Antibody-directed myostatin inhibition enhances muscle mass and function in tumor-bearing mice

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Submitted 9 March 2011; accepted in final form 9 June 2011

Murphy KT, Chee A, Gleeson BG, Naim T, Swiderski K, Koopman R, Lynch GS. Antibody-directed myostatin inhibition enhances muscle mass and function in tumor-bearing mice. Am J Physiol Regul Integr Comp Physiol 301: R716–R726, 2011. First published June 15, 2011; doi:10.1152/ajpregu.00121.2011.—Cancer cachexia describes the progressive skeletal muscle wasting and weakness in many cancer patients and accounts for >20% of cancer-related deaths. We tested the hypothesis that antibody-directed myostatin inhibition would attenuate the atrophy and loss of function in muscles of tumor-bearing mice. Twelve-week-old C57BL/6 mice received a subcutaneous injection of saline (control) or Lewis lung carcinoma (LLC) tumor cells. One week later, mice received either once weekly injections of saline (control, n = 12; LLC, n = 9) or a mouse chimera of anti-human myostatin antibody (PF-354, 10 mg·kg⁻¹·wk⁻¹; LLC+PF-354, n = 11) for 5 wk. Injection of LLC cells reduced muscle mass and maximum force of tibialis anterior (TA) muscles by 8–10% (P < 0.05), but the muscle atrophy and weakness were prevented with PF-354 treatment (P > 0.05). Maximum specific (normalized) force of diaphragm muscle strips was reduced with LLC injection (P < 0.05) but was not improved with PF-354 treatment (P > 0.05). PF-354 enhanced activity of oxidative enzymes in TA and diaphragm muscles of tumor-bearing mice by 118% and 89%, respectively (P < 0.05). Compared with controls, apoptosis that was not of myofibrillar or satellite cell origin was 140% higher in TA muscle cross sections from saline-treated LLC tumor-bearing mice (P < 0.05) but was not different in PF-354-treated tumor-bearing mice (P > 0.05). Antibody-directed myostatin inhibition attenuated the skeletal muscle atrophy and loss of muscle force-producing capacity in a murine model of cancer cachexia, in part by reducing apoptosis. The improvements in limb muscle mass and function highlight the therapeutic potential of antibody-directed myostatin inhibition for cancer cachexia.

Cancer cachexia is a complex metabolic syndrome characterized by skeletal muscle wasting, with or without loss of fat mass and is one of the most common manifestations of cancer (15). Cachexia is present in up to 80% of patients diagnosed with gastrointestinal, pancreatic, and lung cancer (10). Cachexia accounts for >20% of all cancer-related deaths due to respiratory/cardiac failure and is associated with reduced mobility, increased risk of surgery-related complications, impaired response to antineoplastic treatments and increased psychological distress, leading to a reduction in overall quality of life (10). Clearly, there is a profound need for therapies that can ameliorate cancer cachexia. The pathogenesis of cancer cachexia is multifactorial and includes anorexia, inflammation, and metabolic disturbances, leading to reduced muscle protein synthesis and enhanced muscle degradation. Therefore, interventions that can enhance muscle protein synthesis and/or attenuate muscle proteolysis have therapeutic potential for cancer cachexia (34).

Myostatin (GDF-8) is a member of the transforming growth factor-β (TGF-β) superfamily of proteins and negatively regulates skeletal muscle mass. Overexpression of myostatin reduces muscle mass (1, 14, 46), whereas inhibition of myostatin enhances muscle mass (24, 30). Furthermore, inhibition of myostatin activity can enhance muscle mass in several disease models associated with muscle wasting, including the muscular dystrophies (7, 8, 35, 42), sarcopenia (33, 37), and disuse/limb casting (32). Given that muscle myostatin mRNA and protein expression are elevated in experimental cancer cachexia (13), myostatin inhibition represents a potential therapeutic strategy to enhance muscle mass and function in cancer cachexia. Although myostatin blockade induced by injection of RNA oligonucleotides (27) or of soluble activin receptors (4, 45), increased muscle mass, and whole body strength in tumor-bearing mice, it has not been determined whether this translates to improvements in muscle function. Since loss of function of limb muscles impairs functional independence and loss of diaphragm function may be implicated in respiratory failure, it is imperative that studies evaluating the therapeutic potential of an intervention for cancer cachexia include assessments of limb and diaphragm muscle function (34).

The aim was to investigate the therapeutic potential of antibody-directed myostatin inhibition on the mass and functional properties of skeletal muscles from tumor-bearing cachetic mice. We tested the hypothesis that acute myostatin inhibition would attenuate muscle fiber atrophy and enhance muscle functional capacity in limb muscles and diaphragm muscle strips from tumor-bearing mice.

MATERIALS AND METHODS

Experimental animals. All experiments were approved by the Animal Experimental Ethics Committee of the University of Melbourne and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes as stipulated by the National Health and Medical Research Council (Australia). Eleven to twelve-week-old male C57BL/6 mice were allocated randomly into one of three experimental groups: a Lewis Lung Carcinoma (LLC) tumor-bearing group that received a mouse chimera of human myostatin antibody (LLC+PF-354, provided by Pfizer Global Research and Development, Groton, CT; 10 mg·kg⁻¹·wk⁻¹ sc; n = 12); a LLC tumor-bearing group that received an equivalent volume of saline (LLC, 0.1 ml/10 g body mass sc; n = 12); and a control group that also received an equivalent volume of saline (saline, 0.1 ml/10 g body mass sc; n = 12). Saline was used as
a control, as it was used in each of the previously published studies utilizing PF-354 (32, 33, 35). The generation of PF-354 has been described previously (23), and we have demonstrated the dose-dependent neutralization of myostatin activity by PF-354 (35). Three mice in the LLC group and one mouse in the LLC + PF-354 group did not develop tumors. As such, results from these mice were excluded from the study, and results are presented for $n = 9$ and $n = 11$ in the LLC and LLC + PF-354 groups, respectively. All mice were obtained from the Animal Resource Centre (Canning Vale, Western Australia), and housed in the Biological Research Facility at The University of Melbourne under a 12:12-h light-dark cycle. Water was available ad libitum, and standard laboratory chow was provided, changed, and monitored daily. The amount of food consumed per mouse per day was determined, and animals in the Control and LLC + PF-354 groups were pair-fed to the LLC group to account for any anorexia.

**LLC cell line and assessment of PF-354 effects on LLC cell proliferation in vitro.** Frozen LLC cells were kindly donated by Dr. Paul Gregorevic (Baker IDI, Melbourne, Australia), via the NCI-Frederick Cancer DCT Tumor Repository (Frederick, MD). Frozen cells were thawed rapidly in a 37°C water bath and transferred to a 100-mm culture plate (Corning, Corning, New York) containing growth media consisting of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) FBS (Invitrogen) and 2 mM glutamine (Invitrogen), and incubated at 37°C with 5% CO2. Cells were maintained in growth media and passaged when 70–80% confluent. For assessment of the effects of PF-354 on LLC proliferation in vitro, cells were treated for 48 h at 37°C in growth media with 0, 0.1, 1, or 7.2 μg/ml PF-354. LLC proliferation was then assessed using a hemocytometer (Bright Line, Hauser Scientific, Horsham, PA).

**Inoculation of LLC cells and treatment.** Before the injection of LLC cells into mice, cells were counted as described previously, pelleted via centrifugation (1,600 g for 5 min at 25°C) and resuspended at 7.5 × 10^6 cells per milliliter of sterilized saline (0.9%). All mice were anesthetized via an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), such that they were unresponsive to tactile stimuli. Mice were shaved on the dorsal side and given a subcutaneous injection of either 7.5 × 10^5 LLC cells suspended in 100 μl of sterilized saline or 100 μl of sterilized saline only (control). Mice recovered from anesthesia on a heat pad, and body mass and tumor size were monitored daily. One week after the inoculation of LLC cells or saline, mice received an intraperitoneal injection of either saline or PF-354, administered once per week for 5 wk. PF-354 dose was based on previous dose-response data obtained in experiments designed to induce maximal muscle hypertrophy (35).

**Assessment of contractile properties.** Six weeks after inoculation, mice were anesthetized with pentobarbital sodium (Nembutal, 60 mg/kg; Sigma-Aldrich, Castle Hill, NSW, Australia) via intraperitoneal injection. The methods for assessment of the contractile properties of the mouse tibialis anterior (TA) muscle in situ have been described in detail by us previously (17). At the conclusion of the contractile measurements in situ, the TA, extensor digitorum longus (EDL), soleus, plantaris, gastrocnemius, and quadriceps muscles were carefully excised, blotted on filter paper, and weighed on an analytical balance. The right TA muscle was mounted in embedding medium carefully excised, blotted on filter paper, and weighed on an analytical balance. Mice were killed as a consequence of diaphragm and heart excision while anesthetized deeply.

**Skeletal muscle histology.** Serial sections were cut transversely through the TA muscle or diaphragm muscle strip using a refrigerated cryostat (−20°C, CTI Cryostat, International Equipment Cryostat, Needham Heights, MA). Sections were stained (or reacted) with hematoxylin and eosin (H&E) to determine general muscle architecture; laminin (#L9393; Sigma-Aldrich) for determination of total fiber number per cross section, as well as mean myofiber CSA; succinate dehydrogenase (SDH) to determine activity of oxidative enzymes (6); van Giessen’s for assessment of collagen infiltration (33); myosin ATPase activity to determine the percentage of Type I fibers (36); A4.840 or N2.261 (both developed by Dr. Helen M. Blau, obtained from the Developmental Studies Hybridoma Bank developed under auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biology, Iowa City, IA) to assess the percentage of myosin I (21) and myosin IIa isoforms (44), respectively; and p-Smad3 (#1880–1; Epitomics, Burlingame, CA) and DAPI (#D9542, Sigma-Aldrich) to determine the number of nuclei positive for p-Smad3. Optical density (OD) of SDH was determined after 6 min of reactivity for all samples, and SDH-stained sections were captured in full color using bright-field light microscopy and analyzed, as described previously (33).

Additionally, TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) staining was used to detect apoptotic nuclei, according to manufacturer’s instructions (in situ cell death detection kit, fluorescein; Roche, Castle Hill, NSW, Australia). The sections were costained with DAPI (1 μg/ml) and caveolin-3 (1:200 dilution; #ab2912, Abcam, Cambridge, MA), and nuclei were counted as TUNEL-positive only when colocalization of DAPI and TUNEL staining was observed. For quantification of TUNEL staining, the ratio of TUNEL-positive nuclei to the total number of DAPI-positive nuclei was calculated and expressed as a percentage of the total number of nuclei. Caveolin-3 was used to identify the sarcolemma to determine whether apoptotic nuclei were located inside (myofibrillar apoptotic nuclei) or outside the fiber (interstitial apoptotic nuclei) (26). To detect apoptosis in satellite cells, TUNEL sections were costained with an anti-Pax-7 antibody (Developmental Biology Hybridoma Bank) utilizing the mouse on mouse immunodetection kit to reduce background staining (Vector Laboratories, Burlingame, CA). Pax-7 positivity was visualized by Alexa Fluor 594-conjugated streptavidin IgG (Invitrogen), and DAPI was used for counterstaining of nuclei. Nuclei were counted as Pax-7 positive only if colocalization of DAPI and Pax-7 staining was observed. The percentage of Pax-7-positive nuclei to the total number of DAPI-positive nuclei was calculated.

Digital images were obtained using an upright microscope with camera (Axio Imager D1, Carl Zeiss, Wrek, Göttingen, Germany), controlled and quantified by AxioVision AC software (AxioVision AC Rel. 4.7.1, Carl Zeiss).

**Real-time RT-PCR analyses.** Total RNA was extracted from 10–20 mg of TA muscle using a commercially available kit, according to the manufacturer’s instructions (PareLink RNA Mini Kit, Invitrogen). RNA concentration was determined spectrophotometrically at 260 nm, and the samples were stored at −80°C. RNA was transcribed into cDNA using the Invitrogen SuperScript VILO cDNA synthesis kit, and the resulting cDNA was stored at −20°C for subsequent analysis. Real-Time RT-PCR was performed, as described previously (33). The forward and reverse primer sequences used were Myf5, 5’-AACCAGAGACCTCCCA-AGGT-3’ and 5’-AGCTTGGCGAGCGACAC-3’; Myod5, 5’- AGTGAATGAGGGCTCTGAGAG-3’ and 5’-GACATGAGATGCGC-
MYOSTATIN INHIBITION ATTENUATES CANCER CACHEXIA

CACTGTA-3', respectively. Primer sequences for myosin heavy chains MHC1, MHCIIa, MHCIIx, MHCIIb, and calcineurin were as detailed previously (33). The content of single-stranded DNA (ssDNA) in each sample was determined using the Quanti-iT OliGreen ssDNA assay kit (Molecular Probes, Eugene, OR), as described previously (28, 33). Gene expression was quantified by normalizing the logarithmic cycle threshold \((C_T)\) value \((2^{-\frac{C_T}{\text{cDNA content}}})\) to the cDNA content of each sample to obtain the expression 2\(^{-\frac{C_T}{\text{cDNA content}}}\) (ng/ml).

Statistical analyses. All values are expressed as mean ± SE, unless stated otherwise. Groups were compared using a one-way or a two-way ANOVA (group, stimulation frequency), where appropriate. Bonferroni’s post hoc test was used to determine significant differences between individual groups. The level of significance was set at \(P < 0.05\) for all comparisons.

RESULTS

PF-354 has no effect on LLC cell proliferation in vitro.

There was no effect of incubation for 48 h with 0.1, 1, or 7.2 \(\mu g/ml\) PF-354 on LLC proliferation in vitro \((0 \mu g/ml, 1.60 \times 10^6 \pm 0.20 \times 10^6; 0.1 \mu g/ml, 1.60 \times 10^6 \pm 0.17 \times 10^6; 1 \mu g/ml, 1.68 \times 10^6 \pm 0.16 \times 10^6; 7.2 \mu g/ml, 1.66 \times 10^6 \pm 0.19 \times 10^6; n = 6, P = 0.98)\).

PF-354 antibody-mediated myostatin inhibition attenuates the loss of muscle mass in tumor-bearing mice.

Six weeks after injection of saline or LLC cells, there was no difference in absolute body mass between groups \((P = 0.16)\); however, tumor-free body mass was ~9% and ~7% lower in LLC and LLC+PF-354 groups compared with saline-treated controls, respectively \((P < 0.05, \text{Table 1})\). Furthermore, the percentage increase in tumor-free body mass from initial was 5.8% and 6.7% lower in LLC and LLC+PF-354 groups compared with saline-treated controls, respectively \((P < 0.05, \text{Table 1})\). There was no difference between LLC and LLC+PF-354 groups in tumor mass \((P = 0.75, \text{Table 1})\), tumor size \((P = 0.99, \text{Table 1})\), or in tumor mass expressed as a percentage of body mass \((P = 0.50, \text{data not shown})\).

The mass of the TA, EDL, plantaris, and quadriceps muscles from the LLC group were 8%, 10%, 10%, and 12% lower than in control mice, respectively \((P < 0.05, \text{Fig. 1A})\). The reduction in TA, plantaris, and quadriceps muscle mass with LLC cells was prevented with PF-354 treatment \((P < 0.01, \text{Fig. 1A})\). Furthermore, soleus and gastrocnemius muscle mass was 10–12% higher in the LLC+PF-354 group compared with the LLC mice \((P < 0.05, \text{Fig. 1A})\). When normalized for body mass, the mass of the TA, plantaris, and quadriceps muscles were 7–11% higher in PF-354-treated LLC compared with saline-treated LLC mice \((P < 0.05, \text{Table 1})\). There was no effect of either LLC cells or PF-354 treatment on absolute heart mass \((P = 0.36)\) or heart mass normalized for body mass \((P = 0.20, \text{Table 1})\).

PF-354 antibody-mediated myostatin inhibition attenuates the loss of muscle strength in tumor-bearing mice.

There was no effect of LLC cells or PF-354 treatment on the twitch characteristics of the TA muscle in situ \((P > 0.05, \text{data not shown})\), but TA muscle CSA was 11% smaller in LLC \((8.1 \pm 0.3 \text{ mm}^2)\) compared with saline-treated mice \((9.1 \pm 0.3 \text{ mm}^2, P < 0.05)\), and the reduction in TA muscle CSA was prevented with PF-354 treatment \((9.3 \pm 0.2 \text{ mm}^2, P < 0.05)\). The frequency-force relationship revealed a group main effect for TA muscles from the LLC+PF-354 group to produce higher forces than the LLC group \((P < 0.04, \text{Fig. 1B})\). Peak tetanic force \((P_o)\) was 8% lower in the LLC group compared with controls \((P < 0.05)\), but this was prevented with PF-354 treatment \((P > 0.05, \text{Fig. 1C})\). There was no difference in specific (normalized) force \((sP_o)\) between groups \((P > 0.05, \text{Fig. 1D})\).

PF-354 antibody-mediated myostatin inhibition enhances activity of oxidative enzymes in TA muscles from tumor-bearing mice.

Representative H&E-stained sections of TA muscles showed similar myofiber architecture between groups, although there appeared to be greater interstitial spaces between muscle fibers in LLC tumor-bearing mice compared with controls \((\text{Fig. 2A, top})\). However, quantification of van Gieson-stained sections \((\text{Fig. 2A, middle})\) revealed no differences in collagen infiltration between groups \((P < 0.21, \text{Fig. 2B})\). TA muscle sections were also stained for laminin and imaged at 5× to visualize and quantify the individual myofibers per cross section \((\text{Fig. 2A, bottom})\). There was no difference in the total number of muscle fibers per cross section between groups \((P < 0.17, \text{Fig. 2C})\). TA muscle cross sections were reacted for SDH to indicate activity of oxidative enzymes, and for myosin I \((\text{A4.840})\) and myosin IIa \((\text{N2.261})\) to identify Type I and Type IIa fibers, respectively \((\text{Fig. 3A})\). No Type I fibers were detected with the A4.840 reaction in TA muscles of group

### Table 1. Selected morphological parameters from control and LLC tumor-bearing C57BL/6 mice treated with saline or PF-354

<table>
<thead>
<tr>
<th>Morphological Parameter</th>
<th>Saline</th>
<th>LLC</th>
<th>LLC + PF-354</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass, g</td>
<td>29.9 ± 0.6</td>
<td>28.4 ± 0.6</td>
<td>29.2 ± 0.3</td>
</tr>
<tr>
<td>Tumor-free body mass, g</td>
<td>29.9 ± 0.6</td>
<td>27.3 ± 0.7*</td>
<td>27.7 ± 0.4*</td>
</tr>
<tr>
<td>% change tumor-free body mass from initial</td>
<td>8.8 ± 1.5</td>
<td>3.0 ± 1.9*</td>
<td>2.1 ± 1.9*</td>
</tr>
<tr>
<td>Tumor mass, g</td>
<td>0.00 ± 0.00</td>
<td>1.48 ± 0.36*</td>
<td>1.64 ± 0.33*</td>
</tr>
<tr>
<td>Tumor size, cm³</td>
<td>0.00 ± 0.00</td>
<td>2.40 ± 0.72*</td>
<td>2.42 ± 0.47*</td>
</tr>
<tr>
<td>TA mass/body mass, mg/g</td>
<td>2.07 ± 0.04</td>
<td>2.03 ± 0.04</td>
<td>2.17 ± 0.03†</td>
</tr>
<tr>
<td>EDL mass/body mass, mg/g</td>
<td>0.45 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>SOL mass/body mass, mg/g</td>
<td>0.32 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>PLANT mass/body mass, mg/g</td>
<td>0.69 ± 0.02</td>
<td>0.67 ± 0.02</td>
<td>0.73 ± 0.01†</td>
</tr>
<tr>
<td>GAST mass/body mass, mg/g</td>
<td>5.20 ± 0.09</td>
<td>5.21 ± 0.12</td>
<td>5.55 ± 0.11</td>
</tr>
<tr>
<td>QUAD mass/body mass, mg/g</td>
<td>7.69 ± 0.18</td>
<td>7.33 ± 0.24</td>
<td>8.16 ± 0.19†</td>
</tr>
<tr>
<td>Heart mass, mg</td>
<td>140 ± 4</td>
<td>139 ± 5</td>
<td>132 ± 4</td>
</tr>
<tr>
<td>Heart mass/body mass, mg/g</td>
<td>4.72 ± 0.18</td>
<td>5.01 ± 0.19</td>
<td>4.57 ± 0.13</td>
</tr>
</tbody>
</table>

LLC, Lewis lung carcinoma; EDL, extensor digitorum longus; PLANT, plantaris; TA, tibialis anterior; GAST, gastrocnemius; QUAD, quadriceps. Data are expressed as means ± SE; \(n = 9–12\). *\(P < 0.05\) vs. saline; †\(P < 0.05\) vs. LLC.
Tumor-bearing mice treated with PF-354 had a higher proportion of Type IIa fibers (10% and 12% compared with saline and LLC, respectively; \( P < 0.01 \)) and a corresponding smaller proportion of Type IIx/b fibers (10% and 12%, \( P < 0.01 \), Fig. 3B). Treatment with PF-354 increased the CSA of Type IIa fibers (16% and +27%, \( P < 0.05 \)) but not of the Type IIx/b fibers (\( P > 0.05 \)) and did not improve average fiber CSA (\( P = 0.20 \), Fig. 3C). Type IIa fibers in TA muscles from tumor-bearing mice had reduced oxidative capacity (40%, \( P < 0.05 \)). However, PF-354 treatment enhanced the oxidative capacity of Type IIa fibers (60% and +168%, \( P < 0.001 \)) and Type IIx/b fibers.
fibers (38% and 102%, \( P < 0.05 \)), with a corresponding increase in average activity of oxidative enzymes (45% and 118%, \( P < 0.05 \), Fig. 3D). Myosin ATPase histochemistry confirmed the low abundance of Type I fibers, that less than 0.1% of all fibers represented Type I fibers, and that there was no difference in the proportion of Type I fibers between groups (Saline, 0.01 ± 0.01; LLC, 0.04 ± 0.01; LLC+PF-354, 0.05 ± 0.02; \( n = 8, P < 0.12 \), Fig. 3A).

Real-time RT-PCR analysis was used to determine whether the higher proportion of Type IIa fibers in PF-354-treated animals detected with N2.261 corresponded with a higher mRNA expression of MHCIIa and a concomitant lower mRNA expression of the other MHCII isoforms. Muscles from PF-354-treated mice had a 155% (\( P < 0.05 \)) and 507% (\( P < 0.05 \)) higher MHCI mRNA expression compared with saline and LLC, respectively, as well as a 107% (\( P < 0.01 \)) and 300% (\( P < 0.001 \)) higher MHCIIa mRNA expression compared with saline and LLC, respectively (Fig. 5E). There was no difference in MHCIIx (\( P > 0.07 \)) and MHCIIb mRNA expression between groups (\( P > 0.28 \), Fig. 5E).

Consistent with these findings, mRNA expression of calcineurin, a gene implicated in fiber oxidative capacity was 74% (\( P < 0.05 \)) and 68% (\( P < 0.05 \)) higher in TA muscles of tumor-bearing mice treated with PF-354 compared with saline and LLC, respectively, whereas there was no significant difference between saline and LLC groups (Fig. 5F).

PF-354 antibody-mediated myostatin inhibition reduces apoptosis in TA muscles from tumor-bearing mice. The percentage of apoptotic nuclei within cross sections of TA muscles was assessed using TUNEL staining, and costaining with caveolin-3 revealed that for each group, all apoptotic nuclei were outside of the sarcoplasmic membrane (Fig. 4A and inset, and Fig. 4B). The percentage of TUNEL-positive nuclei was 1.4 times higher in TA muscles from tumor-bearing mice compared with controls (\( P < 0.05 \)), but this was prevented with PF-354 treatment (\( P = 0.56 \), Fig. 4D). A Pax-7 antibody was used in conjunction with TUNEL staining and DAPI staining to determine whether the apoptotic nuclei were of satellite cell origin (Fig. 4C). However, no Pax-7 and TUNEL double-positive nuclei were observed for any group (Fig. 4C). There was also no significant difference between groups in the percentage of Pax-7-positive nuclei within TA muscle sections (\( P < 0.24 \), Fig. 4D).
PF-354 antibody-mediated myostatin inhibition reduces p-Smad3 nuclear localization in TA muscles from tumor-bearing mice. TA muscle sections were stained for p-Smad3 and nuclei (DAPI) to assess nuclear p-Smad3 localization (Fig. 5A). TA muscle sections from tumor-bearing mice had a 46% greater nuclear p-Smad3 localization compared with controls (P < 0.001), but this was prevented with PF-354 treatment (P > 0.05, Fig. 5B). Since Smad signaling is known to induce muscle atrophy via inhibition of myogenesis, and muscles from patients with cancer cachexia have reduced expression of myogenic regulatory factors (MRFs) (11), mRNA expression of the MRFs, Myf5, and MyoD were determined. Myf5 mRNA expression was 44% lower in TA muscles of tumor-bearing mice compared with controls (expression was 44% lower in TA muscles of tumor-bearing mice). MyoD mRNA expression was not significantly different between groups (Saline, 179.6 ± 0.51, Fig. 5D). PF-354 antibody-mediated myostatin inhibition does not improve force production of diaphragm muscle strips from tumor-bearing mice. Twitch characteristics of diaphragm muscle strips in vitro were not different between groups (P = 0.70, data not shown). There was a group main effect for the tumor-bearing mice to exhibit lower specific forces as evident from the frequency-force relationship (P < 0.001), but this was not altered with PF-354 treatment (P = 0.11, Fig. 6B), and sPo_{IIa} was not different between groups (Saline, 179.6 ± 4.5; LLC, 168.0 ± 5.6; LLC+PF-354, 174.3 ± 4.8 kN/m²; n = 9–12, P = 0.28).

PF-354 antibody-mediated myostatin inhibition enhances activity of oxidative enzymes in diaphragm muscles from tumor-bearing mice. Cross sections of diaphragm muscle strips were reacted for SDH, myosin I (A4.840), and myosin IIa (N2.261) to indicate oxidative enzyme activity and identify Type I and Type IIa fibers, respectively (Fig. 6). Tumor-bearing mice treated with PF-354 had a higher proportion of Type IIa fibers (+12% and +11% compared with Saline and LLC, respectively; P < 0.05) and a corresponding lower proportion of Type Ix/b fibers (−14% and −12%, P < 0.05, Fig. 6C). There was no difference between groups in the proportion of Type I fibers (P = 0.12, Fig. 6C). Treatment with PF-354 increased average fiber CSA (+82% and +69%, P < 0.05), which was due to an increased CSA of Type IIa fibers (+69% and +67%, P < 0.05) and Type Ix/b fibers (+105% and +84%, P < 0.01), but not of Type I fibers (P = 0.51, Fig. 6D). Compared with controls, diaphragm muscle strips from tumor-bearing PF-354-treated mice had enhanced activity of oxidative enzymes in Type I fibers (+92%, P < 0.01), Type IIa fibers (+90%, P < 0.01), and Type Ix/b fibers (+86%, P < 0.01, Fig. 6E). Consequently, average activity of oxidative enzymes was higher (+89%, P < 0.01) in tumor-bearing mice treated with PF-354 compared with controls (Fig. 6E). However, there was no difference in oxidative capacity of fibers.
DISCUSSION

This is the first study to our knowledge to investigate whether antibody-directed myostatin inhibition can improve the functional properties of limb muscles and diaphragm muscle strips from tumor-bearing mice. PF-354 antibody-directed myostatin inhibition prevented the loss of force-producing capacity of TA muscles from LLC tumor-bearing mice but did not attenuate the loss of force-producing capacity of diaphragm muscle strips from tumor-bearing mice. The findings indicate that antibody-directed myostatin inhibition has therapeutic potential for cancer cachexia and could preserve or improve the functional independence of affected patients.

Antibody-directed myostatin inhibition attenuates wasting in limb muscles from LLC tumor-bearing mice. The LLC tumor-bearing mice used in the present study represents a mild model of cachexia, with a 9% lower tumor-free body mass and an 8–10% lower muscle mass compared with controls at 6 wk post LLC injection. A previous study using the LLC model reported much greater reductions in body mass (22%) and muscle mass (23–38%) at only 15 days following LLC injection (41), but in that study, the LLC tumor cells were injected intramuscularly, which has limited clinical relevance. Another study using subcutaneous injection of LLC cells found an 18% reduction in tumor-free body mass and a 12–14% reduction in muscle mass compared with controls at 14 days postinoculation (4). The greater cachexia reported in that study was associated with a large tumor mass, which represented ~12%
of total body mass, whereas tumor mass in the present study represented ~5% of total body mass. Strict constraints imposed by our institution’s Animal Ethics Committee precluded the growth of tumors in the present study to sizes equivalent to that reported by Benny Klimek et al. (4). The reasons for the slower growth of LLC tumors in the present study are unknown, but different isolates of this line are known to cause variations in the degree of cachexia. However, it is important to highlight that cachexia encompasses a broad spectrum of wasting, from early weight loss (precachexia) to severe cachexia associated with death (16). Although treatment at each stage of cachexia is crucial, the earlier the intervention is initiated, the better the prognosis (16). The LLC tumor-bearing model used in the present study, therefore, represents a mild-cachectic state when treatments should be initiated to maximize the chances of attenuating cancer cachexia and increasing survival (16).

The reduction in TA, plantaris, and quadriceps muscle mass in LLC tumor-bearing mice was attenuated with the myostatin inhibitory antibody, PF-354, which also enhanced the mass of the soleus and gastrocnemius muscles in LLC tumor-bearing mice. These findings are consistent with previous studies reporting an increase in muscle mass in tumor-bearing mice treated with RNA oligonucleotides targeting myostatin (27) or soluble activin receptors (4, 45). However, the increases in muscle mass with soluble activin receptors are substantially greater (25–30%) than the increases in muscle mass with PF-354 (8–12%). These findings demonstrate that greater improvements in muscle mass are achieved when the signaling of multiple TGF-β ligands is inhibited rather than inhibition of myostatin alone (25), and they support the notion that activin A acts in concert with myostatin to induce muscle wasting (45).

The 8–12% increases in muscle mass with 5 wk PF-354 treatment in tumor-bearing mice are smaller than the 35–36% increases in muscle mass with the same duration of PF-354 treatment in healthy C57BL/10 mice (35). These results indicate that PF-354 promotes muscle growth, rather than simply attenuating the loss of muscle mass in conditions of muscle wasting, but that the anabolic effects of PF-254 may be reduced in tumor-bearing mice.

The changes in muscle mass were consistent with the observed changes in total TA muscle CSA. LLC tumor-bearing mice demonstrated a smaller muscle CSA compared with controls, and this loss of muscle was attenuated with PF-354 treatment. There was also no difference in muscle fiber architecture or collagen infiltration between groups.

One of the mechanisms underlying the reduction in muscle mass in LLC tumor-bearing mice is the increased intracellular Smad signaling, which resulted in TA muscles of tumor-bearing mice having a 46% increase in p-Smad3 nuclear
localization. Consistent with the increased nuclear p-Smad3 localization, we observed a reduction in Myf5 mRNA expression in tumor-bearing mice, but both effects were prevented with PF-354 treatment. Myostatin has been shown previously to reduce Myf5 protein expression in C2C12 cells (22). These findings, therefore, suggest that increased intracellular Smad signaling leading to altered transcription of Myf5 contributes to the development of cachexia in LLC tumor-bearing mice but that this effect can be ameliorated with PF-354 treatment. Increased apoptosis within skeletal muscle has been reported in tumor-bearing animals and has been postulated to contribute to the development of cancer cachexia (3, 20, 41). We also found increased apoptosis in the TA muscles of tumor-bearing mice, with all apoptotic nuclei found outside of the sarcoplasmic membrane, but this was prevented completely with PF-354 treatment. Coecting sections with TUNEL and an anti-Pax-7 antibody revealed that none of the apoptotic nuclei represented satellite cells. Therefore, it appears that the increased apoptotic nuclei in TA muscle sections of LLC tumor-bearing mice are not of myofibrillar or satellite cell origin.

Tumor growth was not different between saline-treated and PF-354-treated LLC tumor-bearing mice, which was not surprising, given that we found no effect of varying concentrations of PF-354 on LLC cell proliferation in vitro and because previous studies have reported no effect of administration of an activin receptor extracellular domain fusion protein on tumor mass in mice injected with LLC cells (4). Antibody-directed myostatin inhibition attenuates loss of muscle function in LLC tumor-bearing mice. Cancer cachexia is associated with an impaired functional capacity of the limb muscles, with patients exhibiting reduced isometric and isotonic strength of the quadriceps muscles compared with healthy volunteers (43) and EDL muscles from tumor-bearing mice exhibiting reduced force production in vitro compared with control mice (18, 40). Impaired functional capacity of the limb muscles diminishes the ability of affected patients to perform the activities of daily living and may lead to premature retirement and loss of independence (34). Consistent with the decrease in muscle mass, TA muscles of LLC tumor-bearing mice exhibited reduced absolute force production compared with control mice. Remarkably, this reduction in functional capacity in tumor-bearing mice was completely prevented with PF-354 treatment. Since sPo (force normalized to muscle CSA) was not different between groups, the improvement in muscle mass is the likely main mechanism underlying the restoration of muscle functional capacity with PF-354 treatment. Furthermore, since average fiber CSA and the number of muscle fibers per cross section were similar between groups, changes in fiber size and number did not contribute to the alterations in muscle mass and function with LLC cells and PF-354 treatment.

We have found that PF-354 antibody-directed myostatin inhibition enhances activity of oxidative enzymes in TA muscles of aged mice (33), and in the present study, we found that PF-354 antibody-directed myostatin inhibition enhances activity of oxidative enzymes in TA muscles of tumor-bearing mice. PF-354 treatment increased the proportion of the highly oxidative Type IIA fibers, reduced the proportion of the glycolytic Type IIX/b fibers, increased MHCIIa mRNA expression, and increased activity of oxidative enzymes in Type IIA and Type IIX/b fibers. These findings are consistent with another study, which reported that PF-354 treatment in aged mice improved treadmill running performance and reduced fatigue during tetanic contractions of the plantar flexor muscle group (23). However, the shift in oxidative phenotype with acute myostatin inhibition is in contrast with the findings from studies in knockout mice. Muscles from myostatin knockout mice exhibit a more glycolytic phenotype, increased mRNA expression of the MHCIIx and IIB isoforms, and reduced mRNA expression of MHCIIa isoforms (2, 12, 38). Therefore, it appears that genetic deletion of myostatin results in a more glycolytic muscle phenotype, while acute antibody-directed myostatin inhibition results in a more oxidative phenotype.

Improvements in muscle activity of oxidative enzymes with PF-354 treatment could potentially attenuate fatigue in cancer patients and thus improve their quality of life significantly. The signaling mechanisms responsible for the shift in muscle fiber-type composition with PF-354 are unknown but may involve calcineurin signaling since 1) calcineurin mRNA expression was increased with PF-354 treatment in the current study and also in a previous study (33); and 2) calcineurin signaling induces a slow phenotype, characterized by a shift in fiber type from Type IIB → IIX → IIa and an increase in oxidative enzyme activity (31). The calcineurin-induced changes in muscle phenotype have also been shown to occur within 3 wk (5), and it is, therefore, not surprising that PF-354-induced alterations in muscle fiber-type composition within 5 wk.

Antibody-directed myostatin inhibition does not improve contractile properties of diaphragm muscle strips from LLC tumor-bearing mice. One of the major causes of death in cancer cachexia is respiratory muscle failure (39). This is the first investigation of the efficacy of myostatin inhibition to improve diaphragm muscle function in cachectic tumor-bearing mice. PF-354 antibody-directed myostatin inhibition did not enhance the sPo (force normalized to muscle CSA) of diaphragm muscle strips in vitro, but it did increase the CSA of Type IIA and Type IIX/b fibers. Thus, it is possible that the overall (absolute) force of the diaphragm muscle may have been enhanced with PF-354 treatment, but since sPo (normalized) forces are the only valid measure for diaphragm muscle strips (29), this would not be evident. Similar to the TA muscle, PF-354 treatment enhanced the oxidative capacity of diaphragm muscle strips from tumor-bearing mice, due to the increased proportion of oxidative Type IIA fibers, the reduced proportion of glycolytic Type IIX/b fibers, and the improved activity of oxidative enzymes in all muscle fiber types (Type I, Type IIA, and Type IIX/b).

Perspectives and Significance

PF-354 antibody-directed myostatin inhibition attenuated the atrophy and loss of functional capacity of the TA muscles of tumor-bearing mice exhibiting mild cachexia. The findings are clinically relevant since prognosis is best when treatments are initiated at the early stages of cachexia. Furthermore, improvements in muscle structure and function could enhance the capacity of affected patients to perform the activities of daily living and improve their quality of life. The improvements in muscle structure and function are also likely to increase compliance with other anticancer therapies and so, antibody-directed myostatin inhibition could also provide synergistic benefits for patients with cancer cachexia.
**REFERENCES**


