Increased Muscle Force Production and Bone Mineral Density in ActRIIB-Fc-Treated Mature Rodents

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Myostatin is a highly conserved member of the transforming growth factor-β (TGF-β) superfamily and functions as an inhibitor of muscle growth via activation of activin receptors. A fusion protein consisting of the extracellular ligand-binding domain of activin type IIB receptor with the Fc portion of human immunoglobulin G (ActRIIB-Fc) was used to inhibit signaling through this pathway. Here, we study the effects of this fusion protein in adult, 18-month-old, and orchidectomized mice. Significant muscle growth and enhanced muscle function were observed in adult mice treated for 3 days with ActRIIB-Fc. The ActRIIB-Fc-treated mice had enhanced fast fatigable muscle function, with only minor enhancement of fatigue-resistant fiber function. The ActRIIB-Fc-treated 18-month-old mice and orchidectomized mice showed significantly improved muscle function. Treatment with ActRIIB-Fc also increased bone mineral density and serum levels of a marker of bone formation. These observations highlight the potential of targeting ActRIIB receptor to treat age-related and hypogonadism-associated musculoskeletal degeneration.

Key Words: Sarcopenia—Myostatin—Activin receptor type IIB—Muscle.

Received June 19, 2012; Accepted February 13, 2013

Decision Editor: Rafael de Cabo, PhD
T has been reported to regulate myostatin signaling and one mechanism by which androgens alter lean body mass may be by reducing myostatin expression (23). T has also been reported to induce muscle satellite cell proliferation and to increase muscle cross-sectional area (CSA) in both young (2-month-old) and aged (24-month-old) mice (24). However, the use of T in patients is associated with a number of adverse events such as increased hemoglobin, decreased high-density lipoprotein, and the potential for increased cardiovascular adverse events (25,26). Identification of alternative therapeutics to treat hypogonadism-associated muscle loss is warranted.

To better understand the consequences of pharmacologic intervention in the myostatin signaling pathway, we treated mature, 18-month-old, and orchidectomized (ORX) mice with an ActRIIB-Fc fusion protein and monitored effects on muscle function. We measured muscle size changes by microcomputed tomography, developed and applied a novel in situ muscle contraction assay system to monitor muscle strength, and further assessed treatment effects on bone mineral density (BMD) using densitometry (pDXA).

**Materials and Methods**

**ActRIIB-Fc Fusion Protein**

DNA encoding the human ActRIIB extracellular domain (aa 1–133) was cloned into an Fc fusion vector carrying the gene for the IgG2M4 Fc domain to produce the fusion gene (27). HEK293 cells were transfected with the vector to express the Fc fusion protein, which was purified by size exclusion chromatography. Briefly, the medium was loaded onto a preequilibrated Hi Trap Mab select SuRE column (GE Healthcare, Buckinghamshire, United Kingdom), which was washed and eluted with IgG elution buffer (Pierce, Rockford, IL). Eluted protein was neutralized immediately with ultrapure 1 M Tris-HCl, pH 8.0, then dialyzed against 100 mM Arginine, 100 mM Histidine, 6% (w/v) sucrose buffer overnight at 4°C, and concentrated with an Amicon concentrator. The fusion protein is stable in this buffer and will be diluted using the same buffer in all studies.

**Smad-Binding Element Reporter Assay**

The CellSensor SBE-bla HEK293T cell line (Invitrogen, Carlsbad, CA) was used with minor modifications to the manufacturer’s recommended protocol. Cells were seeded in 384-well plate (Corning Life Sciences, Lowell, MA) at a density of 2.5 × 10^4 cells/well. ActRIIB-Fc inhibitory activity was determined by 10-fold serial dilution in the presence of a fixed ligand, that is, GDF8 and GDF11 (R&D Systems, Minneapolis, MN) concentration (ie, 100 ng/mL for GDF8 and GDF11), and incubated for 72 hours. Crude extracts were prepared and total ATP concentrations were determined with the ViaLight assay kit (Lonza Group, Basel, Switzerland).

**Cell Proliferation Assay**

MPC-11 cells were seeded in a 96-well plate at 5,000 cells/well (~5% confluence), treated with series dilution of ActRIIB-Fc with fixed ligand concentration (ie, 100 ng/mL for GDF8 and GDF11), and incubated for 72 hours. Crude extracts were prepared and total ATP concentrations were determined with the ViaLight assay kit (Lonza Group, Basel, Switzerland).

**Smad3 Phosphorylation Assay**

MPC-11 or HEK293 cells were treated with 600 pM GDF8 or GDF11 and ActRIIB-Fc for 45 minutes. Cells were lysed and were analyzed by immunoblotting using a rabbit monoclonal antibody against Smad3-phospho S423 + S425 (Abcam, Cambridge, MA). Protein bands were quantified by Odyssey scanner (Licor Bioscience, Lincoln, NE).

**Animals**

ORX C57BL/6 mice (5- to 6-month old) and male mice (9- to 11-month old and 18- to 19-month old) were obtained from Taconic Farms (Taconic Farms, Hudson, NY). The animals were fed ad libitum and were housed in 12:12 light/dark cycle for approximately 4 weeks prior to initiation of each study. All animal procedures were approved by Merck Research Laboratories, West Point, Pennsylvania (Institutional Animal Care and Use Committee) in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

**Single-Dose Pharmacokinetic Study**

Mice (10- to 12-month old, 5 mice/group) were treated with a single dose of 10 mg/kg hActRIIB-hIgG2M4 Fc (hActRIIB-Fc) through either tail vein intravenous or intraperitoneal (IP) injection. Blood samples were collected at multiple time points, including predose and at 3 hours, 24 hours, Day 2, Day 3, Day 4, Day 7, and Day 10 after dosing. Briefly, 10 μL of blood (tail vein bleed) was mixed with 1 μL of 55 mM ethylenediaminetetraacetic acid in 96-well plate and was stored at 4°C. After blood collection was completed, 89 μL of phosphate-buffered saline was added to each sample followed by centrifugation at 3,000 g for 15 minutes, and the supernatant was collected and stored at −20°C.

An immunoassay using biotinylated mouse anti-ActRIIB monoclonal antibody (Santa Cruz) was used for capture, and an ALEXA-647-labeled mouse monoclonal antibody specific for human Fc domain (Southern Biotech) was used for detection (Gyros, Uppsala, Sweden). The ActRIIB-Fc concentrations were extrapolated from standard curves.
INCREASED MUSCLE FORCE IN ActRIIB-Fc-TREATED MATURE RODENTS

Animal Treatments

In a proof of concept study, animals (10 mice/group) were treated with either vehicle or 3 or 10 or 30 mg/kg (twice weekly, IP) of hActRIIB-Fc for 30 days (nine injections). Animals body compositions were monitored longitudinally using quantitative nuclear magnetic resonance. At necropsy, histology was performed to determine muscle mass, femoral BMD, and the spleen weight (28). Blood samples were collected to monitor pharmacokinetic of hActRIIB-Fc prior to the second, fifth, and ninth injection.

In a separate study, animals were treated with ActRIIB-Fc either by IP injection or by subcutaneous injection. The pharmacodynamic results following subcutaneous injection were equal or better than that of IP injection. To evaluate muscle function, male mice (10- to 12-month old and 18- to 19-month old) and ORX mice (5- to 6-month old) were treated with 10 mg/kg of ActRIIB-Fc with twice weekly subcutaneous injection. To test the time course of response, animals were also treated with 10 mg/kg of ActRIIB-Fc for different periods of time, for example, 1–9 injections (3–30 days).

Quantitative Nuclear Magnetic Resonance and Microcomputed Tomography Scan and Image Analysis

Mouse whole body composition was monitored longitudinally by quantitative nuclear magnetic resonance (Echo Medical System, Houston, TX). Mice were scanned with microcomputed tomography to measure the tibial muscle CSA (LaTheta LCT-100A; Aloka, Japan). Fifteen slices were scanned from the beginning of hind-limb tibial bone to the merge of tibia and fibula bones. The images were automatically analyzed using the established protocol (29).

In Situ Muscle Contraction Assay

To evaluate muscle function, we custom built an in situ muscle contraction assay system to measure muscle function (29). Briefly, animal left femur bone was exposed by surgery and was clamped with a vertebrae clamp. The animal was transferred onto a custom-built assay stage where the foot is fasten and the Achilles tendon along with a piece of the posterior calcaneous bone were exposed and connected to a muscle lever using a Kevlar thread (305C-LR, Aurora Scientific, Ontario, Canada).

The sciatic nerve was then exposed and stimulated by electrodes. The sciatic nerve was stimulated with tetanic supra maximum currents at 0.1 ms square wave and 100 Hz frequency for 50 ms. Animals were stimulated once every 1.25 seconds for a total of 300 seconds. To compensate for muscle elongation after stimulated contractions, the optimum muscle tension was maintained by a feedback loop in the assay program. The fatigue envelop was generated and analyzed using a double sigmoid curve fitting and the corresponding functional parameters were calculated (29).

After the in situ assay, the hind-limb muscle tissues were collected, and a correlation analysis between muscle function and size was performed.

Histology

After performing the in situ assay, animals were euthanized and hind-limb muscles were collected, weighed, and snap frozen in liquid nitrogen. The frozen quadriceps were immersed in prechilled Tissue-Tek O.C.T. Compound (Miles Kankakee, IL) and frozen in a slurry of ethanol and dry ice for mounting. Cryostat sections (12 μm thick) were transferred onto glass slides (ProbeOn+; Fisher Scientific) and stored at −80°C. Sections were incubated in wheat germ agglutinin conjugated to Alexa fluor 488 labeling reagent (Invitrogen) diluted 1:200 in phosphate-buffered saline for 60 minutes at room temperature. Images were taken using a Nikon (Tokyo, Japan) light microscope at 10× objective to collect images. The fiber size was measured by a script from the Definien program.

BMD Analysis (Densitometry)

Defleshed whole right femora bone was placed on an acrylic base with posterior side down and scanned using pDXA (Norland; Ft. Atkinson, WI) with a resolution of 0.2 x 0.2 mm, field width of 1.4 cm, and speed of 2 mm/s. Regions of interest located at 0–3 mm (distal) and 4–7 mm (central) from the distal end of the femur were isolated using manufacturer’s software. Bone mineral content (mg) and bone area (cm²) were determined for each region of interest and BMD (mg/cm²) was calculated.

Procollagen I N-Terminal Peptide Assay

The mouse procollagen I N-terminal peptide (P1NP) levels were determined using the Rat/Mouse P1NP Enzyme immunoassay kit AC-33F1 according to the manufacturer’s instructions (ImmunodiagnosticSystem, Fountain Hills, AZ). Briefly, fivefold diluted serum samples (50 μL) were added to a 96-well plate coated with a polyclonal rabbit anti-P1NP antibody. Fifty microliters of biotin-labeled P1NP was added and incubated for 1 hour. After 3 washes with buffer, 150 μL of avidin-linked horseradish peroxidase was added and the plate was incubated for 30 minutes. After 3 washes with buffer, 150 μL of TMB substrate was added and incubated for 30 minutes before 50 μL of stop solution was added. Absorbance was measured at 450 nm (reference 659 nm).

Statistics

One-way analysis of variance (α = .05 or .01) was used to determine the differences between the tested groups. The unpaired t test analysis was used to determine the differences between the vehicle- and the ActRIIB-Fc-treated groups.
RESULTS

Design and Purification of ActRIIB-Fc Protein

The ActRIIB-Fc fusion protein was generated by fusing the N-terminal peptide of human ActRIIB (aa 1–133) to a modified version (G2M4) of the human Fc domain (Figure 1A). Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that under nonreducing conditions, the purified hActRIIB-Fc fusion protein consisted of a single dimer (Figure 1B, lane 2). Under reducing conditions, the fusion protein was monomeric with a molecular weight of approximately 51 kD (Figure 1B, lane 3). Size exclusion chromatography confirmed the purity of the fusion protein (Figure 1C).

In Vitro Characterization of the Biological Activity of ActRIIB-Fc

The ActRIIB-Fc fusion protein was tested in a number of cell-based assays to assess its ability to interfere with signaling through the ActRIIB receptor and its endogenous ligands. These assays utilized GDF8 (myostatin) and GDF11, which are two known ligands for ActRIIB. GDF8 and GDF11 signal through ActRIIB to induce β-lactamase activity in a Smad-responsive β-lactamase reporter gene assay in HEK293 cells with comparable efficacy (EC$_{50}$ = 400 and 500 pM, respectively). ActRIIB-Fc fusion inhibited GDF8 and GDF11 activity in a dose-dependent manner with an IC$_{50}$ of 120 and 135 nM, respectively (Figure 2A and B). Similar inhibitory activities were observed in a cell-based proliferation assay using MPC-11 cells (100 and 180 nM for GDF8 and GDF11, respectively; Figure 2C and D). The ActRIIB-Fc fusion also blocked GDF8- and GDF11-induced Smad3 phosphorylation in MPC-11 cells (Figure 2E).

Pharmacokinetic Evaluation of ActRIIB-Fc

C57BL/6 mice treated with a single dose of 10 mg/kg ActRIIB-Fc via IP or intravenous delivery had comparable pharmacokinetic profiles (Figure 3). Following intravenous administration, the ActRIIB-Fc appeared to decline in a biphasic manner, with low clearance (1.9 ± 0.3 mL/h/kg) and long terminal half-life (132 ± 52 h), consistent with the characteristics of an Fc fusion protein (30). Following IP administration, the protein was absorbed quickly and efficiently (T$_{max}$ = 3–48 h) with bioavailability of approximately 77% (Table 1, Figure 3A). No accelerated clearance was observed within the first 10 days of dosing; however, by the fifth week of dosing, there appears to be a decline in circulating levels of the fusion protein.

ActRIIB-Fc Treatment Rapidly Increases Muscle Mass and Enhances Muscle Function in Mature Adult Mice

To study age-related sarcopenia, we focused on mature and aging animals. Retired breeder mice (10- to 12-month old) were treated with 3, 10, and 30 mg/kg of ActRIIB-Fc twice weekly for 30 days. During the first 2 weeks, trough ActRIIB-Fc serum levels were 5–10, 15, and 40 μg/mL for the 3, 10, and 30 mg/kg dose, respectively, consistent with...
predictions from the single-dose pharmacokinetic study. After the first 2 weeks of dosing (fifth dose), trough levels of ActRIIB-Fc were more variable among the animals. By the fifth week (ninth dose), there were significant reductions in the serum levels of the fusion protein compared with previous weeks, suggesting potential development of immunogenicity and increased clearance (Figure 4A–C).

The quantitative nuclear magnetic resonance data showed no obvious change in body weight for any group; however, the 10 and 30 mg/kg groups had significant increases in total lean mass and a trend toward a decrease in total fat mass (Table 1) reminiscent of findings in myostatin deficiency (31). The 3, 10, and 30 mg/kg groups displayed dose-dependent increases in individual muscle weight, that is, 11% (not significant), 30%, and 44% for tibialis anterior; 6% (not significant), 16%, and 19% for gastrocnemius; and 13%, 27%, and 41% for quadriceps, respectively. Within the quadriceps, the average individual fibril size was 2,038 +/- 16 μm² for the vehicle-treated mice and 2,348 +/- 20 μm² for the mice treated with 30 mg/kg ActRIIB. The ActRIIB-Fc-treated mice also had an approximately 12% increase in CSA of the tibial muscle in 30 mg/kg dose group.
ActRIIB-Fc Increased Muscle Mass and Muscle Force in 18-Month-Old Mice

In 18-month-old mice, there was a 21% increase in CSA of tibial muscle size and 18% increase in plantarflexor muscle mass (161 vs 190 mg, respectively) after treatment with 10 mg/kg ActRIIB-Fc for 30 days (data not shown). The fatigue envelop of the ActRIIB-Fc-treated mice showed significantly increased fast fatigable force, whereas the fatigue-resistant muscle force was unchanged, which is in agreement with the findings from young adult mice (Figure 5A). Curve fitting showed significantly increased $F_{\text{max}}$ (15%), $F_{0}$ (17%), and $F_{0} - F_{\text{min}}$ (28%) indicating increased type II fiber function (Figure 7).

The total muscle mass of plantarflexor and quadriceps was 161 and 213 mg, respectively, for vehicle-treated mice and 190 and 259 mg for ActRIIB-Fc-treated mice (Figure 7).

**Table 1. List of Body Composition and Muscle Mass Among the ActRIIB-Fc-Treated Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Fat (g)</th>
<th>Lean (g)</th>
<th>Body Weight (g)</th>
<th>Muscle Mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Endpoint</td>
<td>Baseline</td>
<td>Endpoint</td>
</tr>
<tr>
<td>Vehicle</td>
<td>15.2±0.8</td>
<td>14.5±0.6</td>
<td>23.8±0.3</td>
<td>22.8±0.3</td>
</tr>
<tr>
<td>ActRIIB-Fc, 3mpk</td>
<td>14.7±0.6</td>
<td>13.8±0.7</td>
<td>23.7±0.4</td>
<td>24.0±0.4</td>
</tr>
<tr>
<td>ActRIIB-Fc, 10mpk</td>
<td>15.0±0.8</td>
<td>14.6±0.7</td>
<td>23.7±0.3</td>
<td>25.5±0.3**</td>
</tr>
<tr>
<td>ActRIIB-Fc, 30mpk</td>
<td>15.3±0.7</td>
<td>13.5±0.6</td>
<td>23.6±0.3</td>
<td>26.1±0.3**</td>
</tr>
</tbody>
</table>

**p<0.01**

Time-Dependent Responses to ActRIIB-Fc Treatment

To evaluate the time course of treatment effects on muscle mass and strength, 10- to 12-month-old mice were evaluated at different time points (3 and 8 days) after initiation of ActRIIB-Fc injections (Figure 7). Mice showed significantly increased plantarflexor muscle mass (11%) and function ($F_{\text{max}}$: 8%; $F_{0}$: 2%; $F_{0} - F_{\text{min}}$: −4%) at 3 days of treatment. The mice treated with the ActRIIB-Fc for 8 days (three injections) showed increased mean plantarflexor muscle mass (14%) and function ($F_{\text{max}}$: 4%; $F_{0}$: −1%; $F_{0} - F_{\text{min}}$: 5%). In a separate study (Figure 7), the mice treated with ActRIIB-Fc for 14 days had increased mean plantarflexor muscle mass (18%) and function ($F_{\text{max}}$: 18%; $F_{0}$: 16%; $F_{0} - F_{\text{min}}$: 17%). After 30 days of treatment (nine injections), the results suggested profound increase in fast fatigable muscle strength (Figure 7).
ActRIIB-Fc Increases and BMD and Bone Biomarker in Mice

We found ActRIIB-Fc treatment twice a week for 30 days of 10-month-old male mice increased BMD significantly in whole, distal, and central femur of adult mice (Figure 8A). To gain mechanistic insight into the observed bone phenotype, we measured the bone formation biomarker P1NP in serum (Figure 8B). A dose-dependent increase in P1NP serum levels was found in ActRIIB-Fc-treated mice.

DISCUSSION

Lee and colleagues (10) have shown that systemic delivery of a soluble form of the ActRIIB can result in dramatic increases in muscle size of growing animals (<5 months of age). In agreement with these and other findings in young growing animals (1, 10, 32, 33), we report here adult mice treated with ActRIIB-Fc had enhanced muscle growth, muscle strength, and increased BMD; in addition, both 18-month-old mice and castrated older
Figure 6. Fatigue envelopes of the in situ assays on the ActRIIB-Fc (10 mg/kg) treated 18-mo-old mice (A; n = 10/group) and 5-mo-old ORX mice (B; n = 7 [vehicle] and n = 4 [ActRIIB-Fc]).

Figure 7. In situ force parameters and plantarflexor mass (% change) of 10- to 12-mo-old mice treated with ActRIIB-Fc for 3 (n = 10/vehicle; n = 9/ActRIIB-Fc), 8 (n = 10/group), and 18-mo-old (n = 10/group) and 5- to 6-mo-old ORX (n = 7/vehicle; n = 4/ActRIIB-Fc) mice treated with ActRIIB-Fc for 30 d. The force parameters and plantarflexor mass were converted into percentage with respect to the mean of the corresponding vehicle-treated group. Statistical significance was analyzed using the unpaired t test. *p < .05, **p < .01.

Figure 8. ActRIIB-Fc treatment increased bone mineral density (BMD) in distal and central femur and serum Procollagen 1 N-terminal peptide (P1NP). C57BL/6 mice (10-mo old) were treated with 10 mg/kg (intraperitoneal injection) twice a week for 30 d. (A) BMD of whole, distal, and central femur (n = 10 [vehicle] and n = 7 [ActRIIB-Fc]). (B) Elevated levels of P1NP after treatment with ActRIIB-Fc (n = 10/group).
mice benefit from ActRIIB-Fc treatment with increased muscle mass and improved muscle function. Previous work has shown an ActRIIB-Fc can bind multiple TGF-β ligand family members (34–36). Because the protein used for these studies has minor modifications compared with previous studies, we conducted in vitro evaluation of effects on TGF-β ligand signaling. The ActRIIB-Fc fusion protein exhibited high potency (IC50: 100–200 nM) in vitro as shown in a cell-based reporter and cell proliferation assays.

ActRIIB-Fc protein was well tolerated and exhibited low clearance and relatively long half-life after single dose in mice (37). There was an apparent decline in circulating levels after the fifth week of dosing; because this is a fusion protein with the human amino acid sequence, this decline may be due to immunogenicity and an increase in clearance. The good correlation (r = .7) between increased muscle mass and circulating ActRIIB-Fc levels supports the efficacy of this treatment in 18-month-old mice. Moreover, we provide the first biological demonstration that ActRIIB-Fc treatment in 18-month-old and ORX mice resulted in significantly enhanced muscle strength.

A study has shown whippet dogs heterozygous for a myostatin mutation have significantly enhanced racing performance and were overrepresented in the top racing classes (4). Similarly, decoy receptor proteins for myostatin, for example, soluble ActRIIB-Fc fusions proteins, can also affect a dramatic increase in muscle mass in rodents (10, 11).

Our data showed that the ActRIIB-Fc treatment predominantly enhanced the fast fatigable muscles function, which is in agreement with data from heterozygous whippet racing dogs adapted to short high-intensity races (4, 38). Furthermore, myostatin is expressed in muscle with enriched type II fibers, especially type IIx and IIB fibers, which are also the fiber types that are significantly increased in the myostatin-deficient animals (38–41). Conversely, myostatin upregulation is observed in patients treated with glucocorticoids and in diseases that cause type II fiber degeneration (42, 43). However, it has been reported the soluble ActRIIB can enhance type I fibers in addition to type II fibers (44). Enhancement of type I fiber function is unlikely to be detected in the current in situ assay system, and therefore, we cannot determine whether changes in type I fiber function occurred. Type I fibers comprise only a small portion of the total myofibers in the plantarflexor, and the power of type I fiber contraction is several fold lower than fast-twitching fibers (29, 45). It would be interesting to further characterize the potential contribution of changes in type I fiber function in response to ActRIIB-Fc treatment in these types of studies.

Retired breeder mice showed significantly increased lean mass (~10%), increased hind-limb muscle mass (~15%–30%), and increased myofiber size (~15%) as determined by histogram analysis in response to ActRIIB-Fc treatment (data not shown). Here, we have shown the enhanced plantarflexor muscle strength after administration of ActRIIB-Fc. The in situ assay data support the interpretation that ActRIIB-Fc enhanced plantarflexor strength in part due to the enhancement of fast-twitch fiber (eg, type IIB and type IIX) function because the F0 – Fm phys and the slope 2, but not Fm and slope 1, show the most significant increases (see Table 1 and Figure 5) (46). Treatment of older male mice with 10 mg/kg ActRIIB-Fc for 4 weeks also resulted in an increase of plantarflexor muscle weight by 18% accompanied by an increase in maximal muscle force of 15%, as measured in the in situ assay. Importantly, the positive treatment effects on muscle parameters in 18-month-old mice suggest animals still respond to ActRIIB-Fc even at a stage in life where senescence and increased mortality are observed.

Previous data have suggested myostatin and activin may negatively affect bone mass (47–50). There have been conflicting reports of the role of activin in bone physiology. Some studies report activin promotes bone formation (51, 52), whereas other studies have shown activin inhibits osteoblast differentiation and enhances osteoclast activity (53–55).

Immunohistochemical analyses showed activin beta A, ActRI, and ActRII were localized in proliferating chondrocytes and osteoblasts in tibiae of neonatal rats and decreased with aging in the tibiae and with progressing endochondral bone development indicating that activin/Bone Morphogenetic Protein (BMP) activity is involved in bone modeling (56). More recently, it was shown treatment with ActRIIA-Fc increased bone formation, bone mass, and bone strength in normal and ORX mice (47, 57). Adding to these findings, data from recent studies implied myostatin may negatively affect bone formation (58, 59). Taken together these data suggest ActRIIB-Fc, a potent inhibitor of activin and myostatin activities, might have a positive effect on bone formation (32, 33). In this study, we show mice treated with ActRIIB-Fc for 30 days exhibited a significant increase in BMD at cortical and cortical/trabecular bone regions of the femur. The significant and dose-related increase in serum collagen I synthesis marker (P1NP), indicative of an increase in bone formation, is mechanistically consistent with the significantly increased BMD in aged mice. Further experimentation will be required to better understand the cellular mechanisms and to solidify an increase in bone-forming activity by ActRIIB-Fc treatment. Similar to the increased BMD reported by genetic loss of myostatin function, the efficacy of ActRIIB-Fc could be explained at least in part by a decoy effect on myostatin (60). BMP6 function, or one of its downstream signaling components, was partially inhibited by addition of ActRIIB-Fc in a cell-based assay that monitors endogenous alkaline phosphatase activity using a pluripotent mesenchymal muscle/bone precursor cell line (data not shown); the effect of ActRIIB-Fc on bone may result from antagonizing multiple ligands of ActRIIB such as activin, myostatin, and BMPs.
Aging is associated with upregulated myostatin expression in humans (61). Previous work has demonstrated inhibition of myostatin signaling using a truncated form of myostatin, ant-1, resulted in improved recovery from injury in aged mice (62). LeBrasseur and colleagues showed treatment for 4 weeks with a myostatin neutralizing antibody did not have a detectable effect on peak tetanic force; however, fatigue was significantly attenuated in the plantar flexor muscle group in 24-month-old mice using a similar in situ method for force determination (63). Murphy and colleagues showed a myostatin neutralizing antibody prevented age-related reduction in body mass and enhanced maximum in situ force in the tibialis anterior muscles of 18-month-old mice treated for 14 weeks (64). It is not known whether the differences in efficacy of the myostatin neutralizing antibody are due to differences in the age of the animals examined or in the length of treatment. Our results show treatment of 18-month-old mice with ActRIIB-Fc has a robust effect on muscle mass and muscle force parameters in a short period of time; it is possible inhibition of additional ligands besides myostatin has greater efficacy than the neutralizing antibody. A direct comparison is needed to address this question.

Hormonal changes including reduction in circulating T and growth hormone lead to reduced myoanabolic tone in humans and this contributes to the development of age-related muscle loss (65, 66). Aged rodents also express significantly increased levels of active forms of myostatin, and treatment with T has been demonstrated to repress myostatin levels (23). ORX mice show muscle mass reduction and upregulated myostatin and may represent a hypogonadal model of sarcopenia (67). Koncavevic and colleagues have shown treatment of young growing ORX mice with ActRIIB-Fc for 71 days resulted in a 26% increase in muscle mass (58). Treatment with the ActRIIB-Fc protein had a similar effect in older ORX mice in our study. Here, we show ActRIIB-Fc also improves muscle function. As in 18-month-old mice, the fatigue envelope analysis indicated that type IIb and IIx fibers were predominantly enhanced in ORX mice. It is well documented ORX mice show reductions in lean body mass (68,69). The enhancement of muscle function of ORX mice demonstrated here by ActRIIB-Fc treatment suggests modulation of muscle growth can occur independent of androgen status, thus substantiating the hypothesis of ActRIIB-Fc as a potential therapeutic treatment for sarcopenia in hypogonadal men.

Agents like ActRIIB-Fc target multiple components of the TGF-β pathway. Although they may have benefits on multiple tissues such as bone and muscle, they also may exert direct or indirect effects on other tissues. Further careful characterization of the consequences of pharmacological inhibition of members of this pathway, as well as genetic loss of function or overexpression, will be required to understand the relative role of each ligand in muscle and how this pathway can be best manipulated for therapeutic intervention.

**References**


