Myostatin inhibits IGF-I-induced myotube hypertrophy through Akt

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Myostatin inhibits IGF-I-induced myotube hypertrophy through Akt. Am J Physiol Cell Physiol 297: C1124–C1132, 2009. First published September 16, 2009; doi:10.1152/ajpcell.00043.2009.—Myostatin, also known as growth differentiation factor-8 (GDF-8), is a member of the tumor growth factor-β/TGF-β superfamily that negatively regulates skeletal muscle growth. Loss of functional myostatin in cattle, mice, sheep, dogs, and humans results in increased muscle mass. The molecular mechanisms responsible for this increase in muscle growth are not fully understood. Previously, we have reported that phenylephrine-induced cardiac muscle growth and Akt activation are enhanced in myostatin knockout mice compared with controls. Here we report that skeletal muscle from myostatin knockout mice show increased Akt protein expression and overall activity at baseline secondary to an increase in Akt mRNA. We examined the functional role of myostatin modulation of Akt in C2C12 myotubes, a well-established in vitro model of skeletal muscle hypertrophy. Adenoviral overexpression of myostatin attenuated the insulin-like growth factor-I (IGF-I)-mediated increase in myotube diameter, as well as IGF-I-stimulated Akt phosphorylation. Inhibition of myostatin by overexpression of the NH2-terminal portion of myostatin was sufficient to increase myotube diameter and Akt phosphorylation. Coexpression of myostatin and constitutively active Akt (myr-Akt) restored the increase in myotube diameter. Conversely, expression of dominant negative Akt (dn-Akt) with the inhibitory myostatin propeptide blocked the increase in myotube diameter. Of note, ribosomal protein S6 phosphorylation and atrogin-1/muscle atrophy F box mRNA were increased in skeletal muscle from myostatin knockout mice. Together, these data suggest myostatin regulates muscle growth at least in part through regulation of Akt signaling mechanisms responsible for MSTN’s ability to regulate skeletal muscle hypertrophy are not well understood. C2C12 cells, a myoblast cell line derived from murine satellite cells, have been used extensively as an in vitro model to study both muscle differentiation and hypertrophy. The withdrawal of serum from C2C12 myoblasts causes them to exit the cell cycle and fuse into myotubes. Differentiated myotubes exhibit a hypertrophic, as opposed to a hyperplastic, response to growth factors, such as insulin-like growth factor-I (IGF-I), characterized by an increase in myotube diameter and protein synthesis (13, 26, 27). Whereas these distinct processes may be interrelated, hypertrophy, or a growth in cell size may be more relevant for postnatal modulation of skeletal muscle.

C2C12 myotubes have been used as an in vitro model used to study IGF-I-mediated hypertrophic signaling pathways in skeletal muscle. Phosphatidylinositol 3 (PI3)-kinase/Akt/mammalian target of rapamycin (mTOR) activation downstream of IGF-I has been shown to induce hypertrophy both in C2C12 cells in vitro (26) as well as skeletal muscle in vivo (2). This cell line has also been used to model skeletal muscle atrophy in vitro. Dexamethasone-mediated decreases in myotube diameter and protein synthesis can be blocked by IGF-I (31). Akt, activated downstream of IGF-I, inhibits induction of atrogin-1/muscle atrophy F box (MAFbx) and muscle RING-finger protein 1 (MuRF1) ubiquitin-ligases by forkhead transcription factor FOXO1, thereby preventing muscle atrophy (31). Additionally, a decrease in Akt phosphorylation was seen in myotubes after the addition of recombinant MSTN protein (18). Thus C2C12 cells provide a useful, well-characterized, in vitro model system to examine the effects of MSTN on inhibition of hypertrophy and/or induction of atrophy in skeletal muscle.

Previously, we have shown that MSTN inhibits phenylephrine (PE)-mediated hypertrophy in primary cardiomyocytes in vitro through inhibition of Akt (22). In vivo we found increased Akt activation and hypertrophy in response to PE stimulation in the hearts of MSTN knockout mice compared with controls. To test the hypothesis that Akt may play a role in skeletal muscle hypertrophy as well, we examined whether Akt was altered in these mice and used C2C12 myotubes to determine whether MSTN-mediated regulation of Akt signaling is functionally important for hypertrophy. Here we show a role for Akt in regulating MSTN-mediated inhibition of skeletal muscle hypertrophy.

METHODS

Mice. All animal studies were carried out in accordance with approved IACUC protocols at BIDMC. MSTN null mice (19) were kindly provided by Dr. Se Jin Lee (Johns Hopkins University). Mice were backcrossed to C57Bl6 for ≥9 generations and littermate con-
trols were used in all data presented. Mice aged 8–20 wk old were used for these studies.

Recombinant adenoviruses. Construction of adenoviruses expressing cytochrome c-driven green fluorescent protein (GFP) and myostatin (MSTN), the inhibitory MSTN propeptide, which we term dominant negative MSTN (dnMSTN) (22), constitutively activated (myristoylated) Akt (Akt), and dominant negative (AA mutant) Akt (dnAkt) were described previously (24). Note that all constructs express GFP, which was used to confirm similar expression levels and permit visualization of transduced myotubes for measurement.

Cell culture. C2C12 myoblasts (American Type Culture Collection, Rockville, MD) were grown to confluence in growth media (DMEM/15% FBS) on 60-mm tissue culture dishes and then switched to differentiation media (DMEM/2% HS) on day 0 (D0). Myotubes were infected with adenoviral constructs 48 h after being switched to differentiation media (day 2, D2), and stimulated with IGF-I (10 ng/ml, "Long-R3-IGF-I,” Sigma, St. Louis, MO) after an additional 24 h (day 3, D3). Myotubes were harvested for biochemical analysis or measured on day 4 (D4), 24 h after the addition of IGF-I (D4). Myotubes harvested for biochemical analysis on D4 were switched to serum-free DMEM for 2 h before 30-min stimulation with IGF-I, as indicated.

Myotube diameter measurements. Microscopic images of live GFP-expressing myotubes (D4) were captured using a digital camera mounted on a Leica DM IRB microscope. Myocyte diameter measurements were obtained using NIH Image software. Three short-axis measurements were taken along the length of a given myotube and averaged. At least five myotubes per plate were measured and replicated in three-to eight independent experiments.

Western blot analysis. Heart and skeletal muscle tissue from mice were harvested, rinsed in cold PBS, snap frozen in liquid nitrogen, and homogenized in lysis buffer (Cell Signalling, Beverly, MA) plus 5 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 20,000 g for 10 min at 4°C. Protein concentration was determined by Bradford Assay (Bio-Rad, Hercules, CA), and equal amounts of protein (~100 μg) were loaded in each lane of a 4–15% precast Tris-glycine/sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad). Proteins were separated by electrophoresis, transferred (semidyry, Bio-Rad) to nitrocellulose membranes, and incubated overnight at 4°C with indicated antibody diluted (1:1,000) in 5% nonfat powdered milk/Tris-buffered saline/0.1% Tween. C2C12 myotubes were harvested on ice by scraping in lysis buffer and further processed as tissue samples. Antibodies were the following: anti-Akt, -phospho Akt (p473), -GSK3β, -phospho-S6, -S6, -p70S6K, -phospho-p70S6K (t389, #4225/4242) (Cell Signaling, Beverly, MA), GAPDH (Abcam, Cambridge, MA).

Akt kinase assay. Akt was immunoprecipitated from quadriceps lysates (400 μg) and incubated with substrate, and kinase activity was measured using an anti-phospho-GSK3α/β antibody to detect phosphorylation of a GSK-peptide (Akt substrate) by Western blot analysis, according to the manufacturer’s instructions (Cell Signaling).

Real-time PCR. Samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions. RNA was quantified using a Nanodrop spectrophotometer, and the quality of the RNA was confirmed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). One-step SYBR PCR kit (Stratagene, La Jolla, CA) with predesigned, validated TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) to quantify gene expression of Akt, MuRF-1, atrogin-1, and GAPDH. Analysis of change in gene expression was determined using the 2−ΔΔCt method (17), and a Student’s t-test was performed on the normalized threshold cycle (Ct) values (ΔCt) for each group as this value demonstrated the most normally distributed dataset as determined by a Shapiro-Wilk test compared with the transformed C (2−1/2 ΔCt).

Statistics. Data are represented as means ± SE and compared by two-tailed Student’s t-test or ANOVA with Dunnett or Tukey post hoc analysis as appropriate using GraphPad Prism 4 software. The null hypothesis was rejected for P < 0.05.

RESULTS

Akt protein and activity are increased in skeletal muscle. We have previously reported that MSTN inhibits cardiomyocyte hypertrophy by inhibiting p38 and Akt activation in a stimulus-specific manner without altering total Akt protein (22). To determine whether MSTN regulates Akt in skeletal muscle we examined Akt in skeletal muscle from MSTN-null mice (knockout, KO) (Fig. 1A). In contrast to heart muscle, total Akt expression was increased 2.8-fold in the quadriceps from KO mice (Fig. 1B) (n = 16, P < 0.001 vs. wild type, WT). Additionally, we found a approximately fourfold increase in baseline phosphorylation of Akt (Fig. 1B, n = 4, P < 0.05) that correlated with an overall increase in Akt kinase activity in the quadriceps muscle of KO mice (Fig. 1C, n = 4, P < 0.05). Interestingly, this increase in Akt activity appears predominately due to an increase in protein levels of Akt rather than a change in the percent phosphorylated/total Akt. To further examine the mechanism responsible for the increase in Akt protein, we examined Akt mRNA and found a twofold increase in Akt mRNA in KO compared with WT quadriceps (P < 0.02), suggesting that MSTN deletion regulates Akt expression at the transcript level. We found no increase in total or phosphorylated p38 (data not shown).

MSTN regulates Akt phosphorylation in myotubes. To determine whether MSTN inhibits Akt in myotubes, C2C12 myoblasts were grown to confluence in growth media (DMEM + 15% FBS) and then switched to differentiation media (DMEM + 2% HS) on D0. Fully differentiated myotubes were infected (D2) with adenoviral constructs expressing GFP only, MSTN, or dnMSTN, the NH2-terminal peptide of MSTN that has been previously shown to inhibit MSTN (7). Stimulation of GFP-infected myotubes for 30 min with IGF-I on D4 induced a 6.7-fold increase in Akt phosphorylation (n = 8, P < 0.001) that was blocked by MSTN expression (Fig. 2, n = 8, P < 0.001). dnMSTN overexpression was sufficient to increase Akt phosphorylation by 3.6-fold versus GFP (Fig. 2, n = 8, P < 0.05). Overexpression of membrane-targeted Akt (Akt) resulted in similar Akt phosphorylation (8.6-fold) compared with GFP (Fig. 2, n = 8, P < 0.001) that was blocked by MSTN expression (Fig. 2, n = 8, P < 0.05). Coexpression of MSTN and Akt with (n = 4) or without IGF-I (n = 3) did not significantly change Akt phosphorylation levels compared with GFP + IGF-I or Akt alone. No change in total Akt protein was observed with either MSTN or dnMSTN infection in myotubes.

MSTN regulates Akt activity in myotubes. To confirm that changes in Akt phosphorylation mediated by MSTN and dnMSTN reflect actual Akt activity, we performed direct measurements of kinase activity. IGF-I increased Akt kinase activity 6.3-fold compared with GFP-infected control (n = 3, P < 0.001), and MSTN infection completely blocked activation (Fig. 3, n = 3, P < 0.001), whereas dnMSTN increased activity by 3.6-fold compared with GFP-infected myotubes (Fig. 3, n = 3, P < 0.05).
phy, we examined the ability of MSTN to inhibit IGF-I-mediated hypertrophy of differentiated C2C12 myotubes. Myotubes were cultured and infected (D2) as described above, stimulated with IGF-I on D3, and photographed for measurement 24 h later. GFP-infected myotubes stimulated with IGF-I showed a 4.6-fold increase in myotube diameter (Fig. 4, \( P < 0.001 \)). MSTN infection was sufficient to block IGF-I stimulated hypertrophy (Fig. 4, \( P < 0.001 \)). Increasing Akt activity (Fig. 2) with overexpression of constitutively active Akt was sufficient to induce myotube hypertrophy (Fig. 4, \( P < 0.001 \) vs. GFP alone) that was not inhibited by MSTN overexpression. Additionally, the restoration of Akt activity rescued IGF-I-induced myotube hypertrophy that was blocked by MSTN (Fig. 4, \( P < 0.001 \) vs. GFP alone) suggest that MSTN inhibits myotube hypertrophy through inhibition of Akt.

**Inhibition of MSTN results in Akt-dependent myotube hypertrophy.** Adenoviral overexpression of dnMSTN was sufficient to induce a 3.2-fold increase in myotube diameter (Fig. 5, \( P < 0.001 \) vs. GFP). This hypertrophy was blocked by infection with dnAkt (Fig. 5, \( P < 0.01 \) vs. dnMSTN). IGF-I stimulated hypertrophy was similarly inhibited by dnAkt. Akt activity was reduced by dnAkt to levels seen at baseline in GFP-expressing

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**Fig. 1.** Akt protein, activity, and mRNA are increased in myostatin knockout (KO) muscle. A: representative Western blot analysis of Akt from heart, quadriceps, and gastrocnemius muscle in myostatin wild-type (WT), heterozygous (HET), and homozygous (KO) myostatin KO mice. GAPDH is shown as a loading control. B: representative Western blot analysis from quadriceps of WT and KO mice was probed with anti-phospho-Akt (s437), Akt, and GAPDH antibodies. Bar graphs show quantification of blots from total Akt and phospho-Akt normalized to GAPDH. C: representative Akt kinase assay from WT and KO quadriceps. The graph shows quantification of Akt kinase assay results. Dividing lines on Western blot images depict where bands from the same blot have been juxtaposed. D: quantitative real-time RT-PCR using RNA samples from WT and KO quadriceps shows an increase in Akt gene expression in KO skeletal muscle. Gene expression was normalized to GAPDH. Total Akt, \( n = 16 \) WT:16 KO; p-Akt, \( n = 4 \) WT:4 KO; Akt kinase, \( n = 4 \) WT:4 KO; gene expression, \( n = 3 \) WT:4 KO. *\( P < 0.0001 \) vs. WT, #\( P < 0.05 \) vs. WT.
control cells (Fig. 5, B and C). Thus inhibition of MSTN leads to Akt activation that appears necessary for the observed myotube hypertrophy in vitro.

**Downstream mediators of skeletal muscle mass increase.** To determine possible downstream effectors of Akt that may mediate the observed increase in skeletal muscle mass seen in vivo, we examined changes in phosphorylation of p70S6 kinase, GSK3, S6, and mRNA for the E3 ligases MAFbx and MuRF1, which mediate atrophy (31). Interestingly, we found that only S6 kinase phosphorylation was increased (2.7-fold) in KO skeletal muscle compared with WT (Fig. 6, n = 4, P < 0.05). There was no difference in total p70S6 kinase (Fig. 6A), and baseline phosphorylation of p70S6 kinase was not detected in either group (data not shown). Phosphorylation of GSK3 was not increased in KO skeletal muscle (Fig. 6A, n = 4), suggesting it is not involved in mediating the increase in skeletal muscle mass downstream of Akt. We did not see a change in MuRF1 transcription; however, we found a surprising 2.8-fold increase in MAFbx transcription (Fig. 6B, n = 3, P < 0.01), which would be expected to increase atrophy. Thus modulation of atrophy is unlikely to contribute to the skeletal muscle hypertrophy with MSTN deletion, and the observed increase in MAFbx mRNA may represent a counter-regulatory anti-growth response. Together these data suggest that MSTN modulates myotube hypertrophy through Akt and that the increase in Akt activation seen in MSTN KO skeletal muscle may mediate, at least in part, the observed skeletal muscle hypertrophy without reducing atrophy.

**DISCUSSION**

In this study, we demonstrate that the genetic loss of MSTN in vivo increased expression and overall activity of Akt in skeletal muscle. Furthermore, we show that in vitro overexpression of MSTN can block IGF-I-mediated myotube hypertrophy, whereas inhibition of MSTN results in hypertrophy in an Akt-dependent manner. Together these findings suggest a previously unappreciated mechanism by which MSTN modulates skeletal muscle growth:regulation of Akt signaling.

The current work extends our previous observations in cardiac muscle with some important differences. Previously, we reported that MSTN inhibits cardiomyocyte hypertrophy in response to PE, an α-adrenergic agonist, but not IGF-I (22). In contrast, in C2C12 myotubes, IGF-I-mediated hypertrophy is blocked by MSTN, suggesting MSTN may inhibit hypertrophy in a stimulus- and cell-type-specific manner. We previously demonstrated that PE-stimulated hypertrophy in the hearts of myostatin-null is enhanced in association with an increase in PE-stimulated Akt activity without a change in baseline heart size, total Akt, or Akt activity (22). In contrast, the MSTN null skeletal muscles are hypertrophied and show increased Akt expression and activity at baseline.

Using an in vitro model of skeletal muscle hypertrophy, we further demonstrated that the modulation of Akt activity was mechanistically important for MSTN’s anti-hypertrophic effects. Although the use of adenoviral overexpression in C2C12 myotubes, like many other in vitro models, facilitates the understanding of signaling mechanism through ease of experimental manipulation, caution is warranted in direct extrapolation to the in vivo setting. Of note, in this case, loss of MSTN in vivo resulted in upregulation of total Akt and activity, whereas acute overexpression of dnMSTN or MSTN in C2C12 myotubes altered Akt activity without a change in overall levels of protein. Interestingly, we have previously observed that cardiac-specific overexpression of Akt in vivo increased MSTN expression (4, 22); however, adenoviral overexpression of Akt in cardiomyocytes in vitro did not. These situations may represent secondary adaptations of muscle to the chronic loss or enhancement of specific growth signaling pathways.
absence of increased p70S6 kinase and GSK3 phosphorylation in KO skeletal muscle, although shown to be increased by acute IGF-I/Akt stimulation in C2C12 myotubes (26), may also be subject to homeostatic regulation of chronic activation of growth signaling pathways (negative feedback) in response to chronic in vivo Akt activation. The increase in S6 phosphorylation observed in vivo is consistent with increased Akt activation and growth; however, it is not clear that Akt is directly responsible for this increase. Recent reports have provided further evidence for the importance of S6 regulation in controlling muscle growth downstream of MSTN. One report has shown that acute overexpression of MSTN in skeletal muscle via electroporation of DNA was sufficient to reduce skeletal muscle mass and was associated with a dramatic decrease in S6 phosphorylation (1). Interestingly, they did not observe an increase in mTOR phosphorylation despite the modulation of downstream targets. Acute inhibition of MSTN in vivo by administration of an inhibitory MSTN antibody (JA16) (38) or transfection of a dominant negative MSTN receptor (activin type IIB) (29) selectively into adult mouse skeletal muscle also increased muscle mass in association with increased phosphorylation of S6. These results are consistent with our findings that MSTN can regulate distal downstream effectors of Akt in the mTOR pathway to affect skeletal muscle hypertrophy (1). Surprisingly, we found an increase in MAFbx mRNA. Since Akt is able to inhibit atrophy by blocking FoxO-mediated transcriptional increases in E3 ligases including MAFbx (13, 28, 31), and MSTN has been reported to increase MAFbx transcription in C2C12 myotubes (18), we would have expected a decrease in transcription if the inhibition of atrophy secondary to increased Akt activity in MSTN KO skeletal muscle was involved. The observed increase in MAFbx transcription may represent another homeostatic counter-regulatory response to the increase in muscle

Fig. 4. MSTN inhibits IGF-I-mediated myotube hypertrophy through Akt. A: representative photographs of adenovirally infected, GFP-expressing myotubes. B: quantification of mean myotube diameter ± SE expressed as percent GFP control. A minimum of 5 myotubes per condition from 3 to 9 independent experiments were measured. (GFP, n = 104), (G+I, n = 53), (MSTN, n = 113), (M+I, n = 47), (Akt, n = 76), (MSTN+Akt+I, n = 58), (MSTN+Akt = IGF-I, n = 33) *P < 0.001 vs. GFP, #P < 0.05 vs. GFP, ‡P < 0.001 vs. GFP+IGF-I; scale bar, 50 μm (magnification: ×200).
Fig. 5. Inhibition of MSTN results in Akt-dependent myotube hypertrophy. A: representative photographs showing adenovirally infected, GFP-expressing myotubes. B: quantitation of mean myotube diameter ± SE expressed as percent GFP change (GFP, n = 104, dnMSTN, n = 129, dnMSTN+dnAkt n = 100; dnAkt, n = 42, dnAkt+IGF-I, n = 49). C: quantification of Akt kinase assay Western blots (data from n = 3 independent experiments for all except dnAkt, n = 4). *P < 0.001, †P < 0.01 vs. GFP, ‡P < 0.01 vs. dnMSTN; scale bar, 50 μm (magnification: 200).
mass or perhaps a direct effect of MSTN loss since it has recently been shown that MSTN decreases MAFbx transcription in human skeletal muscle myotubes (35). Overall our data are consistent with published data supporting the idea that MSTN predominantly modulates protein synthesis rather than protein degradation (33, 36, 38).

Our results are also consistent with recent work in C2C12 myoblasts, as opposed to the myotubes employed here, which demonstrated that MSTN modulates myoblast proliferation (hyperplasia) through inhibition of PI3 kinase-Akt and cyclin D1 (40). The current study demonstrates MSTN also modulates myotube growth or hypertrophy (as opposed to proliferation) through modulation of Akt signaling. Although the effects of PI3 kinase-Akt signaling in myoblasts and myotubes are different (27), together these studies place PI3 kinase-Akt signaling at the intersection of MSTN’s effects in both stages of differentiation as well as on both muscle proliferation and growth. The latter effects are likely to provide insight into the postnatal muscle growth seen in the KO mice.

Interestingly, another report in skeletal muscle fibroblasts demonstrates an increase in Akt phosphorylation associated with exposure to exogenous MSTN (16). Although this is the opposite, and likely a cell-type-specific effect, it supports a connection between MSTN and Akt signaling.

In human myotubes MSTN administration was associated with a decrease in myotube diameter and Akt phosphorylation; however, MSTN did not inhibit IGF-1 stimulated Akt phosphorylation (35). Interestingly, pharmacological inhibition of a MSTN receptor (ALK) was sufficient to increase myotube diameter consistent with our own results using dnMSTN to directly inhibit endogenous baseline MSTN signaling. The differences between these studies could be explained by the use of different cell lines, since the human myotubes appear less responsive to IGF-I than C2C12 myotubes, or perhaps by the administration route of MSTN, since MSTN is thought to work via an autocrine factor in vivo and exogenously added MSTN has different cellular effects than MSTN released from the cell in an autocrine fashion (25).

MSTN KO mice also display reduced adipose tissue (21). Interestingly, transgenic overexpression of Akt in skeletal muscle has also been reported to decrease adiposity in mice (8, 11), suggesting the increase in skeletal muscle Akt activity reported here may contribute to the decreased adiposity seen in MSTN KO mice. Similarly, the resistance to diabetes seen after MSTN deletion (21) could relate not only to the increase in muscle mass but the enhanced Akt signaling, which would be predicted to increase glucose uptake, although we have not directly examined this here.

In conclusion, we show that MSTN regulates Akt signaling in vitro and in vivo. In vitro studies suggest this regulation is necessary and sufficient for MSTN’s effects on myotube hypertrophy in response to IGF-1. Moreover, we hypothesize that MSTN’s modulation of Akt signaling could play an important role in other phenotypes seen with genetic manipulation of MSTN in vivo. Understanding the downstream mechanisms responsible for MSTN’s effects on skeletal muscle growth, adipose tissue, and insulin resistance could help guide therapeutic strategies targeting this pathway in a wide variety of conditions.

**Fig. 6.** Increased S6 phosphorylation and atrogin-1/muscle atrophy F box (MAFbx) mRNA in vivo. A: representative Western blots from quadriceps of WT and KO mice was probed with anti-phospho-S6, S6, phospho-GSK3, p70S6K, and GAPDH antibodies. Bar graphs show quantification of blots normalized to S6 or GAPDH. Dividing lines on Western blot images depict where bands from the same blot have been juxtaposed. Quantitative real-time RT-PCR using RNA samples from WT and KO quadriceps shows an increase in MAFbx gene expression in KO skeletal muscle. Gene expression was normalized to GAPDH. (Western blots; n = 3WT:3KO). MURF-1, muscle RING-finger protein-1. Gene expression, n = 3WT:3KO. †P < 0.01 vs. WT, #P < 0.05 vs. WT.
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