

2. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both Myostatin and Activin

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Collaborations:

K Tsuchida kindly provided the FS288 and FSI-I cDNAs used in this work (Nakatani M 2008).

Authors contributions:

H Gilson and JP Thissen designed research.

H Gilson, O Schakman, S Kalista and P Lause performed research.

H Gilson and JP Thissen analyzed data.

H Gilson and JP Thissen wrote the paper.

Abstract

Follistatin (FS) inhibits several members of the TGF- β superfamily, including myostatin (Mstn), a negative regulator of muscle growth. Mstn inhibition by FS represents a potential therapeutic approach of muscle atrophy. The aim of our study was to investigate the mechanisms of the FS-induced muscle hypertrophy. To test the role of satellite cells in the FS effect, we used irradiation to destroy their proliferative capacity. FS overexpression increased the muscle weight by about 37% in control animals, while the increase reached only 20% in irradiated muscle, supporting the role of cell proliferation in the FS-induced hypertrophy. Surprisingly, the muscle hypertrophy caused by FS reached the same magnitude in Mstn KO as in WT mice, suggesting that Mstn might not be the only ligand of FS involved in the regulation of muscle mass. To assess the role of activin (Act), another FS ligand, in the FS-induced hypertrophy, we electroporated FSI-I, a FS mutant which does not bind Act with high affinity. While FS electroporation increased muscle weight by 32%, the muscle weight gain induced by FSI-I reached only 14%. Furthermore, in Mstn KO mice, FSI-I overexpression failed to induce hypertrophy, in contrast to FS. These results therefore suggest that Act inhibition may contribute to FS-induced hypertrophy. Finally, the role of Act as a regulator of muscle mass was supported by the observation that ActA overexpression induced muscle weight loss (-15%). In conclusion, our results show that satellite cells proliferation and both Mstn and Act inhibition are involved in the FS-induced muscle hypertrophy.

Introduction

Increasing size and strength of skeletal muscle represents a promising therapeutic strategy for muscular disorders. One possible new tool is myostatin (Mstn), a TGF- β family member that plays a crucial role in regulating skeletal muscle mass. Mstn, which is expressed almost exclusively in muscle, has been shown to be a potent negative regulator of skeletal muscle growth. Indeed, overexpression of Mstn by transgenesis (31) or gene transfer selectively in skeletal muscle (8) causes muscle atrophy. Conversely, Mstn inhibition or gene deletion increases muscle mass and strength both developmentally (24) and in adult animals (10). Moreover, blockade of Mstn results in functional improvement of dystrophic muscle in the mdx mouse model of Duchenne Muscular Dystrophy (DMD) (4; 45). Thus, Mstn inhibition is an attractive therapeutic approach to treat muscle-wasting diseases such as DMD, cachexia and sarcopenia.

The identification of Mstn binding proteins able to inhibit Mstn activity has led to potential new approaches for postdevelopmental muscle mass enhancement. These Mstn binding proteins include Follistatin (FS) which shows a potent Mstn-inhibiting activity. Indeed, overexpression of FS induces a dramatic increase in muscle mass when overexpressed as a transgene in mice (19) or delivered by Adeno-associated virus (AAV) (11). The increase in muscle mass observed in transgenic mice overexpressing FS in muscle is even significantly larger than that observed in Mstn KO mice (18). The mechanisms involved in the FS effect are however relatively unknown. Since the lack of Mstn results in increased satellite cell activation (16; 38; 43), we investigated the contribution of satellite cells to the FS-induced muscle hypertrophy. FS has been shown to bind other TGF- β family members in addition to Mstn. Therefore, we also assessed whether other FS ligands could act similarly to Mstn in controlling muscle growth.

Materials and methods

Animals

Experiments were performed in rats in order to combine morphological and biochemical analyses on the same muscles. Mice were used in the irradiation experiment and for knock-out models. Six-week-old male Wistar rats (150-160g) provided by Janvier Breeding (Le-Genest-Saint-Isle, France) were used to characterize the muscle hypertrophy induced by FS and the muscle atrophy caused by activin (Act) A. To assess the role of satellite cells, we used 15-week-old adult male FVB mice provided by Janvier Breeding. Finally, to evaluate the role of Mstn and Act inhibition in the FS-induced muscle hypertrophy, we used 8-week-old male FVB wild type mice (WT mice) and Mstn knock-out mice (KO Mstn) harboring a constitutive deletion of the third Mstn exon (10). All animals were housed individually under controlled conditions of lighting (12-h light, 12-h dark cycle) and temperature ($22 \pm 2^{\circ}\text{C}$). The animals were allowed free access to chow and water. The study was conducted in accordance with the directives of the institutional animal care and use Committee of the University of Louvain.

Expression plasmids and DNA preparation

pM1-hFS288, pM1-FSI-I and pM1-ActA-cMyc plasmids were constructed by inserting the hFS288 cDNA, the FSI-I cDNA and the ActA cDNA respectively into the pM1 Expression Vector (Roche Molecular Biochemicals, Indianapolis, IN). hFS288 codes for the human FS containing 288 amino acids and FSI-I codes for a FS-derived Mstn inhibitor which does not affect Act signaling (28). In the pM1-ActA-cMyc plasmid, the mouse ActA cDNA is followed by the tag cMyc. Empty pM1 was used as a control plasmid. Plasmids were amplified in *Escherichia coli* top 10 F' (Invitrogen Corp., Carlsbad, CA) and purified with an EndoFree Plasmid Giga Kit (QIAGEN, Inc., Valencia, CA). Plasmids were stocked at -80°C . The day before injection, plasmids were lyophilized and resuspended in 0.9% NaCl solution.

DNA electrotransfer

Each animal was anesthetized with a mixture of 75 mg/kg ketamine (Ketalar; Pfizer, Oslo, Norway) and 15 mg/kg xylazine hydrochloride (Rompun; Bayer, Fernwald, Germany) administered by IP injection. For rats, the plasmid solution (1 $\mu\text{g}/\mu\text{l}$) was injected into 10 different sites (total volume per muscle = 100 μl) in each tibialis anterior (TA) muscle and the muscles were then electroporated using the electroporation conditions previously described (33). For mice, 30 μl of plasmid solution (1 $\mu\text{g}/\mu\text{l}$) were injected in each TA muscle using a

Hamilton syringe with a 30-gauge needle and the muscles were then electroporated using the electroporation conditions described by Bloquel *et al* (eight pulses of 200V/cm and 20 ms per pulse at 2 Hz (3)).

Gamma irradiation conditions

Local gamma irradiation was achieved with a 250-kV X-ray irradiator (RT 250, Philips Medical System, 0.92 Gy/min) using a 3 cm-diameter circular irradiation field. Mice were anesthetized by an intraperitoneal injection of a mixture of ketamine and xylazine, and placed within a lead shield. The left hindlimb was pulled through a hole in the shield so that only the lower limb containing the TA was exposed to the X-ray. The limb was then subjected to a total gamma irradiation dose of 25 Gy. This dose has been previously shown to prevent satellite cell division (20).

Experimental design

- Characterization of the FS-induced muscle hypertrophy

After one week of adaptation to environment and diet, male Wistar rats (n=7) were electroporated. One TA muscle was injected with the pM1-hFS288 plasmid (left) and the contralateral TA muscle with the pM1 plasmid (right). The rats were sacrificed by decapitation 17 days after electroporation. For biochemical analyses, TA muscles were removed, weighed, deep frozen in liquid nitrogen and stored at -80°C until further analyses. For histological analysis, TA muscles were dissected, weighed and a transverse slice of 0.5 cm thickness was made in the middle belly of the muscle. The transverse slice was further fixed with buffered formol for 48 h and embedded in paraffin.

- Role of satellite cells in the FS-induced muscle hypertrophy

The left legs of adult male FVB mice were first irradiated to block replication of satellite cells and electroporated 5 days later with pM1 or pM1-hFS288. Mice were randomly allocated to one of the four treatment groups: Control (n=7), FS (n=5), Irradiated (n=7), Irradiated+FS (n=7). The left TA were subjected to one of the following treatments: (1) electroporated with pM1 (control or Ctrl), (2) electroporated with pM1-hFS288 (FS), (3) irradiated with 25 Gy (Irr) and electroporated with pM1, and (4) irradiated with 25 Gy and electroporated with pM1-hFS288 (Irr+FS). The right TA of all animals were transfected with the plasmid control pM1. All mice were sacrificed by decapitation 17 days after electroporation. TA muscles were

dissected and a transverse slice of 0.5 cm thickness was fixed with buffered formol for 48 h and embedded in paraffin for morphological analysis.

- Assessment of the muscle hypertrophic effect of FS in Mstn KO mice

The TA muscles of KO Mstn (n=7) as well as WT mice (n=7) were transfected with the plasmid pM1-FS288 (left leg) and the control plasmid pM1 (right leg). The mice were sacrificed by decapitation 17 days after electroporation. TA muscles were dissected and a transverse slice of 0.5 cm thickness was fixed with buffered formol for 48 h and embedded in paraffin for morphological analysis. The remaining ends of the muscles were frozen in liquid nitrogen for biochemical analyses.

- Role of Act inhibition in the FS-induced muscle hypertrophy

In a first experiment, the left TA muscles of WT mice was transfected with the plasmid pM1-FS288 (n=7) or with pM1-FSI-I (n=8) and the right TA muscles received the control plasmid pM1. In a second experiment, the TA muscles of KO Mstn (n=9) as well as WT (n=15) mice were transfected with the plasmid pM1-FSI-I (left leg) and the control plasmid pM1 (right leg). Seventeen days after electroporation, the mice were sacrificed by decapitation. TA muscles were dissected and divided in two parts for morphological and biochemical analyses, as described above.

- Assessment of the muscle atrophic effect of ActA

One TA muscle of male Wistar rats was injected with the pM1-ActA-cMyc plasmid and the contralateral TA muscle with the pM1 plasmid. The rats (n=8) were sacrificed by decapitation 17 days after electroporation and the TA muscles were removed for biochemical and histological analyses as described above.

Muscle protein and DNA concentration measurements

Briefly, 100 mg of TA muscle, previously pestled in liquid nitrogen, were homogenized with Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 1 ml ice-cold lysis buffer (50 mM Tris/HCl [pH 7.5], 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 1 mM phenylmethylsulfonyl-fluoride, 10 µg/mL aprotinin, 10 µg/ml leupeptin, 10 mM β-glycero-phosphate, 1 mM KH₂PO₄, 1 mM vanadate, 50 mM NaF, 10 mM NaPPi). The homogenates were centrifuged for 10 min at 10 000 rpm (Sorvall SS-34 rotor) to pellet myofibrillar proteins. Myofibrillar proteins, resuspended in 8 M urea/50 mM Tris/HCl, pH 7.5, were stored at -80°C as well as

the supernatant containing the soluble proteins. Myofibrillar and soluble muscle protein concentrations were determined using Bradford's protein assay (Bio-Rad, Munich, Germany). The DNA concentration was measured in the myofibrillar and soluble fractions using fluorometry (Jobin Yvon Spectrofluor JY3D).

mRNA analysis by real time quantitative (RTQ)-PCR

Total RNA was isolated from the TA muscles using TRIzol reagent as described by the manufacturer. Recovery was 1 µg/mg of TA muscle. Reverse transcription and RTQ-PCR were done as previously described (7). Accession number for the sequences and primers used were;

Mstn: AY204900 (GGCTTGACTGCGATGAG - ATATAGCATATTAATGGGAGACAT), FS: NM008046 (GGCAGATCCATTGGATTAGCC - TGCCAACCTTGAAATCCCAT), MHCneonatal: XM001080186 (CAGAGGAGGCTGAGGAACAATC - GCCTTTCCTTCAGCCACTTG), MHCIIb: X72590.1 (TAGCTCAATTCCTTCTGTTGAAAGGT - ATTATCTGCAGCTTTTATTTCTTGAT), PCNA: NM022381 (CACCATGTTTGAGGCACGC - GGACATGCTGGTGAGGTTCA), IGF-II: NM031511 (GTCGATGTTGGTGCTTCTCATCT - CGGTCCGAACAGACAAACTGAA, FS288 and FSI-I: NM008046 (GGCTCCGTAAGCGAAGA - CCGTTGAAAATCATCCACTTGAA), ActA (Inhibin beta A): NM017128.1 (GAGGACGACATTGGCAGGAG - TGCAGTGTCTTCCTGGCTGT) , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): AF106860 (TGCACCACCAACTGCTTA - GGATGCAGGGATGATGTTC) used as reporter gene.

Histological analysis of muscle

For the evaluation of the hypertrophic effect of hFS288 and FSI-I, serial sections (5 µm thick) were cut and mounted on glass slides (Superfrost Plus; Menzel-Glaser, Braunschweig, Germany). For immunohistochemistry, sections were deparaffinized and blocked in PBS-BSA (5%) containing normal horse serum (4%) for 30 min at RT. The sections were incubated overnight with a goat polyclonal anti-FS (R&D Systems, 1:20). Primary antibodies were detected by applying for 30 min at RT a biotinylated second antibody which was a horse antigoat conjugated to peroxidase-labeled polymer (Vector Laboratories, Burlingame, CA), followed by applying for 30 min at RT an avidin/biotinylated peroxidase complex (Vectastain ABC kit Peroxidase Standard; Vector Laboratories). Peroxidase activity was revealed with DAB substrate (CHEMICON International, Inc., Temecula, CA), which produces a brown

stain. The sections were counterstained with Mayer's hematoxylin, rinsed, and mounted in Faramount (Dako). Fiber cross-sectional areas (CSAs) were measured with a microscope (Leitz; Leica Microsystems GmbH, Wetzlar, Germany) coupled to an image analyzer system (MOP-Videoplan; Kontron AG, Eching, Germany). To evaluate muscle fiber CSAs, all the positive muscle fibers in the TA transfected with FS gene were counted (pM1-FS288 or pM1-FSI-I). Two hundred negative fibers, randomly chosen and counted in contralateral TA transfected with insert-less plasmid (pM1), were considered as controls.

To detect the fibers transfected with the pM1-ActA-cMyc plasmid, sections were deparaffinized and pretreated in a microwave oven as previously described (34). The primary antibody used was a rabbit monoclonal anti-cMyc (1:800) (Bethyl, Laboratories) incubated for 1 hour.

To evaluate cell proliferation, the deparaffinized and pretreated sections were incubated overnight with a mouse monoclonal anti-BrdU (1:100) (Dako Cytomation, Glostrup, Denmark). The positive nuclei were counted in the whole section of the muscle.

Statistical analysis

Results are presented as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed using a one-way ANOVA followed by a Newman-Keuls multiple comparison test to compare muscles from different animals undergoing different experimental conditions, or a paired *t*-Test to compare muscles undergoing different experimental conditions within the same animal. Interaction between the irradiation and the overexpression of FS was assessed by using two-way ANOVA followed by a Bonferroni post-Test (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego CA, USA).

Results

Postnatal FS overexpression induces muscle hypertrophy

Our results show that FS288 overexpression in TA muscle of rat increased FS mRNA by 22-fold (data not shown) and caused muscle hypertrophy characterized by increased muscle mass (+24%, 711.0 ± 14.0 vs 575.1 ± 10.6 mg; $P < 0.001$) (Fig 1A), fiber CSA (+42%, 1895 ± 100 vs $1337 \pm 45 \mu\text{m}^2$; $P < 0.001$) (Fig 1B) and muscle protein content (+22%, 125.0 ± 4.2 vs 101.2 ± 2.8 mg/muscle; $P < 0.01$) (Fig 1C) 17 days after electroporation. This observation demonstrates that FS288 causes rapid and marked muscle hypertrophy not only when overexpressed early in life (transgenic animals), but also in postnatal life.

As shown in figure 1D, the muscle hypertrophy caused by FS was associated with an increase in DNA content (+28%; 42.0 ± 1.5 vs $32.8 \pm 1.0 \mu\text{g/muscle}$; $P < 0.001$). The hypertrophic muscle contained increased levels of PCNA mRNA (+29%, $P < 0.05$) (Fig 1E), a marker of cell proliferation, and IGF-II (+54%; $P < 0.05$), a potent inducer of myogenesis. Furthermore, FS overexpression increased mRNA levels of neonatal myosin heavy chain (MHC) mRNA (+578%, $P < 0.001$) (Fig 1F), a marker of muscle differentiation, and MHCIib (+31%; $P < 0.05$), the main MHC isoform in TA muscle.

Figure 1

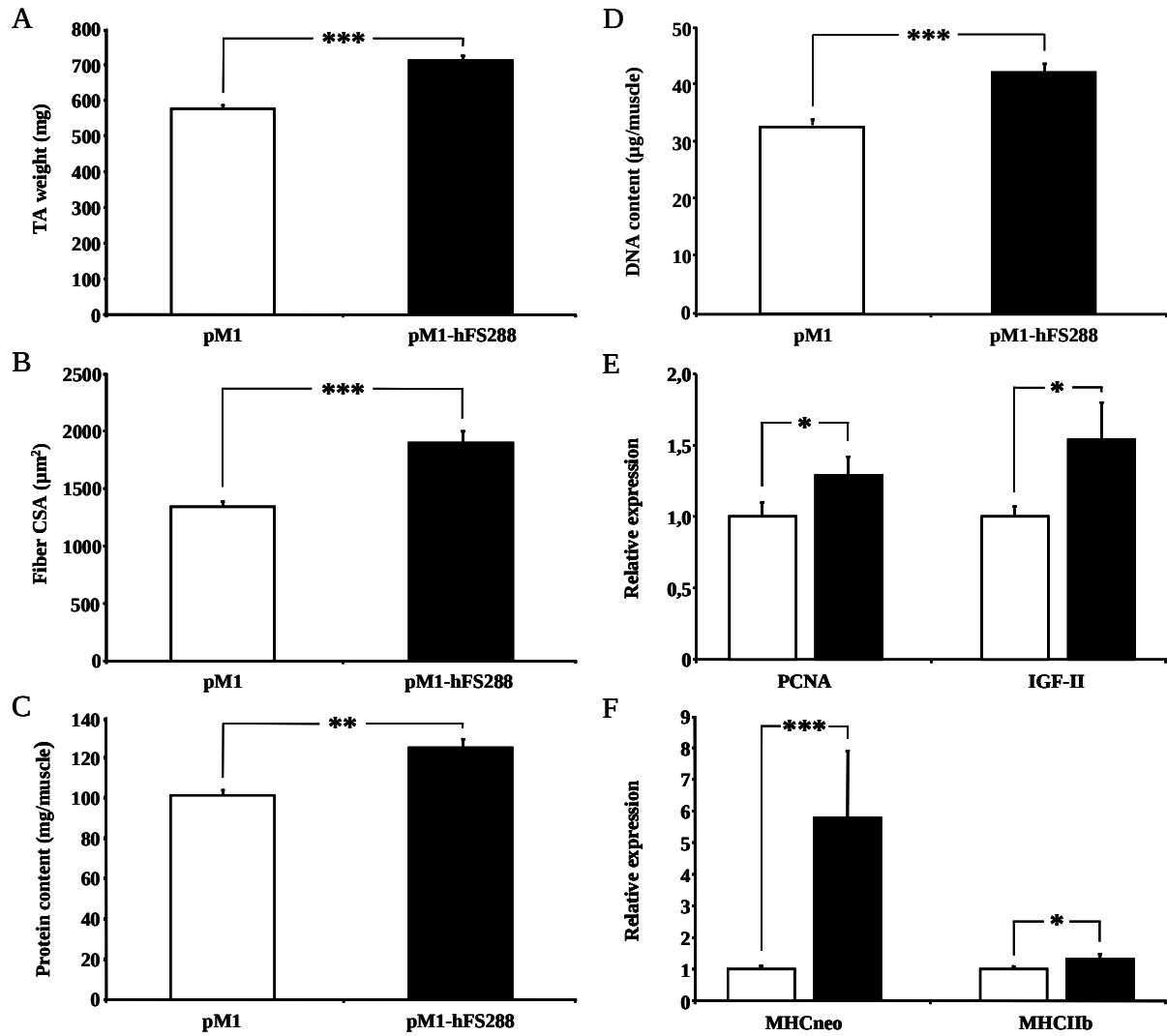


Fig. 1. Overexpression of FS in TA muscle induces muscle (A) and fiber (B) hypertrophy in rat. It also increases the protein (C) and DNA contents (D), and stimulates the expression of PCNA and IGF-II (E), and MHC (neonatal and IIb) (F). All these parameters were measured 17 days after transfection of pM1 (white column) or pM1-FS288 (black column). The results are expressed as mean $\pm$ SEM. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$.

FS-induced muscle hypertrophy is partially mediated by satellite cells proliferation

In order to test whether satellite cells are essential in mediating the hypertrophic effects of FS, we used gamma irradiation to destroy the proliferative capacity of satellite cells in muscle. For technical reasons and to avoid an influence of normal growth on muscle mass, we used adult mice for this experiment. While the muscle weight was increased by 37% after 17 days of FS overexpression in control animals (61.2 ± 2.2 vs 44.9 ± 1.1 mg; $P < 0.001$), the increase was only 20% when muscles had been previously irradiated (55.1 ± 3.3 vs 46.0 ± 2.3 mg; $P < 0.05$) (Fig 2A). Similarly, the increase in CSA of FS-transfected fibers reached 111% in control mice (3856 ± 156 vs $1852 \pm 85 \mu\text{m}^2$; $P < 0.001$) but only 65% in irradiated muscles (3018 ± 206 vs $1843 \pm 63 \mu\text{m}^2$; $P < 0.001$) (Fig 2B). The destruction of the satellite cell proliferative capacity was confirmed by Brdu immunohistochemistry. In our conditions, gamma irradiation indeed decreased the number of positive BrdU cells by 90% in irradiated TA compared to the contralateral muscle (9 vs 90 in Irr group and 6 vs 93 in Irr-FS group, both $P < 0.001$). These results therefore show that satellite cells play a critical role in FS-induced muscle hypertrophy.

Figure 2

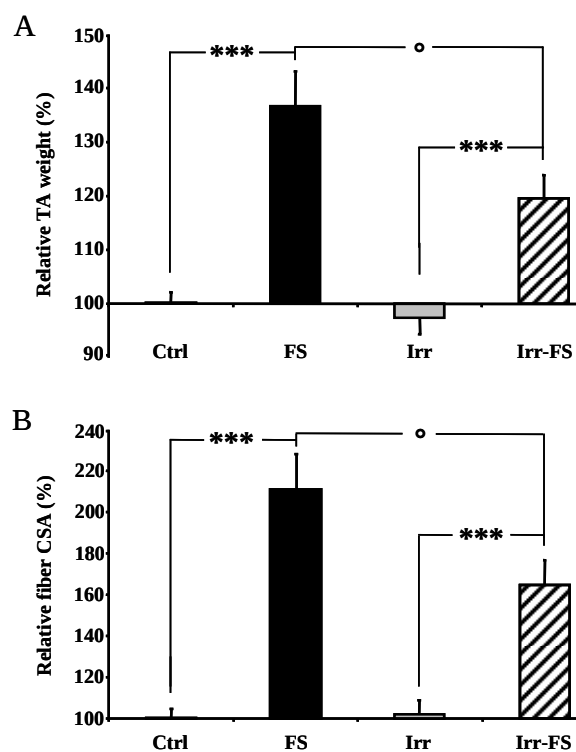


Fig.2. Gamma irradiation partially blunts the FS-induced muscle (A) and fiber (B) hypertrophy in mice. TA mass and the fiber CSA were measured 17 days after transfection of pM1 (white columns) or pM1-FS288 (black and lined columns) and the data are normalized as percentages of the contralateral control muscle. The results are expressed as mean $\pm$ SEM. °, $p < 0.05$ and ***, $p < 0.001$.

ActA inhibition is involved in FS-induced muscle hypertrophy

Since FS is known to bind and inhibit Mstn in skeletal muscle, we evaluated the role of Mstn inhibition in the FS hypertrophic effect. Surprisingly, the muscle hypertrophy obtained by FS overexpression, as assessed by the muscle weight, reached the same magnitude in WT (+41%, 62.0 ± 1.4 vs 44.0 ± 0.9 mg; $P < 0.001$) and KO Mstn mice (+50%, 98.5 ± 4.1 vs 65.5 ± 2.1 mg; $P < 0.001$) (Fig 3A). This result was confirmed by the measurement of the fiber CSA in the two groups (+114%, 3222 ± 218 vs 1509 ± 104 μm^2 ; $P < 0.01$ in WT mice and +87%, 1452 ± 95 vs 2710 ± 76 μm^2 ; $P < 0.001$ in KO Mstn) (Fig 3B).

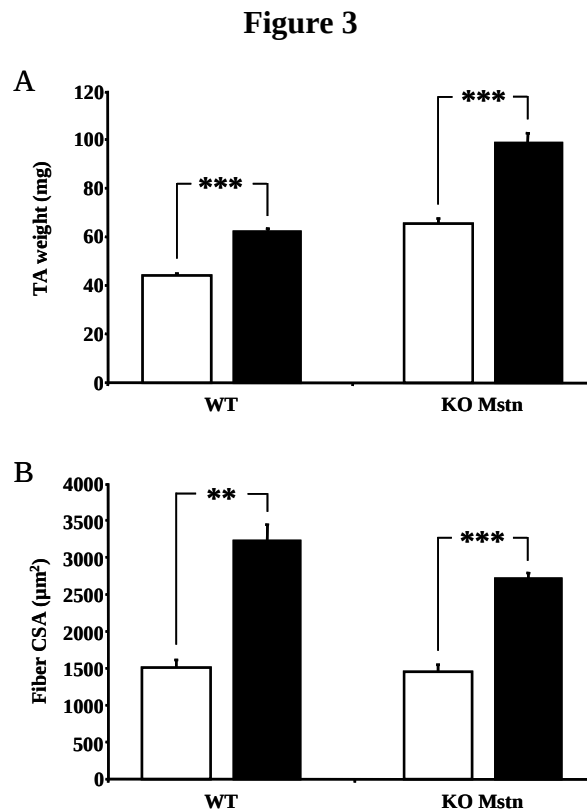


Fig. 3. Overexpression of FS induces comparable muscle (A) and fiber (B) hypertrophy in wild type and KO Mstn mice. TA mass and fiber CSA were measured 17 days after transfection of pM1 (white column) or pM1-FS288 (black column). The results are expressed as mean $\pm$ SEM. **, $p < 0.01$ and ***, $p < 0.001$.

This observation therefore suggests that the FS effect on skeletal muscle is not only due to Mstn inhibition, and that another FS ligand contributes to the FS-induced hypertrophy. We hypothesized that Act could be this FS ligand, because it can be bound by FS and it has been shown to inhibit muscle development *in vitro* (21).

To assess the role of Act inhibition in the FS-induced hypertrophy, we electroporated a FS mutant, FSI-I (28), in wild type mice. Due to the deletion of domain II involved in Act binding, this mutant does not bind Act with high affinity but retains the ability to bind Mstn. Despite a similar overexpression level (132 fold for FSI-I and 87 fold for FS288), FS288 electroporation in WT animals caused a 32% weight muscle increase (65.9 ± 3.2 vs 49.8 ± 1.1 mg; $P < 0.01$), while FSI-I electroporation caused only a 14% weight increase after 17 days (55.3 ± 1.7 vs 48.6 ± 1.4 mg; $P < 0.05$) (Fig 4A). Similarly the 91% muscle fiber hypertrophy induced by FS288 overexpression (3613 ± 271 vs $1883 \pm 97 \mu\text{m}^2$; $P < 0.01$) was reduced to 44% in muscle overexpressing FSI-I (2656 ± 344 vs $1839 \pm 56 \mu\text{m}^2$; $P < 0.05$) (Fig 4B). Considering the similar affinities of FS288 and FSI-I for Mstn, the smaller effect of FSI-I compared to FS288 ($p < 0.01$) suggests that the hypertrophic effect of FS288 is not only due to Mstn inhibition.

Figure 4

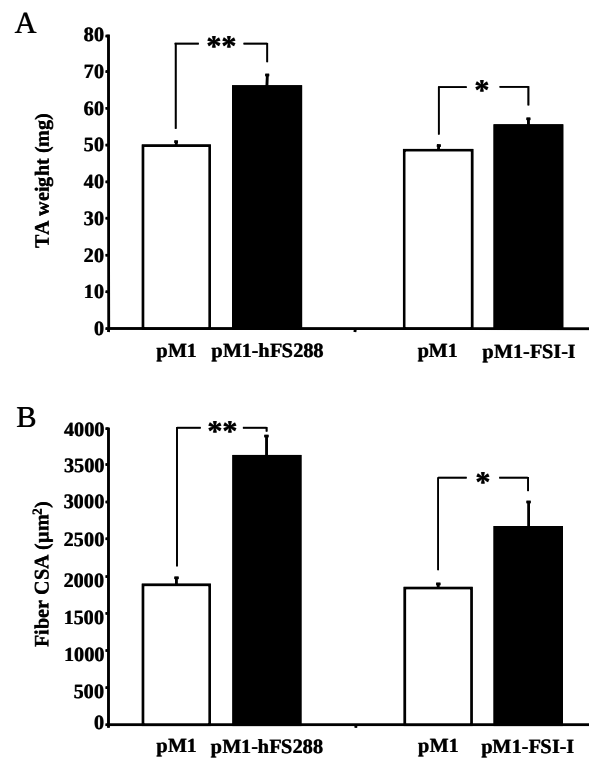


Fig. 4. Overexpression of FSI-I induces a smaller muscle (A) and fiber (B) hypertrophy than FS288 in WT mice. TA mass and fiber CSA were measured 17 days after transfection of pM1 (white column) or pM1-hFS288 / pM1-FSI-I (black column). The results are expressed as mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$.

When electroporated in KO Mstn mice, FSI-I overexpression failed to induce a muscle hypertrophy (+3%, 69.1 ± 3.7 vs $67.0 \pm 3.1 \mu\text{m}^2$; NS) (Fig 5A), in contrast to FS288 which caused a 50% increase in muscle mass in the same animal model (98.5 ± 4.1 vs 65.5 ± 2.1 mg; $P < 0.001$) (Fig 3A). Like for the muscle weight, no effect on fiber size could be detected following the FSI-I overexpression (+6%, 1655 ± 67 vs $1555 \pm 65 \mu\text{m}^2$; NS) (Fig 5B), in contrast to the results obtained with the FS288 (+87%, 1452 ± 95 vs $2710 \pm 76 \mu\text{m}^2$; $P < 0.001$ with FS288) (Fig 3B). These data suggest that most of the hypertrophic effect of FS288 in KO Mstn results from Act inhibition. Therefore, Act may represent a new player in the regulation of the muscle mass.

Figure 5

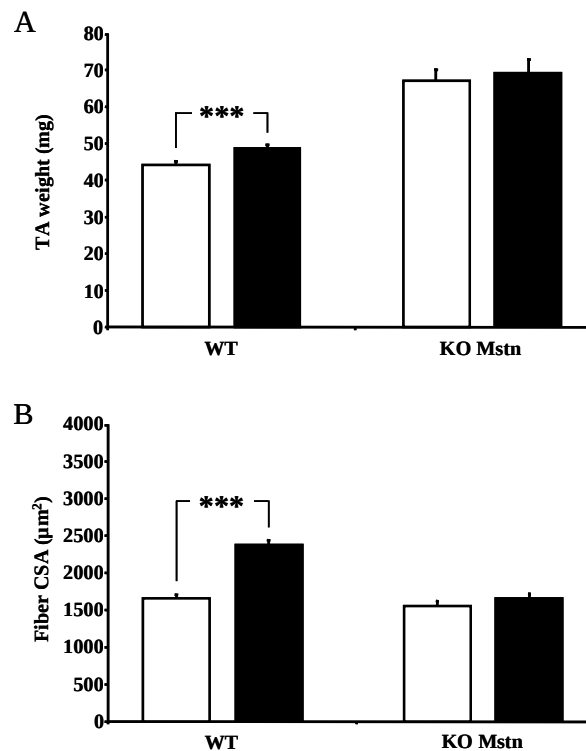


Fig. 5. Overexpression of FSI-I fails to cause muscle (A) and fiber (B) hypertrophy in KO Mstn mice. No increase in muscle mass and in fiber CSA were observed in muscle overexpressing FSI-I in KO Mstn mice compared to WT mice. TA mass and fiber CSA were measured 17 days after the transfection of pM1 (white column) or pM1-FSI-I (black column). The results are expressed as mean $\pm$ SEM. ***, $p < 0.001$.

ActA overexpression induces muscle atrophy

To investigate the potential atrophic effect of Act on skeletal muscle *in vivo*, we overexpressed ActA in TA muscles of rats. Our results show that 17 days after electroporation, the pM1-ActA-cMyc transfection increased ActA mRNA 30-fold (data not shown) and caused a 15% muscle atrophy (519.0 ± 15.3 vs 608.6 ± 18 mg; $P < 0.001$) (Fig 6A). In agreement with muscle weight loss, the CSA was also reduced by 46% in fiber overexpressing ActA (2738 ± 182 vs $1473 \pm 60 \mu\text{m}^2$; $P < 0.001$) (Fig 6B) and the protein content was significantly reduced following ActA transfection (80.7 ± 2.6 vs 92.1 ± 3.7 mg/muscle; $P < 0.01$) (data not shown). These results confirm the fact that like Mstn, ActA is a negative regulator of muscle mass *in vivo*.

Figure 6

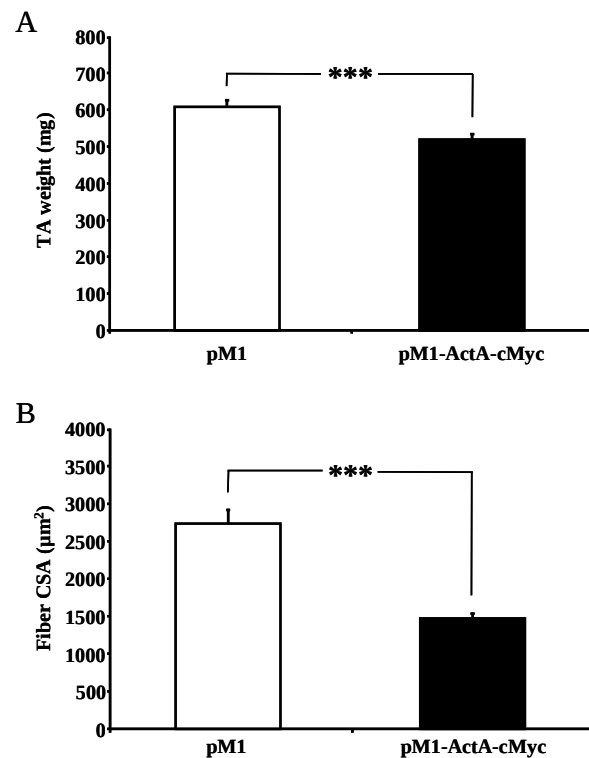


Fig. 6. Overexpression of ActA induces muscle (A) and fiber (B) atrophy in rat. TA mass and fiber CSA were measured 17 days after transfection of pM1 (white column) or pM1-ActA-cMyc (black column). The results are expressed as means $\pm$ SEM. ***, $p < 0.001$.

Discussion

Our study shows that FS overexpression induces skeletal muscle hypertrophy via satellite cell activation and probably increased protein synthesis. Furthermore, our results indicate that FS-induced hypertrophy results not only from Mstn but also from Act inhibition. These observations therefore suggest that, besides Mstn, Act is a crucial player in the regulation of muscle mass.

FS-induced hypertrophy was characterized by increased fiber diameter together with DNA and protein accretion. Muscle DNA content reflects the number of myonuclei, including satellite cell nuclei. Therefore, increased DNA content, as we observed, reflects an increase in the number of myonuclei which is dependent on satellite cell replication. This is entirely consistent with the observation of an increased PCNA expression after FS overexpression. In addition, the differentiation of the satellite cells into new muscle fibers was attested by increased neonatal MHC expression, a marker of myogenesis. On the other hand, increased muscle protein content probably reflects accelerated protein synthesis, in particular of myofibrillar proteins, such as MHCIIB as suggested by increased MHCIIB mRNA. Indeed, infusion of FS has been reported to increase muscle protein synthesis in neonatal rats (41). All these changes that we observed strongly suggest that FS induces muscle hypertrophy by inhibiting Mstn. Indeed, Mstn has been reported to inhibit satellite cell proliferation (38; 43) and differentiation (17) as well as protein synthesis (42; 47; 48), in particular MHCIIB (12). Furthermore, Mstn is abundantly expressed in TA muscle, a muscle mainly composed of IIB myofibers which strongly express Mstn (5; 32). These results are in accordance with previous studies which showed that FS overexpression, either by transgenesis or AAV-mediated gene delivery, causes a dramatic increase in muscle mass (11; 19). In these studies, however, only the long term effect of FS overexpression was considered, in contrast to our study reporting a marked muscle hypertrophy occurring as early as after 17 days.

The implication of satellite cells in the FS-induced muscle hypertrophy, suggested by the increased DNA and PCNA mRNA contents, was directly assessed by destroying their proliferative capacity using gamma irradiation. By combining gamma irradiation with FS gene transfer, we were able to tease apart the mechanisms by which muscle hypertrophy is induced by FS. Our results indicate that satellite cells contribute to the FS-induced hypertrophy, but that FS is still able to stimulate muscle growth even when their proliferative capacity has been previously destroyed. This observation suggests that FS causes muscle hypertrophy by stimulating muscle protein synthesis, a hypothesis recently demonstrated (41).

We are confident that irradiation achieved blockade of satellite cell activation, as DNA synthesis assessed by Brdu staining was dramatically reduced in irradiated muscle. Since the action of Mstn on muscle development relies heavily on its ability to down-regulate satellite cells activity (22; 38), our interpretation is that FS causes muscle hypertrophy by inhibiting Mstn. Interestingly, muscle hypertrophy induced by IGF-I, an anabolic growth factor, is also mediated by a combination of satellite cell proliferation and increased protein synthesis (2). This work is therefore the first to demonstrate the contribution of satellite cells in FS-induced muscle hypertrophy.

The observation that FS overexpression can cause substantial muscle hypertrophy in mice lacking Mstn indicates that FS must exert its effect on muscle growth by targeting ligands other than Mstn. This conclusion is also supported by the quadrupling muscle mass phenotype observed in Mstn KO mice carrying a FS transgene (18), which represents yet another doubling of muscle mass compared to mice lacking only Mstn. Our present findings argue therefore that FS overexpression, even in the postnatal period, could increase muscle growth in the absence of Mstn. The candidate ligands could include Act and GDF-11, the latter sharing 90% homology in amino acid sequence with Mstn (24; 25; 27). Indeed, both are high affinity FS ligands (35; 36; 44) and bind Act type IIB receptor (ActRIIB)(6; 29; 39), the cell surface receptor which is thought to mediate the action of Mstn on muscle cells. Furthermore, GDF-11 and ActA are able to inhibit myogenesis either in chick limb (9; 13) or in C₂C₁₂ myoblasts (21; 39). Thus, we hypothesize that GDF-11 or ActA, together with Mstn, may inhibit muscle growth. To test this hypothesis, we overexpressed FSI-I, a FS mutant which does not affect Act activity. This mutant, characterized by the deletion of FS II domain, which is important for binding to Act, has been reported to retain the ability to bind and inhibit Mstn *in vitro* and to stimulate muscle growth *in vivo* (28). When FS and FSI-I activities were compared, our data show that FSI-I-induced hypertrophy was less marked than that induced by native FS, consistent with the smaller increase in muscle mass observed in FSI-I Transgenic mice (28) compared to FS Transgenic mice (18). This difference in the extent of hypertrophy points to a key role of Act inhibition in the FS effect. On the other hand, since FSI-I, which does not bind Act with high affinity, is still able to cause hypertrophy, our data also support the role of Mstn or GDF-11 inhibition in the FSI-I-induced hypertrophy. Indeed, mutants for Mstn over Act, such as FSI-I, are similarly selective for Mstn and GDF-11. However, the fact that FSI-I overexpression in Mstn KO mice lost its ability to stimulate muscle growth suggests that GDF-11 does not play a major role. In accordance with our results, recent data show that deletion of GDF-11 specifically in skeletal muscle does not

affect muscle size, fiber number, or fiber type (23). Taken together, our observations suggest that inhibition of Act may contribute to the muscle hypertrophy caused by FS.

To directly assess the action of Act on postnatal skeletal muscle, we investigated the effect of ActA overexpression on muscle mass. Our results are the first to demonstrate that increased muscle ActA expression causes muscle atrophy. Although the mechanisms involved are not yet described, it is likely that this atrophy resulted from activation of the ActIIRB and ActIIRA, as described for Mstn. Recent data reported that Mstn causes muscle atrophy by down regulating the AKT/mTOR pathway, leading to blunted protein synthesis (1). Because ActA is expressed in skeletal muscle, the possibility therefore exists that ActA controls muscle growth in an autocrine/paracrine manner. Further work will certainly be necessary to delineate the respective roles of Mstn and ActA in the regulation of muscle mass and development. Nevertheless, the study presented here demonstrates that the capacity of promoting muscle growth by targeting this pathway goes beyond the targeting of Mstn alone. In our work, we demonstrated the hypertrophic effect of FS288. However, two isoforms of FS are generated by alternative splicing: FS288 and FS315. The structural difference between the two forms is the absence of a carboxy-terminal 27 amino acid sequence in the FS288. The FS288 isoform, lacking the C-terminus, shows high tissue binding affinity through heparin sulfate proteoglycans via a highly basic region located in FS I domain (14; 15). However, this basic region is structurally hidden in the FS315 by the C-terminal region, precluding the binding of this FS long isoform to extracellular matrix. As the short form is trapped by the extracellular matrix of the cell transfected (or neighbouring the transfected cells), it is therefore less diluted in the circulation. Thus, for our experiments, the FS288 isoform seemed more suitable to inhibit local Mstn. Although FS288 has been reported to block Act activity more effectively than FS315 (40), the two isoforms seem however equally effective in inhibiting Mstn bioactivity in vitro (36). Up to now, the hypertrophic action of these two FS isoforms has not yet been compared side-by-side. Nevertheless, when delivered by intramuscular injection of AAV virus, FS315 increased the muscle mass and led to functional improvement in dystrophic mice (11). Thus, combining our findings together with the existing data in the literature, we may conclude that muscle overexpression of both FS isoforms enhances muscle mass.

Several observations support the physiological role of FS in the control of muscle mass. Indeed, recent observations suggest that FS may play an essential role in mediating the myogenic effect of androgens (37), a family of very potent anabolic agents. Furthermore, although the role of FS in determining skeletal mass in humans has not yet been directly

explored, recent evidence shows associations between haplotype structures of the FS gene with skeletal muscle mass and strength phenotypes (46). Furthermore, FS may be a target for the pharmacologic treatment of muscle atrophy. Indeed, the beneficial effect of the administration of histone deacetylase inhibitors (26) or nitric oxide (30) in counteracting the progression of muscular dystrophy in the mdx model relies in part on the transcriptional activation of FS. Therefore, in addition to FS itself, FS inducers may represent a new avenue for the treatment of these debilitating conditions. All together, these studies pinpoint the role of FS as a physiological regulator of muscle mass and as a molecular target for future drug development.

In conclusion, we showed that satellite cells proliferation significantly contributes to the FS-induced muscle growth. In addition, we showed that the hypertrophic effect results from inhibition of both Mstn and Act, implicating Act as a novel potent regulator of muscle growth. The striking ability of FS to enhance muscle growth warrants therefore its consideration as physiological regulator of muscle mass and as a molecular target for future drug development.

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