe weakness and premature death in early adulthood. During the progression of the disease, the incidence of cardiac involvement becomes increasingly prevalent (2-4).

Myostatin (GDF-8) is a negative regulator of muscle growth, with gene mutations leading to increased musculature *in vivo*. Increased muscle mass due to myostatin mutations has also been reported in mice (5,6), cattle and and more recently in humans (7). Loss or inhibition of

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myostatin ameliorates the dystrophic phenotype in *mdx* mice (8,9). Specifically, there is a significant decrease in fibrosis in *mdx* mice lacking myostatin (8-10). A phase I/II clinical trial of a myostatin neutralizing antibody in adult muscular dystrophy is currently being conducted in several centers in the US and UK. Recently, myostatin mRNA and protein have been shown to be expressed in low levels in fetal and adult cardiac muscle (11,12). Furthermore, it was shown that myostatin expression is upregulated in the surrounding tissue after myocardial infarcts, implying that it may play a role in cardiac physiology and remodeling (11). However, the functional role of myostatin in cardiac muscle remains to be elucidated. Indeed, no cardiac developmental abnormality has been reported in *myostatin-null* mice (13).

We therefore analyzed the impact of loss of myostatin on cardiac muscle performance in *myostatin-null* mice and on the development of cardiac fibrosis in dystrophin-deficient *mdx* mice.

Materials and Methods

Mice

All mouse protocols were approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine. Mice were obtained from our breeding colonies housed at the Johns Hopkins animal care unit with original C57BL/6 and *mdx* breeding pairs purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Myostatin-null* mice were described previously (13) and bred with *mdx* mice to create double mutant mice (9). Each animal's total weight and the heart weight were recorded before further processing of the tissue.

Histology and cardiomyocyte morphometry

Wild-type, *myostatin-null*, *mdx* and *mdx/myostatin-null* mice at different ages (6 weeks, 10 weeks, 16 weeks, 12 months and 24 months n=6-12 each) were sacrificed by halothane. For morphometric analyses, the heart was flash frozen in cooled isopentane and mounted in Tracacanth (Sigma Aldrich, USA). Subsequently, 10µm sections were stained with hematoxylin and eosin (H&E) or Gomori trichrome. All images were taken using an Eclipse E400 microscope (Nikon Inc.). For quantification of cardiomyocyte size, the cross sectional area of cells stained for laminin-γ1 and DAPI was determined (14). For each genotype, six mice age and sex matched were used and a total of 300 cells from each animal were counted. For quantification of tissue fibrosis, areas of trichrome stained sections including the right and left ventricle, anterior and posterior wall, lateral wall and interventricular septum were analyzed and positive areas were calculated as a percentage of the total heart area using the NIH Scion image software, public domain (15). For all comparisons, age and sex matched animals were used.

Echocardiography

Animals were anesthetized with a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg) administered intramuscularly into the thigh muscle. The chest hair was removed with clippers and depilatory cream. Animals were then placed on a heat-controlled stage with built-in electrocardiography leads. Transthoracic echocardiography was performed using a high resolution ultrasound system (Vevo 660, Visual Sonics, Toronto, Canada) equipped with a high frequency transducer (frequency band 15-45 MHz, center frequency 30 MHz). Parasternal and apical images were obtained and adjustments made in viewing angle and imaging settings so as to optimize epicardial and endocardial definition. Analyzable images were obtained in all animals. Left ventricular dimensions and wall thickness were measured at end diastole and end systole using online electronic calipers. Left ventricular mass was calculated using a validated formula (16). Left ventricular ejection fraction was calculated using the modified Quinones technique.

Statistical analysis

All values are expressed as mean $\pm$ SEM. To determine significance between two groups, comparisons were made using the unpaired Student's *t* tests with $p < 0.05$ considered statistically significant

Results

Morphological evolution of cardiomyopathy in *mdx* mice

Using routine histological H&E as well as Masson's trichrome staining procedures we systematically analyzed hearts of *mdx* mice at different ages (6 weeks, 10 weeks, 16 weeks, 12 months and 24 months $n=6-12$ each). Until the age of 16 weeks only minimal morphological abnormalities were observed. The main histopathological findings during this time were diffuse distribution of small areas of necrotic cardiomyocytes (Figure 1). With increasing age, morphological abnormalities in cardiac muscle of *mdx* mice became more severe as areas of necrosis were replaced with fibrotic lesions. At 12-24 months of age, severe cardiomyopathic changes with large accumulating areas of fibrosis were evident (Figure 1).

Evaluation of heart weight and cardiac cell size

It is well known that loss of myostatin leads to significant increase in skeletal muscle cell size. To determine whether the absence of myostatin has an impact on cardiac muscle size, we analyzed heart weight to body weight ratio in 2-year old wild-type, *myostatin-null*, *mdx* and *mdx/myostatin-null* mice (see Table 1). There was no statistically significant difference between the heart weight/body weight ratios of *myostatin-null* mice when compared to wild-type mice ($5.39 \pm 0.45 \text{ mg/g}$ vs. $5.62 \pm 0.58 \text{ mg/g}$, respectively). In addition, no significant difference was observed between the heart weight/body weight ratios of *mdx/myostatin-null* and *mdx* mice ($5.83 \pm 0.68 \text{ mg/g}$ vs $6.09 \pm 1.53 \text{ mg/g}$)

To quantify the size of cardiomyocytes, we measured the cross section area of these cells. Cryostat sections of heart muscle from 2-year old animals were stained with laminin- $\gamma 1$ to highlight the cell membrane and subsequently costained with DAPI to mark the cell nuclei. Cross sections were analyzed with the NIH scion image program (public domain). There was no significant difference in cell size between wild-type ($113.67 \pm 1.5 \mu\text{m}^2$) and *myostatin-null* ($116.85 \pm 1.9 \mu\text{m}^2$; $P = 0.2$); and between *mdx* ($112.95 \pm 2.2 \mu\text{m}^2$) and *mdx/myostatin-null* mice ($113.56 \pm 2.6 \mu\text{m}^2$; $P = 1.21$) (Figure 2). Taken together, these results demonstrate that absence of myostatin does not lead to myofiber hypertrophy in cardiac muscle as it does in skeletal muscle. We did not detect any differences of heart weight or cardiac cell size between male or female *myostatin-null* mice.

Echocardiography

There was no significant difference in left ventricular end-diastolic (4.04 ± 0.3 versus 4.36 ± 0.2 mm; $n = 7$) and end-systolic dimension (2.82 ± 0.3 vs. 3.22 ± 0.4 mm; $n = 5$), left ventricular mass (0.69 ± 0.02 vs. 0.69 ± 0.01 g) and ejection fraction (0.51 ± 0.05 vs. 0.45 ± 0.12) between wild type and *myostatin-null* mice, respectively (see Table 2). Similarly, no differences were seen in left ventricular end-diastolic (3.74 ± 0.5 versus 3.90 ± 0.4 mm) and end-systolic dimension (2.60 ± 0.5 versus 2.80 ± 0.6 mm), left ventricular mass (0.67 ± 0.02 versus 0.60 ± 0.01 g) and ejection fraction (0.51 ± 0.05 versus 0.48 ± 0.11) between *mdx* and *mdx/myostatin-null* mice, respectively (Figure 3).

Morphological assessment of cardiac muscle in *myostatin-null* and *mdx/myostatin-null* mice

In order to determine whether loss of myostatin has an impact on cardiac muscle cell morphology we analyzed 1-year and 2-year old *myostatin-null* and *mdx/myostatin-null* mice.

Evaluation of H&E and Masson's trichrome stained cardiac sections from *myostatin-null* mice displayed normal histologic features (Figure 4a). In contrast, *mdx* and *mdx/myostatin-null* mice displayed patchy areas of fibrosis at 1 and 2-years of age. Quantification of fibrotic areas showed similar amounts of fibrosis in *mdx/myostatin-null* mice when compared to *mdx* mice (Figure 4b). Thus, our data demonstrate that loss of myostatin does not prevent the development of cardiac fibrosis in dystrophin-deficient *mdx* mice.

Discussion

The current study demonstrates that loss of myostatin is not associated with increased heart mass per body weight or cardiac myocyte hypertrophy. Left ventricular mass and ejection fraction by high resolution echocardiography, heart weight and cardiomyocyte cross-sectional area were similar in *myostatin-null* and wild-type mice. Furthermore, absence of myostatin did not attenuate cardiac fibrosis in the *mdx* mice. These findings were maintained over a 24-month study period. Taken together, these data demonstrate that myostatin does not function as a major regulator of myofiber growth and regeneration in cardiac muscle *in vivo*.

Duchenne muscular dystrophy is the most common form of inherited muscular dystrophy and caused by mutations in gene dystrophin (17). Dystrophin is a 427 kDa protein which together with the syntrophins; the sarcolemmal localized dystroglycans (α and β subunits); the sarcoglycans (α , β , γ and δ subunits) and sarcospan (18), forms a multisubunit complex called the dystrophin-glycoprotein complex (DGC) which serves as a structural linkage between the F-actin cytoskeleton and the extracellular matrix. The *mdx* mouse, which is the murine orthologue of DMD, lacks dystrophin as a result of a nonsense mutation (19,20). The complete loss of dystrophin from the sarcolemma of patients with DMD and the *mdx* mouse perturbs the structural composition of the DGC. *Mdx* mice have continuous rounds of skeletal muscle degeneration and regeneration. Degeneration is most prominent from 3-6 weeks of life (21). During the first few months, only small areas of fibrosis are noted in limb skeletal muscle of *mdx* mice. In contrast, increased connective tissue formation can be detected in skeletal and cardiac muscle of older *mdx* mice resembling the histopathological findings of patients with DMD (22,23). Our morphological analyses of aged hearts from *mdx* mice confirm the previous reports of increased fibrosis. However, our echocardiographic analyses did not show any significant functional impairment of *mdx* mice when compared to wildtype mice as previously reported (24). The apparent discrepancy between our study and the study by Quinlan et al., (24) could potentially be explained by use of different echocardiographic technologies. In our study, high resolution echocardiography machines (25-30 Mhz frequency) was used to evaluate cardiac function. This differs from conventional methods used by most investigators (7-15Hz). The resolution of the machine used in the present study is several fold higher yielding a spatial resolution of 50-70 microns as compared to the conventional machines yielding a resolution of ~500 micron resolution. This disparity in resolution may be a major factor explaining the difference in results.

Most patients with DMD die from cardiac or respiratory failure. In a series of 328 patients longitudinally studied for more than a decade the clinical incidence of cardiomyopathy increased steadily over the teen years with approximately one third of patients being affected by the age of 14, one half of patients by age of 18 and all adult patients (4). Patients with Becker muscular dystrophy (BMD), who have residual expression of dystrophin and a milder skeletal muscle involvement are at high risk of developing cardiomyopathy which often causes death due to heart failure (25). Given the technological improvement of respiratory management via various assist devices in recent years (26); it is predicted that abnormal cardiac function will emerge as an even more common feature of DMD. The incidence may further increase if several of the agents in current clinical trials prove to be successful in ameliorating the skeletal muscle phenotype. Current management options for patients with dystrophin-deficient

cardiomyopathy primarily involves afterload reduction but the efficacy of such treatment in extending lifespan is not known and suggested to be small (27,28).

Myostatin, a member of the TGF β superfamily, is a potent inhibitor of muscle hypertrophy and hyperplasia in skeletal muscle. Its mechanism of action appears to be on resident muscle progenitor cells, satellite cells which are activated to proliferate and differentiate in its absence (6,29). In adult animals, regeneration of skeletal muscle is enhanced in the absence of myostatin as demonstrated in models of acute and chronic injury such as the *mdx mouse* (6).

It is unclear what role, if any, myostatin plays in cardiac muscle. Myostatin is predominantly expressed in skeletal muscle and circulates in the blood stream (13,30). Myostatin mRNA has been demonstrated by Sharma et al., (11) in fetal and adult heart. In addition, the putative receptor to myostatin, the activin receptor IIB is expressed in cardiac tissue (31). A recent study by Morissette et al., (32) demonstrated that myostatin deficient cardiomyocyte cells exhibit a more exuberant growth in response to chronic phenylephrine infusion *in vitro* and *in vivo*. However, our long term observations *in vivo* and previous reports of *myostatin-null* mice suggest that there is no cardiac phenotype in the absence of myostatin (13). As presented here, heart/body weight ratio, morphology and function of hearts from young and senescent *myostatin-null* animals are normal. Moreover, unlike its effects in skeletal muscle, absence of myostatin failed to stimulate regeneration and attenuate the onset and progression of cardiac fibrosis in the *mdx* mice. These results suggest that myostatin is not a major regulator of cardiac myofiber growth *in vivo*. In contrast, Parsons et al. (33), recently reported that mice deficient for δ -sarcoglycan (a mouse model for limb-girdle muscular dystrophy 2F) and myostatin show slight improvement of shortening fraction analyzed via echocardiography, however a detailed analyses including morphology and additional functional parameters over an extended period of time were not shown. Although we were not able to demonstrate a direct beneficial impact on the prevention of cardiac fibrosis in *mdx* mice, it is important to note that double mutant *mdx/myostatin-null* mice did not show any signs of exacerbation of the cardiac phenotype.

Extrapolation of our findings in mice to human patients must be done with caution for several reasons. This includes the fact that the dystrophinopathy in *mdx* mice produces a mild skeletal and cardiac myopathy contrary to the severity of DMD. In addition, genetic deletion of myostatin in the mouse differs from postnatal inhibition of myostatin in development for human therapeutics. In this regard, the present study likely investigates the most dramatic effect of complete myostatin loss throughout development and postnatal life while human therapeutics will likely never lead to complete myostatin blockade. Our data do not support a cardiac effect in the complete absence of myostatin and therefore would not predict a significant impairment nor improvement in the cardiac function of patients treated with myostatin inhibitors such as are now in clinical trials.

Dilated cardiomyopathy has been an increasing cause for morbidity and mortality in DMD patients, due in part to recent improvements in the management of respiratory failure. We demonstrate that the absence of myostatin does not cause increase in cardiac muscle cell size and did not prevent the development of cardiac muscle fibrosis in *mdx* mice. Our data emphasize that monitoring of cardiac function and development of treatment strategies for cardiomyopathy in patients with muscular dystrophy continues to be a critical issue in the future particularly if skeletal muscle abnormalities are ameliorated by agents currently in clinical trial.

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Figure 1.

Histopathological characterization of cardiomyopathy in *mdx* mice During the first 6 months only minimal morphological alterations presenting as single diffuse necrotic cells and fibrotic areas can be observed in cardiac muscle as shown by H&E staining in 6-week and 16-week old mice. In contrast, severe fibrosis can be detected in older mice as shown by Masson's trichrome staining of cardiac muscles from 12 and 24 month old mice. Bar 75 μ m.

Figure 2. Quantification of cardiomyocyte cross section area from 2-year old wild-type, *myostatin-null*, *mdx* and *mdx/myostatin-null* mice (n=3 each) were analyzed. There was no statistical significance in cardiac cell size between either of the genotypes; mean±SEM. $P = 1.02$ for wild-type versus *myostatin-null* and $P = 0.19$ for *mdx* versus *mdx/myostatin-null* mice respectively.

Figure 3.

Echocardiography data. Representative M mode tracing at the mid ventricular level demonstrating measurement of end-systolic (solid line) and end-diastolic (dashed line) dimensions (A). There were no significant differences between groups for LVEDD (B), LVESD (C), LV mass (D) and LV ejection fraction (E).

Figure 4.

A Trichrome staining of 2-year old wild-type, *myostatin-null*, *mdx* and *mdx/myostatin-null* mice demonstrates no visible difference between wild-type and *myostatin-null* mice. In contrast *mdx* mice exhibit patchy areas of fibrosis. A similar amount of fibrosis can be detected in *mdx/myostatin null* mice. **Figure 4 B+C** Quantification of fibrotic areas in 1-year and 2-year old wild-type, *myostatin-null*, *mdx* and *mdx/myostatin-null* mice confirms the histological observations. There is only very minimal amount of cardiac fibrosis in wild-type and *myostatin-null* mice. In contrast, there areas of fibrosis in *mdx* and *mdx/myostatin-null* mice at both 1 and 2-years of age are significantly increased; mean $\pm$ SEM. $P < 0.003$ for wild-type versus *mdx* and *mdx/myostatin-null* at 1 year of age, $P < 0.002$ for wild-type versus *mdx* and *mdx/myostatin-null* at 2 years of age. However, loss of myostatin does not prevent the development of cardiac fibrosis on *mdx* mice.

Table 1

Weight data analyses of 24-month old mice

	Total Body weight (g)		Total Heart weight (g)		Heart/body weight (g)	
	Mean	SD	Mean	SD	Mean	SD
Wild-type n=4	37.4	0.9	0.21	0.02	5.62	0.58
Myostatin-null n=5	40.3	2.1	0.21	0.01	5.39	0.45
mdx n=6	32.8	2.1	0.17	0.04	6.09	1.53
mdx/msm-null n=5	36.2	0.8	0.19	0.03	5.83	0.68

There is no statistical significance of heart/body weight ratio between wild-type and *myostatin-null* ($P = 0.32$) and *mdx* versus *mdx/myostatin-null* mice ($P = 0.68$).

Table 2

Echocardiographic analyses of 24-month old mice

	LVD(s)		LVD(d)		EF		IVS(d)		LVPW(d)		LV Mass	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wild-type n=7	2.82	0.35	4.01	0.35	0.51	0.05	0.80	0.11	0.74	0.09	0.69	0.02
Myostatinnull n=5	3.22	0.49	4.37	0.22	0.45	0.12	0.71	0.05	0.69	0.11	0.69	0.01
mdx n=8	2.61	0.40	3.74	0.55	0.51	0.06	0.71	0.13	0.74	0.08	0.67	0.02
mdx/mstn null n=13	2.81	0.59	3.90	0.45	0.48	0.11	0.81	0.13	0.68	0.09	0.68	0.01

There is no significant difference in left ventricular end-diastolic (LVD d, $P = 0.09$) and end-systolic dimension (LVD s, $P = 0.19$), left ventricular mass (LV mass, $P = 0.75$) and ejection fraction (EF, $P = 0.52$) between wild type and *myostatin-null* mice, respectively.

Similarly, no differences were seen in left ventricular end-diastolic ($P = 0.37$) and end-systolic dimension ($P = 0.54$), left ventricular mass ($P = 0.27$) and ejection fraction ($P = 0.64$) between *mdx* and *mdx/myostatin-null* mice.