Systemic administration of Follistatin288 increases muscle mass and reduces fat accumulation in mice

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The present study describes the physiological response associated with daily subcutaneous injection of mice with recombinant follistatin288. This systemic administration of follistatin288 increases the follistatin levels in serum, indicating that the protein enters the circulation. The data suggest that a dose-dependent increase in body lean mass also occurs, together with an increase in muscle mass, possibly as a result of an increase in the size of the muscle fibers. After thirteen weeks of treatment, metabolic changes were observed; additionally, the switching of muscle fiber types was also apparent through myosin heavy chain remodeling, implying that changes are occurring at the molecular level. Furthermore, an increase in the muscle mass was associated with a significant decrease in the body fat mass. Overall, this study raises the possibility for the use of follistatin288 as an agent to treat muscle wasting diseases and/or to restrict fat accumulation by systemic administration of the protein.

The role of transforming growth factor-β (TGF-β)-mediated signaling has been well established in several essential cellular and developmental processes, including differentiation, migration, proliferation, survival and adult tissue homeostasis1–3. TGF-β is a superfamily of cytokines that are ubiquitously expressed in a range of species, from worms and flies to mammals. Members of this superfamily function by binding specific cell surface receptors (type I & II), which, in turn, activate the Smad proteins. The activated Smad proteins undergo nuclear translocation and, together with other transcriptional co-activators and co-repressors, regulate the expression of downstream target genes4–6. In addition to the canonical Smad mediated signaling, TGF-β functions also mediate other non-Smad pathways, including MAP Kinase, p53, PI3/Akt, JNK and NFκB pathways6,7. Furthermore, the diversity of TGF-β functions arises through its regulation at multiple levels, beginning at the ligand, the receptor and also the level of the transcriptional activation complex formation8–10. Within the TGF-β superfamily, the activity of the growth differentiation factor (GDF) family proteins has drawn increasing attention. The GDF family was discovered to have possible therapeutic applications in the treatment of muscle wasting diseases or muscle loss conditions that are associated with other pathological conditions, including obesity and aging. In this regard, the discovery of GDF-8 (popularly known as myostatin) as a negative regulator of muscle growth raises the possibility of developing new targets to limit its function in the body, thereby facilitating muscle growth. The use of multiple pharmacological inhibitors to block the activity of myostatin11–14, as well as genetic alteration studies15,16 in animals, is very inspiring, and several clinical trials targeting this pathway to treat muscle wasting are ongoing. However, the recent development of endogenous TGF-β inhibitory proteins provides new insight into the regulation of TGF-β function in muscle development. In this regard, Follistatin (FST), a potent myostatin antagonist, seems a good candidate with potential for use as a therapeutic agent. FST antagonizes myostatin activity by binding to it and also by interfering with the binding of myostatin to its receptor17–19, but in vivo studies indicate that myostatin may not be the only regulator of muscle mass and may not be the only target of FST20. Direct interaction between follistatin and myostatin has been established17 and inhibition of TGF-β signaling by follistatin has been reported20. The actual mechanism of action of FST is unclear, but the use of FST to stimulate muscle growth has been considered for therapeutic application13,21,22. In the present study, the strategy was to introduce recombinant FST288 into animals via daily subcutaneous injection. Continuous monitoring of the physiological response associated with the daily injection showed an increase in the lean mass in a dose-dependent manner, and by thirteen weeks, a significant increase in the muscle mass was observed. The results indicate that the increased muscle mass is caused by an increase in the average size of the muscle fiber. Moreover, a switch in the muscle fiber type was observed as a result of myosin heavy chain
Figure 1 | Recombinant FST288 is biologically active. (A) Coomassie blue stained full-length gel image of purified FST288 (left panel), and western blot probed with the anti-FST antibody (right panel). (B) Endotoxin in the protein preparation. The amount of endotoxin was determined (dotted line) from a standard curve in the assay. (C) Activin-induced inhibition of the MPC-11 cell proliferation is blocked by the addition of increasing amounts of recombinant FST288 in the presence of 5 ng/ml activin.
in the lean mass/body mass ratio, and a dose-dependent response was observed in all three experimental groups (figure 3D). In contrast to the lean mass, there was a progressive increase in the fat mass in the FST 0 group during the experiment, and interestingly, the fat mass in the FST 100 group was found to decrease following administration of FST288 (figure 3E). The decrease in the fat mass in the FST 100 group appeared significantly different from that in the FST 0 group from the 9th week onwards; however, the fat mass of the FST 10 and 30 groups appeared significantly different from that of the FST 0 group only at the end of the 13th week. Additionally, the measurement of fat mass/body mass ratio at the end of the course of administration demonstrated an increase in the amount of fat accumulation in the FST 0 group during the 13 week experiment but a decrease in the FST 100 group (figure 3F). The difference in the ratio between the FST 30 & 100 groups and the FST 0 group is statistically significant, and the average values indicate a dose-dependent effect on fat loss in the three experimental groups (figure 3F). A comparison of the ratio of lean mass/body mass and fat mass/body mass data (figure 3D & 3F) demonstrated that the administration of FST288 stimulates lean mass gain while reducing fat accumulation in the body at the same time in a dose-dependent manner.

**Postmortem dissection of tissues indicates muscle growth with decreased fat accumulation.** At the end of 13 weeks, FST288 administration was stopped, and the animals were euthanized. Individual tissues were removed and weighed and then preserved for further analyses. As mentioned earlier, animals receiving 100 µg/day FST288 showed the maximum effect of all tested doses on the body composition measurements; this result prompted the decision to analyze tissue specific changes between the FST 100 and FST 0 groups (control). Analysis of the individual muscle weights of the hind limbs of the animals demonstrated a significant increase in weight in the levator ani (approximately 34%), gastrocnemius (approximately 41%), and extensor digitorum longus muscle (EDL) (approximately 29%) in the FST 100 group compared to the control group (figure 4A). Additionally, a 8–18% weight increase in the tibialis anterior, soleus and quadriceps muscles in the FST 100 group was observed. Although weight gain was observed in the kidney and prostate tissues in the FST 100 group animals, the variability rendered the difference non-significant. The combined data indicate that there is an increase in the muscle mass in the FST 100-treated animals. The increase in the dissected muscle mass was validated by a visual comparison between the FST 100–treated mice and control mice (figure 4B–J). A comparison of the abdominal visceral fat accumulation in the FST 100 group shows a marked difference (figure 4) from the control animals, consistent with the fat mass data obtained by EchoMRI analysis.

**FST288 administration induces muscle fiber hypertrophy with myofiber remodeling.** To understand the anatomical changes in the muscle fiber organization, a histological analysis was performed on three isolated muscle tissues, namely, the gastrocnemius, tibialis anterior and EDL. Frozen cross-sections (8 µM) from the midbelly of each muscle were immunostained with the anti-laminin antibody, and the area within the laminin stained fibers was determined using Vision assistant software. Approximately two thousand fibers randomly selected from tissues belonging to the same group were analyzed, and the frequency distribution of the fiber area for all three muscles was plotted (figure 5A, B & C). Compared to the FST 0 group, the FST 100 animals demonstrated an increase in the average number of fibers with higher cross-sectional area in the distribution of all three muscles. Moreover, the average fiber area of the FST 100 group was significantly higher than that of the FST 0 group muscles (figure 5D, E & F) (approximately a 11%, 13% and 23% increase in the tibialis, gastrocnemius and EDL muscles, respectively). This observation indicates that there was hypertrophy of the muscle following FST288 treatment and that caused the increase in muscle mass. The total number of fibers was not significantly different in the control and FST 100 group muscles (tibialis and EDL) (data not presented).

Skeletal muscle fibers are heterogeneous in terms of their size, fiber type and activity. This heterogeneity fulfills a variety of functions by a variety of muscles in the body; however, in response to changes in the metabolic activity and/or performance demand, skeletal muscles undergo remodeling of the fibers to meet functional requirements. To understand the changes in the myofiber type in the FST 100 treated animals, immunohistochemistry was performed using myosin heavy chain (MHC) isoform specific antibodies in the three muscles gastrocnemius, tibialis and EDL. Skeletal muscles are composed of four types of myosin heavy chain isoforms (type I and IIA, B
& X), and isoform-specific mouse monoclonal antibodies were used for immunodetection. It should be noted that the isoform specific antibodies were raised in mice, thereby restricting the ability to co-stain a single muscle section for multiple isoforms. As a result, consecutive sections from the same muscle were stained with MHC I and II A, B & X-specific monoclonal antibodies separately and co-stained with laminin and DAPI. As shown in figure 6, a change in the distribution of the MHC isoforms was observed in the muscles from the FST 100 group compared to the FST 0 group (see Supplementary figure S1–S3). Thus, the data suggest that in the gastrocnemius and tibialis, MHCI, MHCIIA & X are present in clusters in specific regions of the muscle, whereas MHCIIIB is ubiquitous. However, EDL presents an almost homogenous distribution for all MHC isoforms tested. Careful observation indicates that compared to the FST 0 group, the number of MHCI & IIA fibers decreased and the number of MHCIIIB fibers increased in all three muscles tested in the FST 100 group. However, the numbers of MHCIIIX fibers either increased or were not affected by FST 100 administration.

FST288 administration alters the metabolic rate and activity of mice. To understand changes in the physiochemical status and activity of animals that result from FST288 treatment, metabolic measurements were performed on four animals from each of the FST 0 & 100 groups. The volume of oxygen consumption (VO2) and the volume of carbon dioxide (VCO2) production were calculated based on the fraction of gas passing through the input

Figure 3 | Growth and change in body composition in response to FST288 administration to mice. (A) Time course of the average body weight from all four groups, as indicated, represented as the percent of baseline. (B) Lean mass/fat mass ratio of animals, as indicated, after 13 weeks of treatment with FST288. (C & E) Time courses of lean mass and fat mass measurement, respectively, during the period of FST288 administration, plotted as the percent of body weight. (D & F) Lean mass/body mass and fat mass/body mass, respectively, of animals, as indicated, after 13 weeks of administration of FST288, plotted as the change (difference) from the baseline values. *, ** and *** indicate p < 0.05, < 0.01 and < 0.001, respectively. p-value measurements in A, C and E are between FST 0 and FST 100.
and at the exhaust point along with the air flow through the cage. An integrated program conducted the experimental operation and data collection. The data analysis indicated that the VO₂ of the FST 100 group was significantly lower than that of the control animals (figure 7A). The respiratory exchange ratio (RER) is an indicator of metabolism that describes which fuel (carbohydrate or fat) is utilized to supply the body with energy, and it is calculated as the ratio of the volume of CO₂ produced over the volume of O₂ consumed (VCO₂/VO₂). The RER value is 1.0 when only carbohydrates are utilized, and the ratio is 0.7 when fat is predominantly utilized as the fuel source to supply the body with energy. The average RER value for the FST 100 group animals was significantly increased compared to that for the FST 0 group animals, as shown in figure 7B. This observation indicates that after 13 weeks of administration, the FST 100 group animals use glycolytic metabolism more than oxidative metabolism, which is consistent with the observed decrease in fat mass in the body. Total body energy expenditure is calculated as the equivalent of heat produced, which is determined from the RER and O₂ consumption values by a method known as indirect calorimetry. These data indicate that the energy expenditure of the FST 100 group animals was significantly lower (approximately 15%) than that of the control animals (figure 7C). To correlate the metabolic rate with physical activity, spontaneous locomotor activity measurements (X-axis ambulatory activity) were performed. As shown in figure 7D, the X-axis ambulatory activity of the FST 100 animals was significantly higher (approximately 21% higher) than that of the control animals.

**Discussion**

The present study describes the expression of the protein follistatin 288 (FST288) in *E. coli*. This protein was found to be biologically active and enters the systemic circulation after subcutaneous injection. The increased amount of FST288 in the serum of animals receiving a higher dose of FST288 indicated that the exogenous recombinant protein level in the serum can be altered by varying the amount of protein administered and that, more importantly, the
injected FST288 circulated in the body without undergoing degradation (as revealed by gel electrophoresis mobility) (figure 1 and 2). Many physiological responses resulted from FST288 administration. First, a progressive effect was observed over the course of administration. However, continuous monitoring of the mice indicated that the weight gain by the control group (which did not receive FST288) was greater than that of the experimental groups. The lean mass/fat mass ratio became progressively lower for the control group (FST 0) over the course of the experiment, whereas this ratio increased in a dose-dependent manner for the experimental groups (Figure 3). More specifically, the data indicate that the loss of fat mass is associated with muscle mass gain.

The function of follistatin as a stimulator of muscle mass has been established in transgenic animals, but the current study indicates that the muscle mass gain is also associated with a concomitant fat loss. The fat loss was revealed by EchoMRI measurements, in addition to the visual difference in the visceral fat accumulation between the control and the FST 100 group animals. Furthermore, this study clearly indicates that follistatin is also a regulator of fat metabolism. The increase in muscle mass is possibly due to the increase in the average muscle fiber size (figure 5). Additionally, the data suggest that follistatin treatment modifies the muscle contraction machinery at the molecular level. Skeletal muscle is composed of four types of muscle fibers, which can be distinguished based on the expression of four MHC isoforms (MHC I, IIA, IIB and IIX). Type I and IIA fibers are employed for slow twitches, whereas type IIB and IIX fibers are utilized for the fast movement of the muscle. The ratio of the four types of fibers present in the skeletal muscle varies in the muscle groups present in the body. Furthermore, the dynamic nature of the fiber types is due to alteration of their relative ratios in response to muscle activation, such as exercise, electrical stimulation, or in response to hormonal stimulation. MHC isoform types have been reported to switch in skeletal muscles depending on the physical demands, and the remodeling of MHC compositions has been established to occur, by multiple experimental approaches, although the underlying mechanism is not yet clear. A combination of in vivo and in vitro studies indicates that intracellular calcium release in response to motor neuron stimulation plays a vital role in regulating calcineurin activity. Slow twitch fibers maintain a higher level of muscle mass.
The most important observation of the current study is the physiological response detected to the injected recombinant protein, which leads us one step closer to the therapeutic administration of follistatin. The use of steroids as a stimulator of muscle mass is common amongst athletes, but the associated adverse effects restricts steroid use for the treatment of muscle degenerative diseases as well as age related mobility limitations. While scientists are trying to develop Selective Androgen Receptor Modulators (SARMs), follistatin could be a new candidate for future therapeutic application. Moreover, systemic administration as utilized in the current study has several advantages for clinical applications. With a daily injection, the doses can be controlled, and the drug can be administered as needed. Thus, overall, the study opens up the possibility of testing FST288 as a therapeutic agent to reverse muscle wasting conditions and aids our understanding of the functional mechanism of follistatin.

**Methods**

**Expression and purification of human FST288.** Follistatin 288 (FST288, amino acid residue no. 30–317) cDNA was cloned into the SalI/XhoI site of pET30 and expressed with a His-tag in E. coli BL21(DE3) by induction with IPTG. The cells were then resuspended in buffer containing 100 mM Tris-HCl, pH 8.0, and 10 mM NaCl and were sonicated on ice. The insoluble portion of the lysed cell suspension was separated by centrifugation, and the pellet was solubilized with a buffer containing 50 mM Tris-HCl, pH 8.0. FST288 was purified with a HisPur cobalt spin column (Thermo scientific, Rockford, IL) according to the manufacturer’s instructions. The purified protein, in elution buffer containing 8 M urea, was diluted (1:4) with 200 mM Tris-HCl, pH 10.0, and 2 mM DTT and incubated on ice for 4–5 hours. The diluted protein was dialyzed against Tris buffer stored at −80°C with 15–20% glycerol.

**Activity assay of the recombinant FST288.** The biological activity of the recombinant FST288 was assessed by testing its ability to neutralize the growth inhibitory effect of activin on mouse MPC-11 cells, as described previously by Phillips et al. Briefly, 1000 viable cells were seeded per well in a 96-well plate in a volume of 0.1 ml DMEM supplemented with 10% fetal calf serum, Pen-Strep and 25 µM β-mercaptoethanol. Activin (Stemgent, MA) or recombinant FST288 in the presence of activin was added, as indicated, to the culture medium, and cells were allowed to grow for 48 hours. Finally, 0.25 µCi of [3H] thymidine (6.7 Ci/mmol) was added to each well, and the cells were incubated for another 24 hours. The incorporation of [3H] thymidine into the cells was measured with a Cerenkov counter.

**Animal experiments.** Animal experiments were conducted in accordance with the guidelines established and approved by the Institutional Animal Care and Use Committee (IACUC) of Boston University. Eight weeks old C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and housed in the Laboratory Animal Science Center (LASC) of Boston University Medical Center. Animals were allowed to recover for one week in the animal facility prior to the start of the experiments. The recombinant FST288 in PBS containing 20% glycerol was subcutaneously injected into each animal daily for 13 weeks in a volume of 100 µl for all doses. The experiment was performed on four groups of animals with six individuals per group. The four groups were FST 0, 10, 30 and 100, with a daily FST administration of 0, 10, 30 and 100 µg per animal, respectively.

**Body composition.** The body composition was assessed by two methods: 1) NMR using the EchoMRI-700 instrument and 2) postmortem dissected tissue weights. An EchoMRI-700 (Body Composition Analyzer) from Echo Medical System (Houston, TX) was used to determine the fat mass and lean mass on a weekly basis in conscious, un-anesthetized animals. The body weight of each animal was measured, and the animals were placed individually into a plastic holder with limited restraint. Each scan took approximately 1–2 min. For postmortem measurement of muscle and other tissue weights, the individual muscles/tissues from each animal were dissected at the end of the 13 week administration, and the average weight was presented.

**Muscle morphology.** Dissected muscle, frozen with OCT compound, was cut at the midbelly, and 8 µm sections were transferred to glass slides. The sections were subjected to immunohistochemistry on rabbit anti-laminin Ab-1 antibody from NeoMarkers, Fremont, CA) to outline the muscle fibers for the measurement of fiber areas. For fiber typing, myosin heavy chain specific monoclonal antibodies were obtained from Developmental Studies Hybridoma Bank, University of Iowa. Type I (BA-D5), type IA (SC-71), type IIB (BF-F3) and type IIX (H61) antibodies were used, along with laminin for co-staining. Laminin were stained with APL, and the images were acquired using the Eclipse TE 2000-U fluorescence microscope (Nikon Instruments Inc., Melville, NY). The fiber area measurements were performed using the Vision assistant software (National Instruments, Austin, TX).
Metabolic assessments. Indirect calorimetry was utilized to examine oxygen consumption (VO₂) and carbon dioxide production (VCO₂) of individual mice for 24 hours in the fed status using a comprehensive laboratory animal monitoring system (CLAMS) equipped with an Oxymax Open Circuit Calorimeter (Columbus Instruments, Columbus, OH). VO₂ and VCO₂ values were used to calculate the respiratory exchange ratio (RER) and energy expenditure (heat - kcal/hr).

Spontaneous locomotor (ambulatory) activity was recorded at the same time with indirect calorimetry using the Opto M5 multi-channel activity monitor (Columbus Instruments, Columbus, OH). The data were analyzed using Oxymat for Windows (V4.2) software.

Western blotting. From each animal, at 13 weeks of FST288 administration, 0.5 μl of serum was electrohoresed on a 12% SDS-PAGE gel, and the separated proteins were transferred to a PVDF membrane. The blot was probed with an anti-FST-24 hours in the fed status using a comprehensive laboratory animal monitoring (Graphpad). The body weight and body composition values obtained at the beginning of the experiment were considered a baseline for subsequent analyses. Body weight gain was represented as the percent of the respective baseline values, and lean mass or fat mass was presented as the percent of the body weight. Dissected tissue weights were presented as the percent of control tissue (FST 0) weights.

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Acknowledgments
The author thanks Dr. Gianluca Toraldo and Prabhuha Chauhan for their help and support in conducting the study. The work was supported by the Department of Medicine, Boston University School of Medicine. SSG is currently supported by a pilot project funded by Children’s Hospital Boston George O’Brien Urology Research Center (5P50DK065298-10). The MHC monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Author contributions
S.S.G. designed and performed the experiments, prepared figures and wrote the manuscript.

Additional information
Supplementary information accompanies this paper at https://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Gangopadhyay, S.S. Systemic administration of Follistatin288 increases muscle mass and reduces fat accumulation in mice. Sci. Rep. 3, 2441; DOI:10.1038/srep02441 (2013).

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