Molecular Medicine

Myostatin Regulates Cardiomyocyte Growth Through Modulation of Akt Signaling

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Abstract—Myostatin is a highly conserved, potent negative regulator of skeletal muscle hypertrophy in many species, from rodents to humans, although its mechanisms of action are incompletely understood. Transcript profiling of hearts from a genetic model of cardiac hypertrophy revealed dramatic upregulation of myostatin, not previously recognized to play a role in the heart. Here we show that myostatin abrogates the cardiomyocyte growth response to phenylephrine in vitro through inhibition of p38 and the serine–threonine kinase Akt, a critical determinant of cell size in many species from drosophila to mammals. Evaluation of male myostatin-null mice revealed that their cardiomyocytes and hearts overall were slightly smaller at baseline than littermate controls but exhibited more exuberant growth in response to chronic phenylephrine infusion. The increased cardiac growth in myostatin-null mice corresponded with increased p38 phosphorylation and Akt activation in vivo after phenylephrine treatment. Together, these data demonstrate that myostatin is dynamically regulated in the heart and acts more broadly than previously appreciated to regulate growth of multiple types of striated muscle. (Circ Res. 2006;99:15-24.)

Key Words: myostatin ■ Akt ■ p38 ■ hypertrophy

Akt is a serine–threonine kinase whose role in controlling cell growth has been conserved from Drosophila to mammalian species.1–4 To study the effects of chronic Akt activation in the heart, we generated 2 transgenic murine lines with cardiac-specific expression of activated Akt.1 Both lines develop substantial cardiac hypertrophy characterized by an increase in cardiomyocyte size with preserved cardiac function,1 without the “fetal” transcriptional profile characteristic of pathologic cardiac hypertrophy.5 Together these data suggest constitutive Akt activation in the heart induces an exaggerated growth response, consistent with its role in other species.2,3 One of the transgenic lines generated exhibited exaggerated growth response, consistent with its role in other species.2,3 One of the transgenic lines generated exhibited X-linked inheritance and, in the hearts of female mice, the expected chimeric transgene expression caused by X inactivation.5 As expected, transgene-expressing cardiomyocytes from these mice were larger than littermate control cardiomyocytes. However, cardiomyocytes not expressing the transgene following chromosomal inactivation were notably smaller than control cardiomyocytes,1 raising the possibility that a negative regulator of cardiomyocyte growth may be induced, perhaps as a counter-regulatory response to the overall increase in heart size.

To identify possible inhibitors of cardiomyocyte growth, we performed transcript profiling of Akt-transgenic hearts in comparison to transgene-negative littermates.5 The transcript most highly upregulated in both lines (65- and 18-fold)5 was myostatin (MSTN), a highly conserved transforming growth factor (TGF)-β family member and potent negative regulator of skeletal muscle growth. Although expression of MSTN in the heart has been previously reported,6 a functional role for myostatin in the heart has not been appreciated.7 MSTN was not directly induced by Akt activation in cardiomyocytes,5 suggesting induction occurs as an indirect consequence, perhaps in response to the dramatic cardiac hypertrophy manifest in these mice.

Although targeted deletion of MSTN in mice produces impressive skeletal muscle hypertrophy and resistance to diabetes, the responsible signaling mechanisms have not been fully delineated. A detailed analysis of hearts from MSTN−/− mice has not been reported.

To explore the role of MSTN in cardiomyocyte growth, we examined the effects of cardiomyocyte expression of MSTN or the inhibitory pro-domain (dnMSTN) in vitro on the response to hypertrophic stimuli. We found that MSTN regulates cardiomyocyte growth in a stimulus-specific manner while inhibiting p38 and Akt phosphorylation. Studies in MSTN−/− mice suggest these findings have in vivo relevance as well. Together these data demonstrate that MSTN regu-

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lates not only skeletal but also cardiac muscle growth. The clinical relevance of these findings has recently been underscored by the discovery of MSTN mutations in people,\textsuperscript{8,9} as well as interest in inhibiting MSTN in skeletal muscle diseases.\textsuperscript{10}

**Materials and Methods**

**Recombinant Adenoviruses Expressing Full-Length and Truncated Forms of MSTN**

Mouse cDNA encoding MSTN and truncated forms of MSTN (dnMSTN) were prepared from total heart cDNA by PCR. Recombinant adenoviruses (Ad.MSTN and Ad.dnMSTN) expressing cyto-megalovirus (CMV)-driven green fluorescent protein (GFP) and MSTN or dnMSTN were generated by homologous recombination. Adenovirus expressing GFP (Ad.GFP), myristoylated Akt (Ad.myr-Akt), and dnAkt (Akt-AA) have been described previously.\textsuperscript{11} Inactive mutant (duaI phosphorylation site TGY changed to AGF) p38α (DNp38α) and activated MKK3b (MKK3bE) were kind gifts from Dr Yibin Wang (University of California, Los Angeles).\textsuperscript{12} Animals were handled in strict accordance to the guidelines of the Massachusetts General Hospital Subcommittee on Research Animal Care.

**Neonatal Cardiomyocytes**

Primary cultures of neonatal rat ventricular cardiomyocytes were prepared from Sprague–Dawley neonates as previously described.\textsuperscript{13}

**Mice**

MSTN\textsuperscript{+/+} mice\textsuperscript{7} were kindly provided by Dr Se Jin Lee (Johns Hopkins University, Baltimore, Md). Mice were backcrossed to C57B6 for ≥6 generations, and littermate controls were used in all data presented.

**Ex Vivo MSTN Assay**

Hearts from Akt transgenics and littermates were harvested and washed in PBS. Atria were removed and hearts were minced. Resulting tissue was incubated (48 hours, 37°C) in 3.5 mL of DMEM containing 10% FCS. Media were removed and centrifuged (2000g, 15 minutes), and supernatants were collected for analysis.

**Immunoblotting**

Cardiomyocyte proteins were lysed as described,\textsuperscript{13} after stimulation (30 minutes, 100 μmol/L phenylephrine [PE] [Sigma], 1 nmol/L leukemia inhibitory factor [LIF] [Chemicon], or 10 nmol/L insulin-like growth factor-I [IGF-I] [Chemicon]). SB239063 (10 μmol/L; Calbiochem) was added 1 hour before stimulation to inhibit p38. Protein from 8- to 12-week-old mouse hearts was obtained after atria removed. After concentration determination by the Bradford method (Bio-Rad), proteins (30 μg) were separated by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes (Bio-Rad) by semidyay transfer. Blots were incubated with anti-Akt, anti–phospho-Akt (473), anti–phospho-GSK3β(9/21), anti-Erk, anti–phospho-Erk, anti-Jnk, anti–phospho-Jnk, anti-p38, or anti–phospho-p38 (1:1000; Cell Signaling), overnight at 4°C and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; DAKO), and detected by chemiluminescence (Cell Signaling).

**Measurement of Protein Synthesis**

Media were changed to serum-free DMEM containing [3H]-leucine (1 μCi/mL), and cells were stimulated with PE (100 μmol/L; Sigma), LIF (1 nmol/L; Chemicon), or IGF-I (10 nmol/L; Chemicon) for 24 hours before washing with PBS, H_2O, harvesting in 0.25 N NaOH, and determining [3H]-leucine incorporation by scintillation counting.

**Cell Proliferation**

Media were changed to serum-free DMEM with or without PE (100 μmol/L, 24 hours) as indicated. Cells were washed with PBS, trypsinized, resuspended in fresh medium, and counted with a hemocytometer.

**Akt Kinase Assay**

Akt was immunoprecipitated from heart lysates with anti-Akt antibody and kinase activity was measured according to the manufacturer’s instructions (Cell Signaling).

**Echocardiography**

Echocardiography was performed on nonanesthetized mice using a 13L high-frequency linear (10 MHz) transducer (VingMed 5, GE Medical Services) with depth set at 1 cm and 236 frames per second for 2-D images. M-mode images used for measurements were taken at the papillary muscle level.

**Adult Cardiomyocyte Measurement**

Adult mouse CMs from MSTN\textsuperscript{+/+} and MSTN\textsuperscript{−/−} were isolated, fixed on slides, and measured as described previously.\textsuperscript{1}

**Acute Phenylephrine Infusion**

Under isoflurane anesthesia, 6- to 8-week-old mice were injected (via inferior vena cava) with 50 μL of 2 mmol/L PE dissolved in sterile 0.9% NaCl containing 0.02% ascorbic acid. After 3 minutes, mice were euthanized and hearts were excised, washed in cold PBS, and atria were removed and snap frozen in liquid nitrogen for analysis.

**Chronic Phenylephrine Infusion**

Under isoflurane anesthesia, Alzet microosmotic pumps (DURECT Corp, model no. 1002) were implanted subcutaneously in 6- to 8-week-old age-matched MSTN\textsuperscript{+/+} and MSTN\textsuperscript{−/−} mice dorsally to deliver PE for 14 days (75 mg/kg per day; dissolved in sterile 0.9% NaCl/0.02% ascorbic acid). Vehicle-treated, gender/age-matched mice served as controls.

**Statistics**

Data are represented as mean±SEM and compared by 2-tailed Student’s t test or ANOVA where appropriate. The null hypothesis was rejected for P<0.05.

An expanded Materials and Methods section can be found in the online data supplement, available at http://circres.ahajournals.org.

**Results**

Because commercially available antibodies failed to reliably detect MSTN protein by Western blotting in either heart or skeletal muscle, we established an ELISA sensitive to nanomolar concentrations of MSTN (data not shown). We used this ELISA to determine whether the observed transcriptional changes resulted in an increase in MSTN secretion from isolated hearts. We found a 3.1-fold increase in MSTN secretion from Akt transgenic hearts compared with littermate transgene-negative controls (P<0.001). Interrogation of a publicly available microarray database\textsuperscript{14} revealed that cardiac MSTN mRNA also increased after 2 weeks of intensive exercise (4-fold, P<0.004) and in mice with cardiac expression of constitutively active (but not dominant negative) phosphoinositide 3-kinase (PI3K) (2.3-fold, P<0.04), the upstream activator of Akt. Thus, MSTN expression is dynamically regulated in the heart, especially under hypertrophic conditions that would be expected to activate Akt.

To examine the biological effects of MSTN in cardiomyocytes, we generated recombinant adenoviral vectors encoding
either full-length MSTN (Ad.MSTN) or the amino-terminal propeptide previously demonstrated to inhibit the actions of MSTN\(^{15}\) (Ad.dnMSTN). Both viruses mediated expression of the appropriate transgene in cardiomyocytes (data not shown).

To model the hypertrophic response, we stimulated cardiomyocytes in vitro with the \(\alpha_1\)-adrenergic agonist PE.\(^{16}\) After PE stimulation, both cell size and protein synthesis increased in control virus-infected cardiomyocytes (Figure 1A, 1C, and 1D). Whereas MSTN expression did not alter size or protein synthesis in unstimulated cardiomyocytes, it completely abrogated the increase in both seen after PE stimulation (Figure 1A, 1C, and 1D). Coexpression of dnMSTN in MSTN-infected cells restored the growth response to PE (Figure 1A, last panel), suggesting dnMSTN was able to antagonize the effects of the expressed MSTN. Moreover, in unstimulated cardiomyocytes, dnMSTN expression increased both cell size and protein synthesis to levels comparable to that seen after PE stimulation (Figure 1B through 1D). dnMSTN also enhanced sarcomere prominence (Figure 1B), another characteristic of cardiomyocyte hypertrophy. Neither MSTN nor dnMSTN altered cardiomyocyte number 24 hours after treatment, confirming that changes in protein synthesis were caused by cell growth rather than proliferation (Figure 1E).

Although MSTN has not been postulated to act through Akt signaling, our observations in transgenic Akt mice suggested this might be the case. In chimeric Akt hearts, cardiomyocytes not expressing the transgene were smaller than those from nontransgenic littermates, whereas cardiomyocytes expressing myr-Akt were comparable to those from nonchimeric transgenic mice,\(^{1}\) suggesting Akt may lie downstream of the effects of MSTN. We examined this hypothesis in neonatal cardiomyocytes. Control cells treated with PE manifested a dramatic inhibition of PE-mediated cardiomyocyte hypertrophy. In contrast, MSTN-expressing cardiomyocytes manifested a dramatic inhibition of PE-stimulated phosphorylation of both Akt (\(P<0.001\)) and glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)), a downstream target relevant to hypertrophy.\(^{17,18}\) In contrast, MSTN-expressing cardiomyocytes revealed enhanced sarcomere organization in dnMSTN-expressing cardiomyocytes. C, Cell size. Cell size in cardiomyocytes transduced and stimulated as above was quantified from digital micrographs analyzed with NIH Image (\(* * * P<0.001\) vs unstimulated, Ad.GFP transduced; \(### P<0.001\) vs PE stimulated, Ad.GFP transduced). Cumulative data shown from 7 independent experiments with 250 to 750 cells quantitated for each condition from N independent observations: GFP (\(N=7\)); MSTN + PE (\(N=3\)); MSTN + PE (\(N=4\)); dnMSTN (\(N=5\)); D, Protein synthesis. Protein synthesis was measured by \(^{3}H\)-leucine incorporation in cells treated as above and normalized to unstimulated controls (\(* * P<0.01\), \(* P<0.05\) vs unstimulated, Ad.GFP infected, unstimulated; \(### P<0.001\) vs Ad.GFP transduced, PE stimulated). Cumulative data from 7 independent experiments shown with each condition tested N times: GFP (\(N=11\)); GFP + PE (\(N=11\)); MSTN (\(N=9\)); MSTN + PE (\(N=9\)); dnMSTN (\(N=4\)). E, Cell proliferation. Cell number was quantitated in 4 independent experiments for each of the treatment conditions at the same time point used for cell size and protein synthesis.

![Figure 1. MSTN inhibits PE-mediated cardiomyocyte hypertrophy. A, Confocal microscopy. Cardiomyocytes transduced (multiplicities of infection [MOI]=100) with Ad.GFP, Ad.MSTN, or Ad.MSTN + Ad.dnMSTN were PE stimulated (100 \(\mu\)mol/L, 24 hours) where indicated before fixation, staining with rhodamine-phalloidin, and examination by \(\times 40\) laser-scanning confocal microscopy in at least 6 independent experiments for each condition. B, Confocal microscopy. Laser-scanning confocal microscopy (\(\times 80\)) of cardiomyocytes transduced with Ad.GFP or Ad.dnMSTN reveals enhanced sarcomere organization in dnMSTN-expressing cardiomyocytes. C, Cell size. Cell size in cardiomyocytes transduced and stimulated as above was quantified from digital micrographs analyzed with NIH Image (\(* * * P<0.001\) vs unstimulated, Ad.GFP transduced; \(### P<0.001\) vs PE stimulated, Ad.GFP transduced). Cumulative data shown from 7 independent experiments with 250 to 750 cells quantitated for each condition from N independent observations: GFP (\(N=7\)); MSTN + PE (\(N=3\)); MSTN + PE (\(N=4\)); dnMSTN (\(N=5\)); D, Protein synthesis. Protein synthesis was measured by \(^{3}H\)-leucine incorporation in cells treated as above and normalized to unstimulated controls (\(* * P<0.01\), \(* P<0.05\) vs unstimulated, Ad.GFP infected, unstimulated; \(### P<0.001\) vs Ad.GFP transduced, PE stimulated). Cumulative data from 7 independent experiments shown with each condition tested N times: GFP (\(N=11\)); GFP + PE (\(N=11\)); MSTN (\(N=9\)); MSTN + PE (\(N=9\)); dnMSTN (\(N=4\)). E, Cell proliferation. Cell number was quantitated in 4 independent experiments for each of the treatment conditions at the same time point used for cell size and protein synthesis.]
protein synthesis and cell size (Figure 3A and 3B; \( P < 0.001 \)). Thus, Akt activation appears necessary for PE-induced cardiomyocyte hypertrophy, and MSTN inhibition of Akt could contribute to its inhibition of the growth response to PE.

To test this, we coexpressed either activated or dnAkt with MSTN or dnMSTN expression, respectively. Expression of activated Akt substantially restored PE-stimulated cell growth in MSTN-expressing cardiomyocytes (Figure 3C; \( P < 0.001 \) versus GFP and MSTN+PE). Conversely, Akt inhibition in dnMSTN-expressing cardiomyocytes inhibited the cell growth seen with dnMSTN alone (Figure 3C; \( P < 0.001 \) versus dnMSTN; \( P = \text{NS} \) versus GFP). Together these data demonstrate that MSTN modulates cardiomyocyte growth in vitro, at least in part, through inhibition of Akt activation.

To further explore the mechanisms involved, we stimulated cardiomyocytes with LIF and IGF-I, hypertrophic agonists that activate Akt through distinct mechanisms, and examined the ability of MSTN to inhibit hypertrophy and Akt activation. Although the increase in protein synthesis induced by LIF (\( P < 0.05 \)) appeared slightly reduced by MSTN infection, this was not statistically significant compared with LIF-stimulated, GFP-infected cardiomyocytes (Figure 4A). Similarly, MSTN expression appeared to slightly but not significantly reduce LIF-induced Akt phosphorylation (Figure 4C). In contrast, MSTN did not inhibit IGF-I-induced protein synthesis at all (Figure 4B), which remained significantly increased above baseline (\( P < 0.01 \)). Similarly, MSTN did not inhibit IGF-I-induced Akt phosphorylation (Figure 4D; \( P < 0.01 \)). Thus, MSTN-mediated inhibition of Akt paralleled its effects on hypertrophy and appears to be stimulus specific, presumably because of differences in the upstream pathways mediating Akt activation after PE as compared with LIF or IGF-I.

We next examined the effects of MSTN on activation of mitogen-activated protein (MAP) kinases (extracellular signal-regulated kinase [Erk], c-Jun N-terminal kinase [JNK], and p38) both because of crosstalk between these pathways and Akt\(^{20,21} \) as well as their independent regulation of cardiomyocyte growth.\(^{22-24} \) The pattern of Erk phosphorylation in response to PE, LIF, and IGF-I was not significantly altered by MSTN expression (Figure 5A). None of the agonists induced substantial JNK phosphorylation (Figure 5B). JNK phosphorylation appeared modestly suppressed in MSTN-infected cardiomyocytes, although neither this difference nor the baseline induction of JNK phosphorylation was statistically significant (Figure 5B). In contrast, PE induced significant phosphorylation of p38 (Figure 5C; \( P < 0.01 \)), which was completely inhibited by MSTN expression. LIF-mediated p38 phosphorylation was only partially attenuated by MSTN. IGF-I did not increase p38 phosphorylation, and MSTN expression did not alter this. These data demonstrate that, in addition to inhibiting Akt activation, MSTN inhibits PE-mediated p38 phosphorylation. Consistent with this, MSTN also inhibited PE-induced atrial natriuretic factor
stream of MKK and not directly on p38 or Akt (Figure 6B). dnMSTN infection alone did not induce p38 phosphorylation. This could reflect the involvement of other pathways in the effects MSTN or interaction of dnMSTN with other TGF-β family members. In addition, it seems likely that p38 phosphorylation seen in whole-cell lysates is an imperfect reflection of the specific subcellular p38 that may be important in these signaling events.

To see whether MSTN regulates cardiomyocyte growth in vivo, we examined hearts from MSTN−/− mice.7 Surprisingly, hearts from 8-week-old male MSTN−/− mice were modestly (9.6%) smaller than those from wild-type littermates, although this difference did not achieve statistical significance (P < 0.06). Cardiomyocytes from MSTN−/− mice were significantly smaller than those from wild-type littermates (P < 0.001). Baseline cardiac function and chamber dimensions as assessed by echocardiography in 8- to 10-week-old mice were not different in MSTN−/− mice compared with littermates (Table). To determine whether the in vivo response to PE mirrored the changes observed in vitro, we infused PE via miniosmotic pump for 2 weeks. Given the dramatic effect of MSTN genotype on body habitus and composition, we compared the heart weight in each genotype to age-matched, vehicle-treated controls at the end of this period. MSTN-deficient mice exhibited greater cardiac growth in response to PE infusion (Figure 7A; P < 0.05). Interestingly, this difference was most dramatic for male MSTN−/− mice (14% versus 2.4%) (Figure 7A; P < 0.05 versus wild-type littermates). After PE infusion, male MSTN−/− hearts were comparable in weight to age-matched wild-type littermates (124.0 ± 5.6 versus 123.1 ± 6.8 mg) and significantly larger than vehicle-treated, age-matched MSTN−/− controls (108.8 ± 4.0 mg). PE did not induce substantial cardiac growth in female mice independent of genotype (Figure 7A; P = NS).

Whereas there was no difference in baseline Akt expression or activity in MSTN−/− hearts (data not shown), acute infusion of PE resulted in greater Akt activation in hearts from MSTN−/− mice (Figure 7B; 5.4 ± 1.6-fold; P < 0.04). This Akt activation was accompanied by an increase in PE-stimulated p38 phosphorylation (Figure 7C; 4.5 ± 1.0-fold; P < 0.05). Together, these data suggest that endogenous MSTN also plays a role modulating cardiac p38 phosphorylation, Akt activation, and growth in vivo.

Discussion

We found that MSTN, a potent negative regulator of skeletal muscle growth, is dynamically regulated in the heart and regulates cardiomyocyte growth in vitro. These data suggest that MSTN may play a broader role than previously appreciated, regulating growth of striated muscle cells of both skeletal and cardiac lineages. Moreover, the unanticipated finding that MSTN modulates activity of Akt, a critical determinant of cell size in many systems, as well as p38 phosphorylation, may have implications for our understanding of the actions of MSTN in other settings.

The observation that cardiomyocytes in chimeric Akt-transgenic hearts that did not express the transgene were smaller than wild-type cardiomyocytes and that MSTN was

(ANF) expression, known to be increased by PE and p3822 but not by Akt (Figure 5D; P < 0.01).

To examine whether MSTN-mediated inhibition of p38 was connected to its inhibition of Akt, we treated cardiomyocytes with 10 μmol/L SB239063, a p38-specific inhibitor. We found that treatment with SB significantly inhibited PE-induced Akt phosphorylation in GFP- or MSTN-infected cardiomyocytes (P < 0.01 versus GFP+PE; Figure 6A). Moreover, adenoviral expression of DNP38c blocked PE-induced phosphorylation of both p38 and Akt (Figure 6B). Conversely, p38 activation with constitutively activated MKK3bE induced Akt phosphorylation, even in MSTN-expressing cardiomyocytes, suggesting that MSTN acts up-

Figure 3. Role of Akt signaling in PE and MSTN effects. A, PE-induced protein synthesis. Protein synthesis was measured by [3H]-leucine incorporation as above and normalized to maximal (100%) PE-stimulated incorporation (**P<0.001 vs Ad.GFP; ###P<0.001 vs Ad.GFP PE-stimulated). Cumulative data from 11 (GFP±PE) or 4 (dnAkt±PE) independent experiments are shown. B, PE-induced cell size. Cell size was measured as above (**P<0.001 vs unstimulated Ad.GFP, ###P<0.001 vs Ad.GFP PE stimulated). Cumulative data from 2 independent experiments with 183 to 221 cells measured for each condition shown. C, MSTN-inhibited cell size. Cell size was quantitated in cardiomyocytes treated as above (**P<0.001, +++P<0.001, ###P<0.001). Cumulative results from 7 independent experiments are shown.
induced in these hearts, raised the possibility that MSTN regulates cardiomyocyte growth through Akt. Induction of MSTN mRNA in transgenic Akt mice was confirmed by quantitative RT-PCR, and an increase in cardiac secretion was documented by ELISA. Interrogation of a publicly available microarray database revealed that cardiac MSTN mRNA is also increased by 2 weeks of intensive exercise and in mice with cardiac activation of PI3K, the upstream activator of Akt. Nevertheless, whether the reduction in nontransgenic cardiomyocyte size seen in chimeric Akt hearts can be fully attributed to MSTN is currently unknown.

The effects of MSTN expression on cardiomyocyte growth were examined first in vitro. MSTN expression completely suppressed the increase in cell size and protein synthesis induced by the α-adrenergic agonist PE. Conversely, expression of the inhibitory propeptide dnMSTN increased cardiomyocyte size and protein synthesis to a degree comparable to that seen with PE stimulation alone. These effects correlated with modulation of Akt activation as well as downstream target phosphorylation (GSK3β) and the functional importance of this was demonstrated by the ability of appropriate activating or inhibitory Akt constructs to significantly reverse the effects of MSTN or dnMSTN expression, respectively. Thus we suggest a previously unappreciated mechanism by which MSTN regulates cell size is through modulation of Akt activity. Although these observations were initially made in cardiomyocytes, they likely also apply to skeletal muscle, where baseline MSTN expression is higher than in heart.

Interestingly, MSTN did not suppress cardiomyocyte growth or Akt phosphorylation induced by LIF or IGF-I. These observations suggested MSTN modulates PE-induced cardiomyocyte growth through effects on upstream pathways different from those used by LIF or IGF-I. An examination of MAP kinase signaling revealed that MSTN also inhibited p38 phosphorylation. Previous work in other cell types has demonstrated p38 can function upstream of Akt, More-
over, p38 activation is both necessary and sufficient for PE-induced cardiomyocyte hypertrophy, whereas LIF-mediated hypertrophy depends less on p38 activation. IGF-I does not activate p38 and activates Akt through p38-independent mechanisms. In cardiomyocytes, p38 inhibition with a pharmacological inhibitor or DNp38 blocked PE-induced Akt phosphorylation. Expression of activated MKK3b, which preferentially activates p38α, rescued Akt phosphorylation in PE-stimulated, MSTN-expressing cardiomyocytes. Although causality is difficult to definitively establish, taken together these data suggest that MSTN inhibits PE-induced cardiomyocyte growth and Akt phosphorylation by inhibiting p38α activation upstream of
appears to be prohypertrophic,22,28 whereas the in vivo growth response thus reinforcing our overall model.

Importantly, studies in MSTN knockout mice revealed en-
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tion after dnMSTN expression. First, this may
possible that dnMSTN interacts with other homologous
members of the TGF-β superfamily, 1 of which was recently
reported to modulate cardiac Akt signaling,26 or other activin
type II receptor ligands postulated to regulate cell growth in
conjunction with MSTN.27 Thus additional effects of
MSTN may obscure effects on p38. For all of these
reasons, we chose to focus additional efforts on the MSTN
knockout model, which is not confounded in these ways.
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The role of p38 in cardiomyocyte hypertrophy is complex.
In vitro activation of p38 in neonatal rat cardiomyocytes appears to be prohypertrophic,22,28 whereas the in vivo situation is less clear. Transgenic overexpression of Dnp38α and DNMKK3b result in cardiac hypertrophy,29 suggesting an antihypertrophic role for p38 in vivo. However, cardiac overexpression of TGF-β-activated kinase-1 (TAK1) induces p38 activation and cardiac hypertrophy,30 and expression of

Figure 7. MSTN−/− mice. A, Heart weight. PE (75 mg/kg per
day) was infused for 14 days in MSTN−/− and MSTN−/− mice
before the animals were euthanized and heart weight was deter-
mined. Percentage increase in heart weight after PE infusion is
shown compared with age- and genotype-matched controls
receiving vehicle only. Number of mice studied: MSTN+/+
(N=27); MSTN−/− (N=21); male MSTN+/+ (N=18); male MSTN−/−
(N=12); female MSTN+/+ (N=9); female MSTN−/−
(P<0.04 vs unstimulated MSTN−/−); #P<0.05 vs unstimulated,
male MSTN−/−). B, Akt activation. Three minutes after PE injec-
tion, hearts from male MSTN−/− and MSTN−/− littermates were
harvested. Representative immunoblots and cumulative quantifi-
cation from Akt kinase assays (whole-heart lysates) are shown
(N=8 mice; #P<0.04 vs MSTN+/+). C, p38 phosphorylation.
Whole-heart lysates from male mice treated as above immuno-
blotted with anti-phospho-p38 antibody. Representative immu-
noblot and cumulative quantitative data from 6 mice shown
(P<0.05 vs MSTN+/+).

MAP kinase phosphatase-1 (MKP-1) reduces p38 activity while mitigating hypertrophy after pressure overload.31 Thus the functional role of p38 in cardiac hypertrophy may be context dependent, consistent with the observation that

MKK3. Nevertheless, we recognize that other pathways
likely also contribute.

There are several possible explanations for the lack of p38
phosphorylation after dnMSTN expression. First, this may
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Finally, although dnMSTN effectively inhibits MSTN, it is
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It remains unclear why 8 week-old male MSTN−/− hearts were slightly but paradoxically smaller at baseline. However, a comparable reduction in heart size was also seen in another line of MSTN−/− mice. This could reflect a distinct role for MSTN during development or secondary effects of systemic MSTN deletion. Similarly, in vivo inactivation of modulatory calcineurin-interacting protein (MCIP), which inhibits cardiomyocyte hypertrophy, results in smaller hearts at baseline.

Interestingly, we found that male MSTN−/− mice had enhanced cardiac growth after PE infusion in vivo, whereas females did not. This is consistent with prior work demonstrating that α₁-adrenergic signaling is critical for physiological cardiac growth in male but not female mice. Moreover, in a model of adrenergic-induced cardiac hypertrophy and death, female mice were relatively resistant, apparently because of an estrogen-inducible increase in MAP kinase phosphatase-1 (MKP-1) counteracting adrenergic-mediated phosphorylation of p38. Thus, the lack of an effect in female MSTN−/− could reflect gender-specific modulation of p38 as well as its involvement in both MSTN and adrenergic signaling. In addition, it is also possible that MSTN has p38-independent, gender-specific effects. Of note, muscle-specific transgenic MSTN overexpression decreases muscle mass in male but not female mice, and MSTN has been reported to inhibit expression of ARA-70, an androgen receptor cofactor.

These findings may have clinical implications for ongoing efforts to inhibit MSTN in skeletal muscle diseases or as an unapproved means for enhanced athletic performance. The experiments described here do not accurately model inhibitory binding proteins of myostatin in normal serum. It remains unclear why 8 week-old male MSTN−/− hearts were slightly but paradoxically smaller at baseline. However, a comparable reduction in heart size was also seen in another line of MSTN−/− mice. This could reflect a distinct role for MSTN during development or secondary effects of systemic MSTN deletion. Similarly, in vivo inactivation of modulatory calcineurin-interacting protein (MCIP), which inhibits cardiomyocyte hypertrophy, results in smaller hearts at baseline.

In summary, we have found that MSTN, a potent negative regulator of skeletal muscle growth, is dynamically regulated in the heart and acts to modulate cardiomyocyte growth in a stimulus-specific manner. Inhibition by MSTN of the growth response to the α₁-adrenergic stimulus, PE, appears mediated through inhibition of p38α and Akt. Together these studies suggest MSTN acts more broadly than initially postulated to regulate growth of cardiac, as well as skeletal, striated muscle, which may have implications for the design of clinical trials with MSTN inhibitors. Moreover, MSTN modulation of Akt and p38 signaling could provide insight into its mechanism of action in other systems.

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Disclosures
None.

References


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a. Expanded Materials And Methods

Generation of Recombinant Adenoviruses Expressing Full-Length and Truncated Forms of MSTN

Forms of MSTN — Mouse cDNA encoding MSTN and truncated forms of MSTN (dnMSTN) were prepared from total heart cDNA by PCR (MSTN, forward primer: AATGATGCAAAAAACTGCAAATGTATG, reverse primer: GAAGACCTTCCATGACTTGGGAAG; dnMSTN, forward primer: AATGATGCAAAAAACTGCAAATGTATG, reverse primer: GTCAAGGTGACAGACACACACACCAAGTG). Amplicons were subcloned to pCR2.1-TOPO (Invitrogen) and directly sequenced. Recombinant adenoviruses (Ad.MSTN and Ad.dnMSTN) expressing CMV-driven green fluorescent protein (GFP) and MSTN or dnMSTN, under the control of a separate CMV cassette, were generated by homologous recombination. Adenovirus expressing GFP with β-galactosidase (Ad.GFP), myrisoylated Akt (Ad.myr-Akt), and dn-Akt (Akt-AA) have been described previously1.

Preparation of Neonatal Cardiomyocytes — Primary cultures of neonatal rat ventricular Cardiomyocytes were prepared from the cardiac ventricles of Sprague-Dawley neonates as previously described2.

Neonatal Cardiomyocyte Infection — To study the effects of transient transgene expression, myocytes were transduced with adenoviral vectors at a MOI of 50-200 for 24 h in DMEM containing 10% FBS and subsequently serum-starved in DMEM for 16 h prior to experimentation.

MSTN Enzyme Linked Immunoassay (ELISA) — Capture antibody (bovine anti-C-terminal human MSTN, 4 µg/ml; Biovendor) was incubated (1 h, 20 °C) in binding
buffer (0.2 M sodium carbonate-bicarbonate, pH 9.4) in black 96-well plates (Maxisorp; Nunc). Wells were washed (3 × 150 µl) with wash buffer (TBS, 0.05% Tween20) and incubated (3 × 3 min) with 5% BSA (w/v) in PBS. A standard curve was created using serial dilutions of C-terminal human MSTN (Biovendor) in 0.5% BSA (w/v) in PBS. Standards and samples (100 µl) were incubated (1 h, 20 °C) in the pre-coated wells. Wells were washed (3 × 150 µl) with wash buffer and incubated (1 h, 20 °C) with primary antibody (rabbit anti-myostatin-C-terminus peptide B, 0.125 ng/ml; Bethyl laboratories) in TBS superblock (Pierce) containing 0.05% Tween20. Wells were washed (3 × 150 µl) with wash buffer and incubated (1 h, 20 °C) with HRP-linked anti-rabbit antibody (1:4000; Dako) in TBS superblock containing 0.05% Tween20. Wells were washed (3 × 150 µl) with wash buffer and incubated (30 min, 20 °C) with Quantablu (Pierce) and fluorescence emission determined (λ = 325).

**Neonatal Cardiomyocyte Size Analysis** — Cardiomyocytes were isolated and subsequently purified on pre-formed Percoll density gradients as previously described³. Briefly, following isolation, cardiomyocytes were resuspended in high density Percoll (1.12 g/ml) and underlayed to an equal volume (15 ml) of low density Percoll (1.06 g/ml). Myocytes were centrifuged on this discontinuous Percoll gradient (45 min, 1,800 × g) after which non-myocytes had migrated to the surface of the upper layer of Percoll and cardiomyocytes had migrated to the interface of the discontinuous gradient. Cardiomyocytes were recovered, washed × 2 in 30 ml plating medium (DMEM:M199 4:1 containing 10% HS, 5%FCS) and plated at 310 cells/mm² in 35 mm dishes.

Following experimental conditions cells were fixed in 3.7% formaldehyde (10 min, 20 °C) washed in PBS (2 × 3 ml) and visualized by phase-contrast microscopy. Digital
photographs were taken from 6 representative fields (~120 myocytes/condition) and myocyte surface area was determined using NIH image.

**Confocal Microscopy** — Cardiomyocytes were prepared and purified by Percoll density centrifugation as described. Following experimental conditions cells were fixed in 3.7% formaldehyde (10 min, 20°C) washed in PBS (3 × 4 ml) and stained with rhodamine-phalloidin (1:100 in PBS, 10 min; Molecular Probes). In some experiments nuclei were counterstained (5 min) with propidium iodide (10 µg/ml) in PBS containing RNase (0.5 mg/ml; Qiagen). Sarcomere organization and nuclei were visualized by laser-scanning confocal microscopy (Biorad).

**ANF expression** — Neonatal cardiomyocytes were homogenized in TRI reagent (Sigma) and total RNA was prepared according to the manufacturer's instructions. Quantitative PCR for rat ANF was performed using sense (5'-CGGACTAGGCTGCAACAGCT-3') and antisense (5'-CCAGGAGGGTATTCACCACCT-3') oligonucleotides and an ANF probe (FAM-CGGTACCGAAGATAACAGCCAAATCTGCT-TAMRA-3'). Results were normalized to rodent GAPDH control kit (Applied Biosciences) according to the manufacturer's recommendations.

**Measurement of heart weight** — Hearts were excised, rinsed in cold PBS, blotted dry, and weighed after removal of atria.
References:

