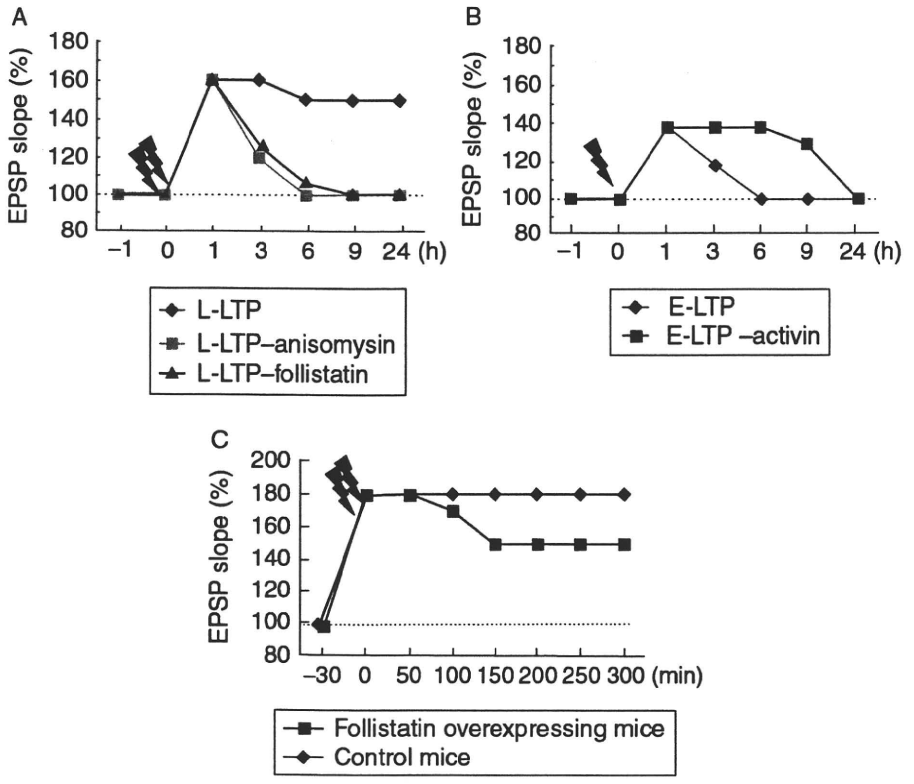

Figure 9.4 **Activin is required for L-LTP. (A) Effect of follistatin, antiactivin A, and anisomycin on dentate gyrus LTP persistence in urethane-anesthetized rats. (B) Effect of activin on dentate gyrus LTP persistence in urethane-anesthetized rats. (C) Maintenance of CA1 L-LTP in hippocampal slices from follistatin overexpressing mice and control mice. Two lightning symbols represent strong high-frequency stimulations produced by L-LTP. One lightning symbol represents a weak high-frequency stimulation elicited by E-LTP.**

hippocampal slices from follistatin transgenic mice (FBItTA, see below) is significantly reduced compared with control mice (Fig. 9.4C; Ageta *et al.*, 2010).

In the marine snail *Aplysia*, TGF-β induces long-term, but not short-term, facilitation at synapses between sensory and motor neurons (Zhang *et al.*, 1997). Similarly, treatment with TGF-β2, another isoform of TGF-β, affects synaptic strength and induces CREB phosphorylation in rat cultured hippocampal neurons (Fukushima *et al.*, 2007). Therefore, the TGF-β family of proteins, namely, activins and TGF-β1/2, participate not only in development, but also in neuronal plasticity, of the mature CNS.

E. Activin influences reconsolidation and extinction

LTM consists of several distinct processes—acquisition (training), maintenance, and retrieval (recall) phase—through which memory is consolidated. Two recent studies revealed that retrieval of consolidated memory leads to

two opposing processes: one that weakens old memory and another that strengthens it (Eisenberg *et al.*, 2003; Suzuki *et al.*, 2004). The former process is known as "extinction learning." The latter process has recently been referred to as "reconsolidation"; memory could be vulnerable following retrieval, so it is reconsolidated in a protein synthesis-dependent manner (Fig. 9.5; Nader, 2003; Nader *et al.*, 2000; Tronson and Taylor, 2007). The study of reconsolidation has extended to numerous learning tasks, such as fear conditioning test (Nader *et al.*, 2000), Morris water maze (Suzuki *et al.*, 2004), and object recognition test (Kelly *et al.*, 2003) in various species such as crabs (Pedreira *et al.*, 2002), chicks (Anokhin *et al.*, 2002), Medaka fish (Eisenberg et al., 2003), rodents (Nader *et al.*, 2000), and humans (Walker *et al.*, 2003). These results suggest that "reconsolidation" is an evolutionarily conserved memory system. Because there is a possibility that enhanced extinction learning or disrupted reconsolidation could be particularly efficacious when treating strong traumatic memory-related disorders, such as posttraumatic stress disorder (PTSD) or phobias, studies have investigated the molecular mechanisms of these processes. In addition, results from our laboratory recently demonstrated that activins in the brain affect both extinction and reconsolidation (Ageta *et al.*, 2010).

To examine the role that activin plays in fear memory formation, spatiotemporal-regulated activin (ABItTA) and follistatin (FBItTA) transgenic mice were generated (Ageta *et al.*, 2010). When follistatins are continuously expressed in the brain during training, maintenance, and retrieval phases (Fig. 9.6, Exp. A), FBItTA mice are impaired in LTM but not short-term memory (STM). These results were consistent with activin requirements for L-LTP (see above). Furthermore, a 1-week memory test was performed on FBItTA mice to determine how reconsolidation processes are regulated by activin inhibition (Fig. 9.6, Exp. B). When follistatins were expressed in the brain during maintenance and retrieval phases, there was no significant genotype effect on Test 1 between the FBItTA and control mice (Fig. 9.6, Exp. B). In addition, when the same animals were retested for freezing behavior 24 h later (Test 2 in Exp. B), the FBItTA mice exhibited significantly fewer freezing responses compared with the control mice. When follistatin expression was suppressed during all phases in FBItTA mice, significant genotype effects were not observed in either Test 1 or 2. Therefore, inhibition of activin signals during retrieval resulted in suppressed reconsolidation.

Three-week memory testing was also performed on the ABItTA mice, which induces extinction (Fig. 9.7). When activins were upregulated in the brain during maintenance and retrieval phases, there was no significant genotype effect in Test 1 between the ABItTA and control mice (Fig. 9.7, Exp. C). In this experimental paradigm, the freezing level was significantly less in Test 2 compared with Test 1 in control mice. However, there was no significant change between Test 1 and 2 in ABItTA mice.

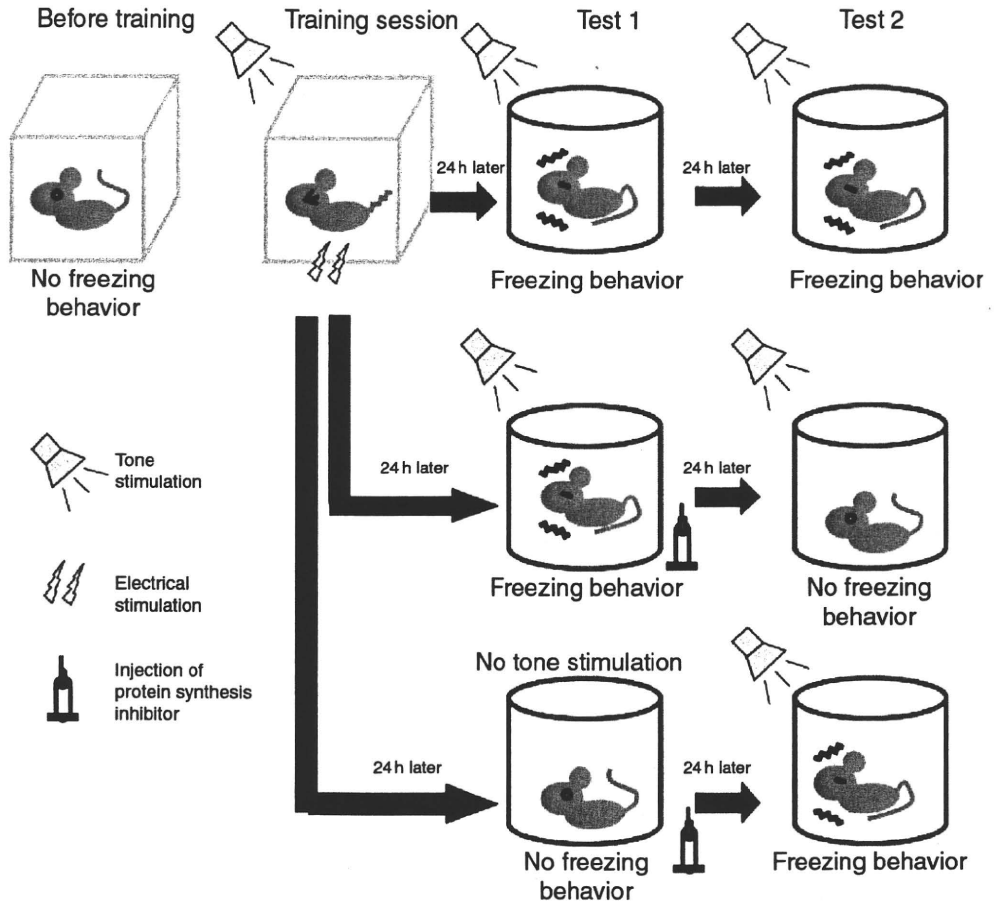

Figure 9.5 Memory reconsolidation in 2000, Nader *et al.* (2000) demonstrated reconsolidation in rats using a cued-fear conditioning test. The illustrations represent the Nader experiment. In brief, rats are placed in chamber A (gray box) and then receive tone and electrical stimulation. After 24 h, rats are placed in chamber B (red columnar box), whose shape is different from that of chamber A, and the rats are exposed to tone (Test 1 in upper row). If the rats associate tone with danger (electrical stimulation), they exhibit freezing behavior. After 24 h, the same rats were retested for freezing behavior (Test 2 in upper line). In the experiment paradigm that triggers reconsolidation process, the rats exhibited freezing behavior again in Test 2 (Test 2 in upper row). In this experimental paradigm, Nader *et al.* demonstrated that intra-amygdala inhibition of protein synthesis, following retrieval of a previously consolidated memory, resulted in amnesia (no freezing behavior) for the retrieved memory (Test 2 in middle row), but not for consolidated memories that were not retrieved (Test 2 in lower row). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)

ABItTA mice also exhibited significantly more freezing than control mice in Test 2. These findings demonstrated that activin upregulation in the brain inhibits the extinction learning.

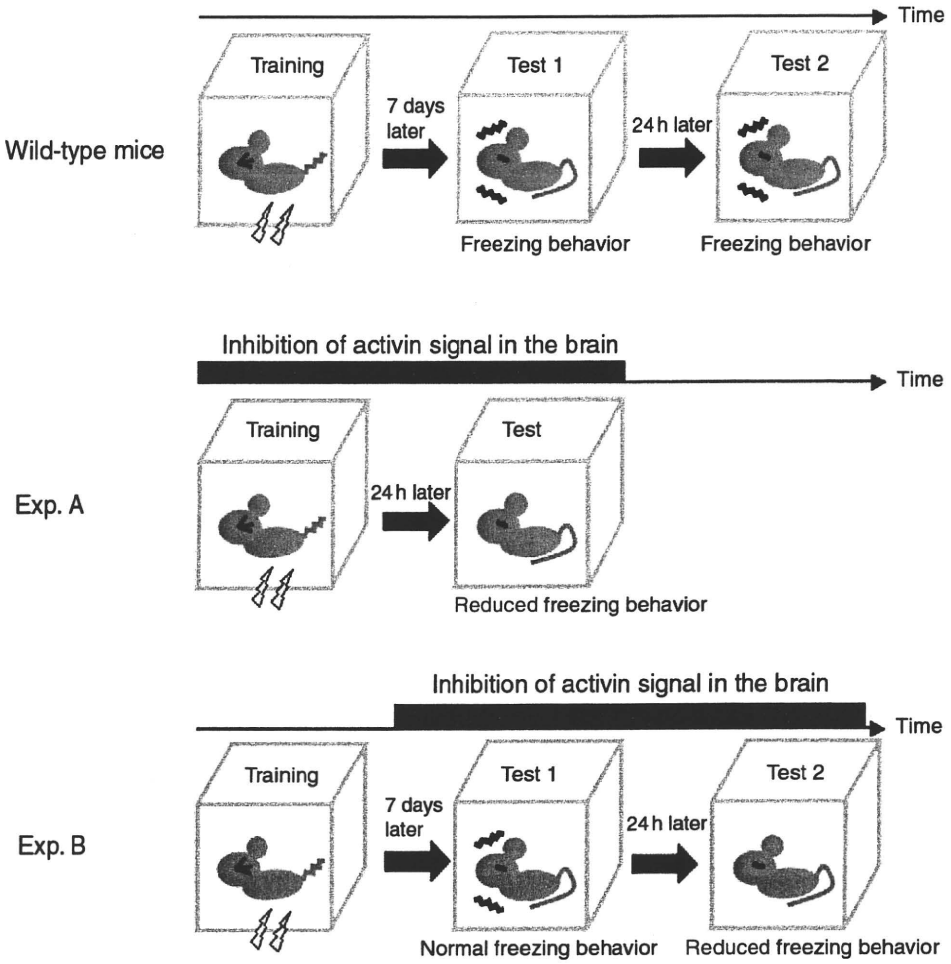

Figure 9.6 Contextual fear-conditioning test triggers reconsolidation in FBItTA mice. Utilizing the contextual fear-conditioning test, mice were placed in chamber A (gray box) and were exposed to electrical stimulation. During the retrieval phase, the mice were placed in the same chamber (Test). If the mice associate the chamber with danger, they will exhibit a freezing behavior during the Test phase. In the experiment paradigm that triggers reconsolidation, wild-type mice exhibited freezing behavior again during Test 2 (Test 2 in upper line). In this experimental paradigm, when mice were injected with protein synthesis inhibitor 30 min after Test 1, the mice exhibited reduced freezing behavior in Test 2. In experiment A (Exp. A, middle row), follistatins were continuously expressed during training, maintenance, and retrieval phases in the brain of FBItTA mice. FBItTA mice exhibited reduced freezing behavior during testing in Exp. A. In experiment B (Exp. B, lower row), follistatins were expressed in the brain during maintenance and retrieval phases in FBItTA mice. FBItTA mice exhibited reduced freezing behavior during Test 2, but not during Test 1, of Exp. B. Two lightning symbols represent strong electrical stimulation.

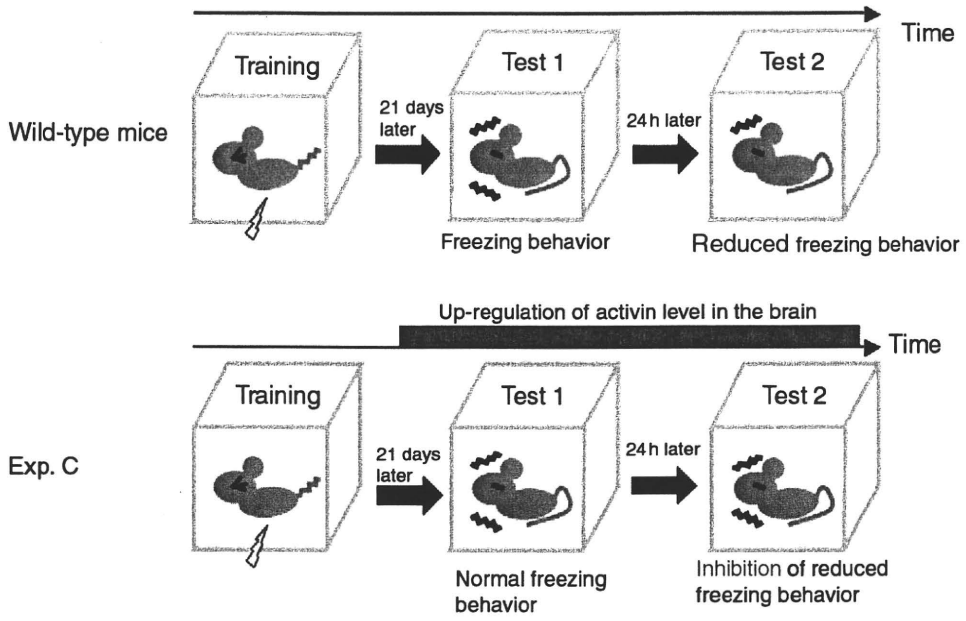

Figure 9.7 **Contextual fear-conditioning test triggers extinction learning in ABItTA mice. In an experiment paradigm that triggers extinction learning, wild-type mice exhibited reduced freezing behavior during Test 2 (Test 2 in upper line). The level of electrical stimulation and length of maintenance phase in Exp. C were different from Exp. B. In experiment C (Exp. C, lower row), activin levels were elevated during maintenance and retrieval phases in the brains of ABItTA mice. ABItTA mice did not exhibit reduced freezing behavior during Test 2 of Exp. C. One lightning symbol represents weak electrical stimulation.**

Results from our studies demonstrated that activin inhibition during memory retrieval suppresses previously consolidated fear memories. Therefore, activin signaling could be a promising target for the treatment of disorders that are based on strong traumatic memories, such as PTSD and phobias.

V. Conclusion and Perspectives

Activins are involved in various brain functions, including spine formation, anxiety, neurogenesis, L-LTP, LTM, extinction, and reconsolidation (Fig. 9.8). Recent studies have also shown that activins exhibit neurotrophic and neuroprotective effects (Hughes *et al.*, 1999; Tretter *et al.*, 2000; Wu *et al.*, 1999). For these reasons, activin-related compounds have therapeutic potential for candidate drugs to treat CNS disorders.

Because antibodies and peptides cannot cross the blood–brain barrier, activins, antiactivin antibodies, and follistatins are not suitable for treating the above-mentioned CNS disorders. Recently, small molecules, such as

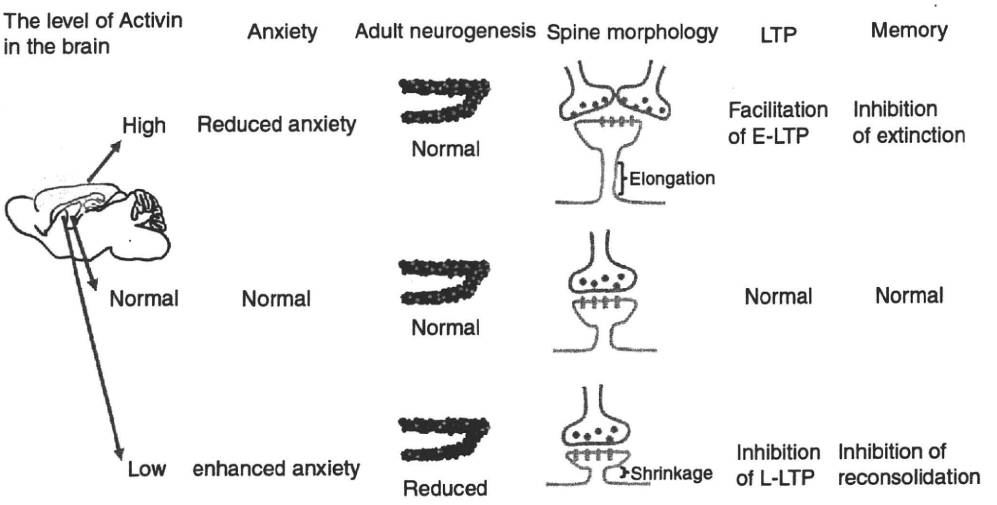

Figure 9.8 **Summary of the role of activin in the brain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)**

SB-431542 and GW788388, were identified as specific inhibitors of type I activin receptor-like kinase (ALK) receptors, including ActRIB/ALK4, ALK5, and ALK7 (Inman *et al.*, 2002). In addition to small molecule compounds, orally active ALK5 inhibitors were also developed (Gellibert *et al.*, 2006). If these compounds cross the blood–brain barrier, they may also be useful for treating CNS disorders. Because activin-signaling cascades exist in various peripheral tissues, activin-related compounds could result in unpredictable side effects in various tissues. To minimize side effects, it could be essential to combine the development of novel activin-related compounds with brain-specific drug delivery systems. We believe that the development of activin and follistatin transgenic mice could be useful for these drug developments.

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Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin

Hélène Gilson,[1] Olivier Schakman,[1] Stéphanie Kalista,[1] Pascale Lause,[1] Kunihiro Tsuchida,[2] and Jean-Paul Thissen[1]

[1]*Unité de Diabétologie et Nutrition, Université Catholique de Louvain, Brussels, Belgium; and* [2]*Division for Therapies against Intractable Diseases, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi, Japan*

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Gilson H, Schakman O, Kalista S, Lause P, Tsuchida K, Thissen JP. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin. *Am J Physiol Endocrinol Metab* 297: E157–E164, 2009. First published May 12, 2009; doi:10.1152/ajpendo.00193.2009.—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, but 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 that does not bind Act with high affinity. Whereas 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. Therefore, these results 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 cell proliferation and both Mstn and Act inhibition are involved in the FS-induced muscle hypertrophy.

INCREASING SIZE AND STRENGTH of skeletal muscle represents a promising therapeutic strategy for muscular disorders. One possible new tool is myostatin (Mstn), a transforming growth factor-β (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.

Address for reprint requests and other correspondence: H. Gilson, Unité de Diabétologie et Nutrition, Université Catholique de Louvain, 54 Ave. Hippocrate, B-1200, Brussels, Belgium (e-mail: helene.gilson@uclouvain.be).

The identification of Mstn-binding proteins that are able to inhibit Mstn activity has led to potentially 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-knockout (KO) mice (18). However, the mechanisms involved in the FS effect are 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 to combine morphological and biochemical analyses on the same muscles. Mice were used in the irradiation experiment and for KO models. Six-week-old male Wistar rats (150–160 g) 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-wk-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-wk-old male FVB wild-type (WT) mice and Mstn-KO mice harboring a constitutive deletion of the third Mstn exon (10). All animals were housed individually under controlled conditions of lighting (12:12-h light-dark cycle) and temperature (22 ± 2°C). The animals were allowed free access to chow and water. The study was conducted in accordance with the directives of and approved by the Institutional Animal Care and Use Committee of the University of Louvain.

Expression Plasmids and DNA Preparation

pM1-hFS288, pM1-FSI-I, and pM1-activin A (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 that does not affect Act signaling (28). In the pM1-ActA-c-Myc plasmid, the mouse ActA cDNA is followed by the tag c-Myc. Empty pM1 was used as a control plasmid. Plasmids were amplified in *Escherichia coli* top 10 F′ (Invitrogen, Carlsbad, CA) and purified with an EndoFree Plasmid Giga kit (Qiagen, Valencia, CA). Plasmids were stocked at −80°C. On 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 intraperitoneal injection. For rats, the plasmid solution (1 μg/μl) was injected into 10 different sites (total volume/muscle = 100 μl) in each tibialis anterior (TA) muscle, and the muscles were then electroporated using the electroporation conditions described previously (33). For mice, 30 μl of plasmid solution (1 μg/μl) was injected into 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. (8 pulses of 200 V/cm and 20 ms/pulse at 2 Hz) (3).

γ-Irradiation Conditions

Local γ-irradiation was achieved with a 250-kV X-ray irradiator (RT 250, 0.92 Gy/min; Philips Medical System) 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 γ-irradiation dose of 25 Gy. This dose has previously been shown to prevent satellite cell division (20).

Experimental Design

Characterization of the FS-induced muscle hypertrophy. After 1 wk 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 euthanized 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 and weighted, 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), and irradiated + FS (n = 7). The left TA muscles were subjected to one of the following treatments: *1*) electroporation with pM1 (control), *2*) electroporation with pM1-hFS288 (FS), *3*) irradiation with 25 Gy (Irr) and electroporation with pM1, and *4*) irradiation with 25 Gy and electroporation with pM1-hFS288 (Irr-FS). The right TA muscles of all animals were transfected with the plasmid control pM1. All mice were euthanized 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 Mstn-KO (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 euthanized 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 were 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 Mstn-KO (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 euthanized 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-c-Myc plasmid and the contralateral TA muscle with the pM1 plasmid. The rats (n = 8) were euthanized 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, was homogenized with Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 1 ml of 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-glycerophosphate, 1 mM KH_2PO_4, 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, as well as the supernatant containing the soluble proteins, were stored at −80°C. 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 Spectrofluo JY3D).

mRNA Analysis by Real-Time Quantitative PCR

Total RNA was isolated from the TA muscles using TRIzol reagent as instructed by the manufacturer. Recovery was 1 μg/mg TA muscle. Reverse transcription and real-time quantitative PCR were done as described previously (7). Accession numbers for the sequences and primers used were Mstn: AY204900 (GGCTTGACTGCGATGAG-ATATAGCATATTAATGGGAGACAT), FS: NM008046 (GGCAGA-TCCATTGGATTAGCC-TGCCAACCTTGAAATCCCAT), MHCneonatal: XM001080186 (CAGAGGAGGCTGAGGAACAATC-GCCTTTC-CTTCAGCCACTTG), MHCIIb: X72590.1 (TAGCTCAATTCCTTC-TGTTGAAAGGT-ATTATCTGCAGCTTTTATTTCCTTGAT), PCNA: NM022381 (CACCATGTTTGAGGCACGC-GGACATGCTGGTGAG-GTTCA), IGF-II: NM031511 (GTCGATGTTGGTGCTTCTCATCT-CGGTCCGAACAGACAAACTGAA), FS288 and FSI-I: NM008046 (GGCTCCGTAAGCGAAGA-CCGTTGAAAATCATCCACTTGAA), ActA (Inhibin-β A): NM017128.1 (GAGGACGACATTGGCAGGAG-TGCAGTGTCTTCCTGGCTGT), and glyceraldehyde-3-phosphate dehydrogenase: AF106860 (TGCACCACCAACTGCTTA-GGATGCAGG-GATGATGTTC), 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 (1:20; R & D Systems). Primary antibodies were detected by applying for 30 min at RT a biotinylated second antibody that was a horse anti-goat conjugated to peroxidase-labeled polymer (Vector Laboratories, Burlingame, CA), followed by application of an avidin/

biotinylated peroxidase complex (Vectastain ABC kit Peroxidase Standard; Vector Laboratories) for 30 min at RT. Peroxidase activity was revealed with DAB substrate (Chemicon International, 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, Wetzlar, Germany) coupled to an image analyzer system (MOP-Videoplan; Kontron, Eching, Germany). To evaluate muscle fiber CSAs, all of 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-c-Myc plasmid, sections were deparaffinized and pretreated in a microwave oven, as described previously (34). The primary antibody used was a rabbit monoclonal anti-c-Myc (1:800) (Bethyl Laboratories) incubated for 1 h.

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 ± SE. 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 posttest (GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA).

RESULTS

Postnatal FS Overexpression Induces Muscle Hypertrophy

Our results show that FS288 overexpression in TA muscle of rat increased FS mRNA 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. 1*A*), fiber CSA (+42%, 1,895 ± 100 vs. 1,337 ± 45 μm^2, $P < 0.001$; Fig. 1*B*), and muscle protein content (+22%, 125.0 ± 4.2 vs. 101.2 ± 2.8 mg/muscle, $P < 0.01$; Fig. 1*C*) 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 Fig. 1*D*, the muscle hypertrophy caused by FS was associated with an increase in DNA content (+28%, 42.0 ± 1.5 vs. 32.8 ± 1.0 μg/muscle, $P < 0.001$). The hypertrophic muscle contained increased levels of PCNA mRNA (+29%, $P < 0.05$; Fig. 1*E*), 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. 1*F*), a marker of muscle differentiation, and MHCIIb (+31%, $P < 0.05$), the main MHC isoform in TA muscle.

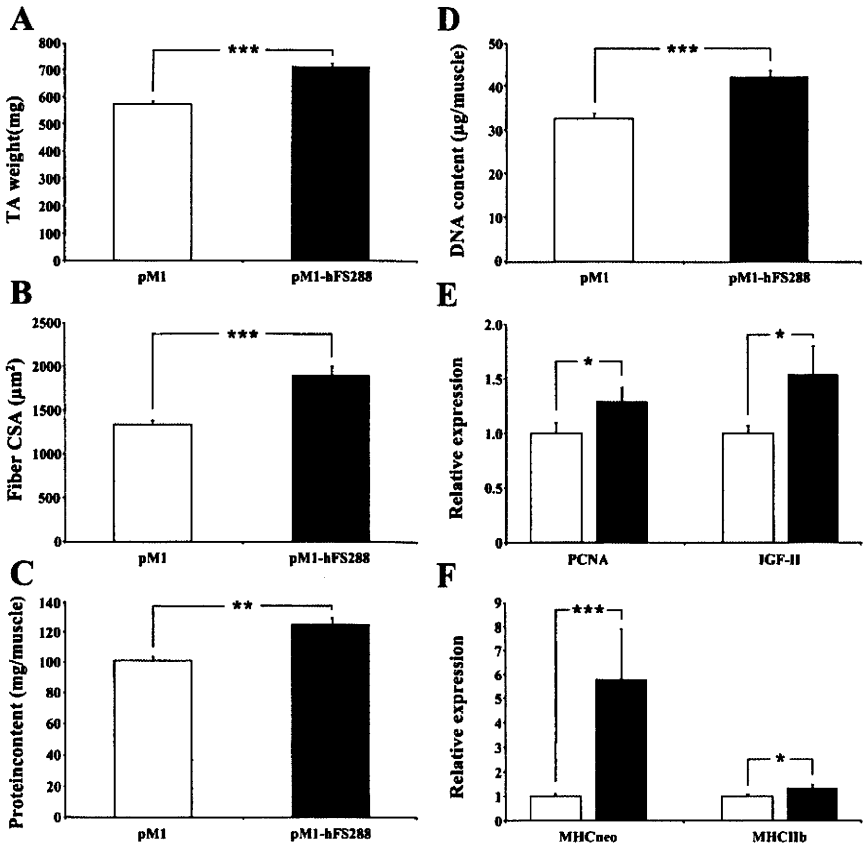

Fig. 1. Overexpression of follistatin (FS) in tibialis anterior (TA) muscle induces muscle (*A*) and fiber (*B*) hypertrophy in rat. It also increases the protein (*C*) and DNA (*D*) contents and stimulates the expression of PCNA and IGF-II (*E*) as well as myosin heavy chain (MHC) [neonatal (MHCneo) and IIb (MHCIIb); *F*]. All of these parameters were measured 17 days after transfection of pM1 (open bars) or pM1-FS288 (filled bars). The results are expressed as means ± SE. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. CSA, cross-sectional area.

FS-Induced Muscle Hypertrophy is Partially Mediated by Satellite Cell Proliferation

To test whether satellite cells are essential in mediating the hypertrophic effects of FS, we used γ-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. Whereas 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. 2*A*). Similarly, the increase in CSA of FS-transfected fibers reached 111% in control mice (3,856 ± 156 vs. 1,852 ± 85 μm^2, $P < 0.001$) but only 65% in irradiated muscles (3,018 ± 206 vs. 1,843 ± 63 μm^2, $P < 0.001$) (Fig. 2*B*). The destruction of the satellite cell proliferative capacity was confirmed by Brdu immunohistochemistry. In our conditions, γ-irradiation indeed decreased the number of positive BrdU cells by 90% in irradiated TA compared with the contralateral muscle (9 vs. 90 in Irr group and 6 vs. 93 in Irr-FS group, both $P < 0.001$). Therefore, these results show that satellite cells play a critical role in FS-induced muscle hypertrophy.

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 Mstn-KO mice (+50%, 98.5 ± 4.1 vs. 65.5 ± 2.1 mg, $P < 0.001$) (Fig. 3*A*). This result was confirmed by the measurement of the fiber CSA in the two groups (+114%, 3,222 ± 218 vs. 1,509 ± 104 μm^2, $P < 0.01$, in WT mice; and +87%, 1,452 ± 95 vs. 2,710 ± 76 μm^2, $P < 0.001$, in Mstn-KO mice; Fig. 3*B*). Therefore, this observation suggests that the FS effect on skeletal muscle is not due only 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 has been shown to inhibit muscle development in vitro (21).

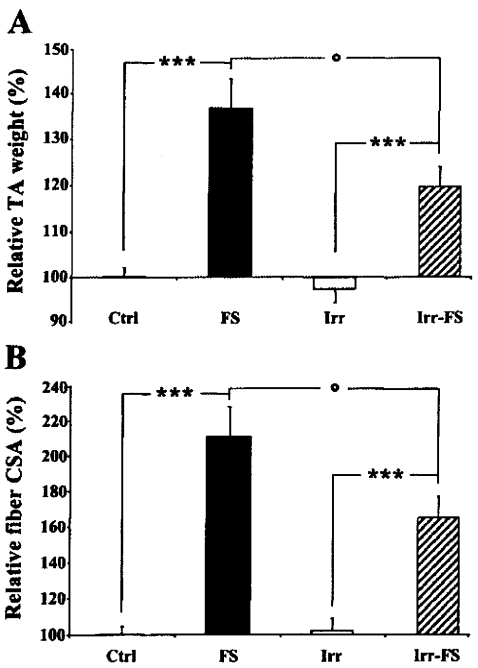

Fig. 2. γ-Irradiation partially blunts the FS-induced muscle (*A*) and fiber (*B*) hypertrophy in mice. TA mass and the fiber CSA were measured 17 days after transfection of pM1 (open bars) or pM1-FS288 (black and hatched bars), and the data are normalized as percentages of the contralateral control muscle. The results are expressed as means ± SE. °$P < 0.05$ and ***$P < 0.001$. Ctrl, control; FS, electroporation with pM1-hFS288; Irr, irradiation with 25 Gy (gray bars); Irr-FS, irradiation with 25 Gy and electroporation with pM1-hFS288.

To assess the role of Act inhibition in the FS-induced hypertrophy, we electroporated a FS mutant, FSI-I (28), in WT 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, $P < 0.01$), whereas 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. 4*A*). Similarly, the 91% muscle fiber hypertrophy induced by FS288 overexpression (3,613 ± 271 vs. 1,883 ± 97 μm^2, $P < 0.01$) was reduced to 44% in muscle overexpressing FSI-I (2,656 ± 344 vs. 1,839 ± 56 μm^2, $P < 0.05$) (Fig. 4*B*). Considering the similar affinities of FS288 and FSI-I for Mstn, the smaller effect of FSI-I compared with FS288 ($P < 0.01$) suggests that the hypertrophic effect of FS288 is not due only to Mstn inhibition. When electroporated in Mstn-KO mice, FSI-I overexpression failed to induce a muscle hypertrophy [+3%, 69.1 ± 3.7 vs. 67.0 ± 3.1 μm^2, not significant (NS); Fig. 5*A*], 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. 3*A*). Like for the muscle weight, no effect on fiber size could be detected following the FSI-I overexpression (+6%, 1,655 ± 67 vs. 1,555 ± 65 μm^2, NS; Fig. 5*B*), in contrast to the results obtained with the FS288 (+87%, 1,452 ± 95 vs. 2,710 ± 76 μm^2, $P < 0.001$, with FS288; Fig. 3*B*). These data suggest that most of the hypertrophic effect of FS288 in Mstn-KO mice results from Act inhibition. Therefore, Act may represent a new player in the regulation of the muscle mass.

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-c-Myc 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. 6*A*). In agreement with muscle weight loss, the CSA was also reduced by 46% in fiber overexpressing ActA (2,738 ± 182 vs. 1,473 ± 60 μm^2, $P < 0.001$; Fig. 6*B*), 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.

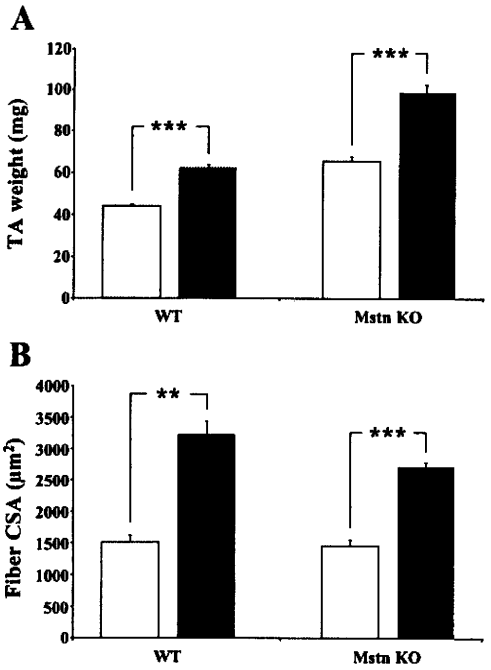

Fig. 3. Overexpression of FS induces comparable muscle (*A*) and fiber (*B*) hypertrophy in wild-type (WT) and myostatin-knockout (Mstn-KO) mice. TA mass and fiber CSA were measured 17 days after transfection of pM1 (open bars) or pM1-FS288 (filled bars). The results are expressed as means ± SE. $^{**}P < 0.01$ and $^{***}P < 0.001$.

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. Therefore, these observations 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 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 of 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 (16) as well as protein synthesis (42, 47, 48), in particular MHCIIb (12). Furthermore, Mstn is abundantly expressed in TA muscle, a muscle composed mainly of IIb myofibers that strongly express Mstn (5, 32). These results are in accordance with previous studies that showed that FS overexpression, either by transgenesis or AAV-mediated gene delivery, causes a dramatic increase in muscle mass (11, 19). However, in these studies 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 the destruction of their proliferative capacity using γ-irradiation. By combining γ-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 destroyed. This observation suggests that FS causes muscle hypertrophy by stimulating muscle protein synthesis, a hypothesis that was demonstrated recently (41). We are confident that irradiation achieved blockade of satellite cell activation, since 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 downregulate satellite cell 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). Therefore, this work is the first to demonstrate the contribution of satellite cells in FS-induced muscle hypertrophy.

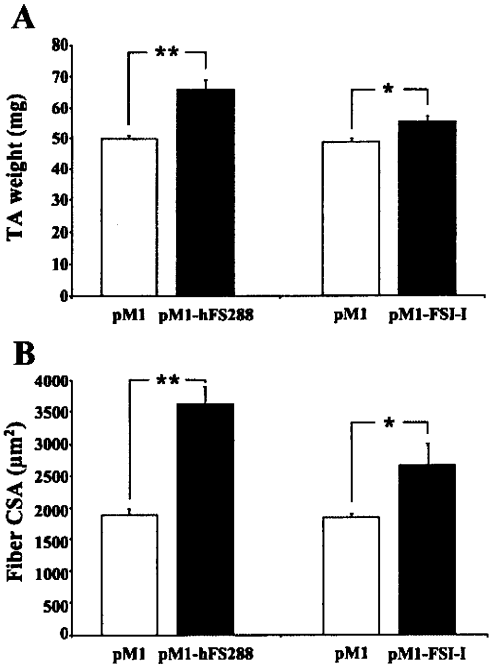

Fig. 4. Overexpression of FSI-I induces a smaller muscle (*A*) and fiber (*B*) hypertrophy than FS288 in WT mice. TA mass and fiber CSA were measured 17 days after transfection of pM1 (open bars) or pM1-hFS288/pM1-FSI-I (filled bars). The results are expressed as means ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$.

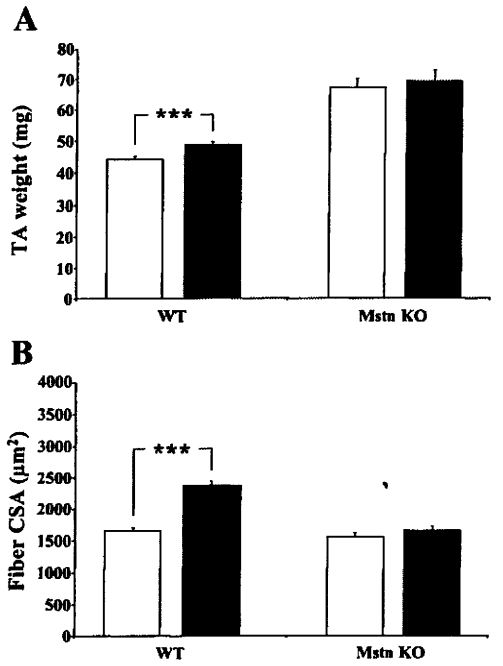

Fig. 5. Overexpression of FSI-I fails to cause muscle (*A*) and fiber (*B*) hypertrophy in Mstn-KO mice. No increase in muscle mass or in fiber CSA was observed in muscle overexpressing FSI-I in Mstn-KO mice compared with WT mice. TA mass and fiber CSA were measured 17 days after the transfection of pM1 (open bars) or pM1-FSI-I (filled bars). The results are expressed as means ± SE. ***$P < 0.001$.

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 with mice lacking only Mstn. Therefore, our present findings argue that FS overexpression, even in the postnatal period, could increase muscle growth in the absence of Mstn. The candidate ligands could include Act and growth differentiation factor 11 (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 (ActIIRB) (6, 29, 39), the cell surface receptor that 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 C_2C_{12} 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 that 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 showed 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 with 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 downregulating 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, FS288 and FS315, are

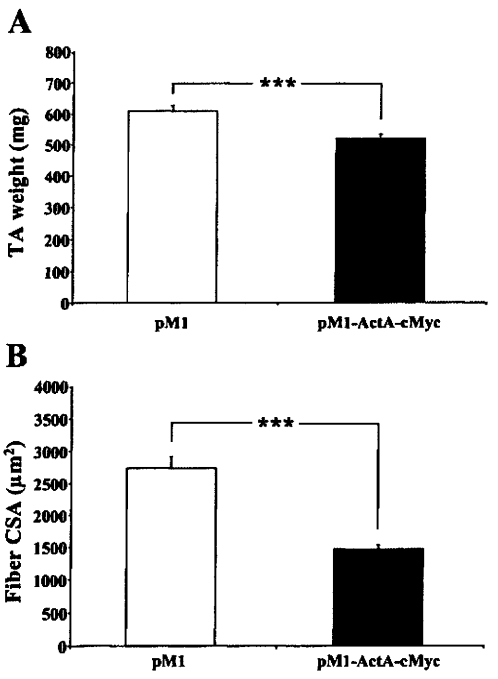

Fig. 6. Overexpression of activin A (ActA) induces muscle (*A*) and fiber (*B*) atrophy in rat. TA mass and fiber CSA were measured 17 days after transfection of pM1 (open bars) or pM1-ActA-c-Myc (filled bars). The results are expressed as means ± SE. ***$P < 0.001$.

generated by alternative splicing. 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 COOH 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 COOH-terminal region, precluding the binding of this FS long isoform to extracellular matrix. Because the short form is trapped by the extracellular matrix of the cell transfected (or the neighboring 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 equally effective in inhibiting Mstn bioactivity in vitro (36). Up until 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, 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 explored directly, 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 pharmacological 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. Altogether, 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 cell 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. Therefore, the striking ability of FS to enhance muscle growth warrants its consideration as a physiological regulator of muscle mass and as a molecular target for future drug development.

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GRANTS

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Activin A and Follistatin-Like 3 Determine the Susceptibility of Heart to Ischemic Injury

Yuichi Oshima, MD, PhD; Noriyuki Ouchi, MD, PhD; Masayuki Shimano, MD, PhD; David R. Pimentel, MD; Kyriakos N. Papanicolaou, BSc; Kalyani D. Panse, MSc; Kunihiro Tsuchida, MD, PhD; Enrique Lara-Pezzi, PhD; Se-Jin Lee, MD, PhD; Kenneth Walsh, PhD

Background—Transforming growth factor-β family cytokines have diverse actions in the maintenance of cardiac homeostasis. Activin A is a member of this family whose regulation and function in heart are not well understood at a molecular level. Follistatin-like 3 (Fstl3) is an extracellular regulator of activin A protein, and its function in the heart is also unknown.

Methods and Results—We analyzed the expression of various transforming growth factor-β superfamily cytokines and their binding partners in mouse heart. *Activin βA* and *Fstl3* were upregulated in models of myocardial injury. Overexpression of activin A with an adenoviral vector (Ad-actβA) or treatment with recombinant activin A protein protected cultured myocytes from hypoxia/reoxygenation-induced apoptosis. Systemic overexpression of activin A in mice by intravenous injection of Ad-actβA protected hearts from ischemia/reperfusion injury. Activin A induced the expression of Bcl-2, and ablation of Bcl-2 by small interfering RNA abrogated its protective action in myocytes. The protective effect of activin A on cultured myocytes was abolished by treatment with Fstl3 or by a pharmacological activin receptor-like kinase inhibitor. Cardiac-specific *Fstl3* knockout mice showed significantly smaller infarcts after ischemia/reperfusion injury that was accompanied by reduced apoptosis.

Conclusions—Activin A and Fstl3 are induced in heart by myocardial stress. Activin A protects myocytes from death, and this activity is antagonized by Fstl3. Thus, the relative expression levels of these factors after injury is a determinant of cell survival in the heart. (***Circulation.*** **2009;120:1606-1615.)**

Key Words: activin A ■ apoptosis ■ follistatin-like 3 ■ myocytes ■ reperfusion

The transforming growth factor-β (TGF-β) family comprises a large number of multifunctional proteins that can be divided into subfamilies including activins, bone morphogenic proteins, growth and differentiation factors (GDFs), and TGF-βs. These secreted proteins have diverse roles in cell proliferation, differentiation, apoptosis, and immune responses.[1] TGF-β1, the founding member of the TGF-β superfamily, is a mediator of cardiac hypertrophy and remodeling.[2,3] It has also been reported that bone morphogenic protein-2,[4,5] GDF-15,[6,7] and myostatin (GDF-8)[8] influence the growth and survival of cardiac myocytes. However, the majority of TGF-β superfamily members have not been examined for their potential cardiac-regulatory functions.

Clinical Perspective on p 1615

The follistatin family proteins function as extracellular antagonists of TGF-β superfamily cytokines. Follistatin and follistatin-like 3 (Fstl3) bind directly to TGF-β superfamily cytokines to inhibit their biological activities.[1] Recently, Lara-Pezzi et al[9] reported that *Fstl3* transcript expression is upregulated in end-stage failing myocardium and its expression is correlated with molecular markers of disease severity. They also reported that transcripts encoding follistatin-like 1 (Fstl1), a distant member of the follistatin family, are upregulated in heart failure and expression is positively correlated with better functional recovery after implantation of a left ventricular assist device. We have shown that Fstl1 is secreted from cardiac myocytes after injury in animal models and that it functions to promote cardiac myocyte survival.[10]

To better understand the regulation of secreted factors from the heart, we performed gene array transcriptome analyses on murine hearts that were subjected to injury and other stimuli.[11,12] These analyses revealed that members of the follistatin family of secreted factors were upregulated on injury or Akt

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From the Molecular Cardiology Unit, Whitaker Cardiovascular Institute (Y.O., N.O., M.S., K.N.P., K.W.), and the Myocardial Biology Unit (D.R.P.), Boston University Medical Campus, Boston, Mass; Imperial College London, Heart Science Centre, Harefield, United Kingdom (K.D.P., E.L.-P.); Division for Therapies Against Intractable Diseases, Institute for Comprehensive Medical Sciences, Fujita Health University, Toyoake, Japan (K.T.); and Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Md (S.L.).

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Correspondence to Kenneth Walsh, PhD, Molecular Cardiology Unit/Whitaker Cardiovascular Institute, Boston University Medical Campus, 700 Albany St, W611, Boston, MA 02118. E-mail kxwalsh@bu.edu

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